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Optimization of the nanofabrication by acid hydrolysis of bacterial cellulose nanowhiskers

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ABSTRACT

This work aims at examining the various factors that affect cellulose nanowhiskers (CNWs) extraction from bacterial cellulose (BC). Specifically, the effect of sulfuric acid hydrolysis time and further treatments such as neutralization and dialysis on the properties of the obtained nanoparticles was studied. The morphology of BCNWs was examined by TEM, showing a decrease in the nanowhiskers' length when increasing hydrolysis time as expected. The XRD patterns of the different samples showed a crystalline structure characteristic of the cellulose I allomorph. From the calculated crystallinity indexes it was deduced that long hydrolysis times, such as 48 h, are required when intending to digest a significant fraction of amorphous material and thus, obtaining a significant increase in crystallinity by comparison with the native BC. Nevertheless, as a consequence of this extensive acid hydrolysis treatment, the thermal stability of the material is significantly decreased, making it unsuitable for most melt-compoundable polymer-based nanocomposites applications. On the other hand, neutralization produced a slight increase in the crystallinity index, and, most importantly, it led to a remarkable increase on the BCNWs thermal stability, as determined by TGA. Furthermore, it was found out that dialysis applied after neutralization did not present any additional improvement on the BCNWs' properties.

1. Introduction

Cellulose is one of the most abundant biopolymers on earth. Vegetal resources such as wood, cotton and linter are the most commonly raw materials employed to extract cellulose. Nevertheless, cellulose can be also synthesized by some bacterial species, such as *Gluconacetobacter xylinum*. In a static culture medium rich in polysaccharides, this bacterial species is able to produce a layer of bacterial cellulose (BC) in the liquid/air interface. This highly hydrated pellicle consists of a random assembly of ribbon-shaped fibrils, less than 100 nm wide, which are composed of a bundle of nanofibrils (Yamanaka et al., 1989). Although plant-derived cellulose (PC) and BC have the same chemical structure, they have different structural organization and mechanical properties. BC shows a finer web-like network structure, higher water holding capacity and higher crystallinity (Iguchi, Yamanaka, & Budhiono, 2000; Wan et al., 2007). Furthermore, while PC is naturally associated with other kinds of biopolymers such as hemicelluloses and lignin, BC is practically pure cellulose. Due to its outstanding properties, i.e. high purity, high crystallinity, high mechanical strength, low density and biocompatibility, bacterial cellulose has become an interesting material with applications in biomedicine (Czaja, Krystynowicz, Bielecki, & Brown, 2006; Klemm, Schumann, Udhardt, & Marsch, 2001; Svensson et al., 2005), paper industry and, more recently, as a reinforcement agent for polymeric matrixes (Gindl & Keckes, 2004; Millon & Wan, 2006; Park, Kang, Kim, & Jin, 2007; Wan et al., 2009).

For their application as nanofillers, cellulosic materials are usually subjected to hydrolysis with strong acids such as sulfuric acid or hydrochloric acid, which produce a preferential digestion of the amorphous domains of the material and cleavage of the nanofibril bundles (Rånby, 1949), therefore breaking down the hierarchical structure of the material into crystalline nanofibres or nanocrystals, usually referred to as cellulose nanowhiskers (CNWs). The morphology of the obtained CNWs depends on the cellulose source and the hydrolysis conditions. While CNWs extracted from vegetal resources such as cotton or wood typically have a length of 100–300 nm and width of 5–20 nm (Araki, Wada, Kuga, & Okano, 1998; Favier, Chanzy, & Cavaille, 1995; Siaueira, Bras, & Dufresne, 2009), CNWs obtained from tunicin and bacterial cellulose may have several micrometres in length and a width of 5–50 nm (Araki & Kuga, 2001; De Souza Lima, Wong, Paillet, Borsali, & Pecora, 2003; Hirai, Inui, Horii, & Tsuji, 2009). Regarding the hydrolysis conditions, the acid concentration, cellulose/acid ratio, temperature and hydrolysis time are factors which determine the CNWs' morphology. The CNWs' aspect ratio (*L*/*D*) is a crucial parameter which has

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a remarkable influence on the reinforcing capacity of the nanofiller when incorporating it into a polymeric matrix (Eichhorn et al., 2010). Therefore, the acid hydrolysis conditions must be carefully studied and controlled in order to obtain a material with the desired morphology.

The most widely used procedure for the extraction of cellulose nanowhiskers consists of sulfuric acid treatment followed by filtration or centrifugation. Sulfuric acid hydrolysis leads to stable aqueous suspensions of cellulose nanocrystals which are negatively charged and thus, do not tend to aggregate. During the hydrolysis process, esterification of the surface hydroxyl groups from cellulose takes place and as a consequence, sulfate groups are introduced (Rånby, 1949). Despite the advantage of obtaining stable suspensions, the presence of sulfate groups in the outer surface of the material has been proved to strongly decrease the thermal stability of the material (Roman & Winter, 2004), which is also a key factor when intending to use CNWs as nanoreinforcement.

Typical CNWs extraction methods involve centrifugation after hydrolysis with the purpose of removing acid and the degraded material. After several centrifugation cycles CNWs are usually obtained from the turbid liquid supernatant, while bigger cellulosic material fractions and some impurities remain in the solid precipitate. Taking into account that in the case of BC there is no hemicellulose or lignin to remove, previous studies proposed an extraction method in which BCNWs are obtained in the centrifugation precipitate instead of the supernatant and, thus, the yield can be as high as 89% based on the dry weight of bacterial cellulose (Martínez-Sanz, Olsson, Lopez-Rubio, & Lagaron, 2010; Olsson et al., 2010) vs. yields around 1–5% when the whiskers are obtained from the liquid supernatant. In contrast with this great advantage, the highly crystalline network structure of BC requires strong hydrolysis conditions in order to break down the morphology of fibril bundles and individual nanofibrils cannot be yielded without partial carbonization and degradation of the material (Olsson et al., 2010).

In a previous study, it was found that BCNWs with a crystallinity index of ca. 86% were obtained after applying a relatively strong sulfuric acid hydrolysis. Nevertheless, the thermostability of the material was significantly diminished with respect to the untreated BC and BCNWs started to degrade at approximately 100 °C (Martínez-Sanz et al., 2010), which is far below the typical temperatures for processing most thermoplastics. In the present study, the effects of sulfuric acid hydrolysis time and post-treatments on the morphology, crystallinity and thermostability of BCNWs were studied for the first time. The hydrolysis time ranged from a typical duration of 2 h to a long treatment of 69 h, similar to the previous work mentioned above. The effect of neutralization and dialysis after the acid treatment on the thermal stability was also studied. The objective was to optimize the BCNWs extraction process in order to obtain a material with a high crystallinity index and high thermal stability and having a proper morphology which makes it suitable for nanocomposites applications.

2. Materials and methods

2.1. Preparation of bacterial cellulose mats

The bacterial strain *G. xylinum* 7351, obtained from the Spanish type culture collection (CECT) was incubated in a static culture medium composed of 20 g glucose, 5 g yeast-extract, 1.15 g citric acid, 5.7 g $MgSO_4 \cdot 7H_2O$ and 12.25 g buffered peptone water, per litre of water, at 30 °C. All of the cells were pre-cultured in a test tube containing 5 mL of media. When a thin layer of cellulose was detected on top of the surface, they were transferred to 200 mL bottles and subsequently to the final culture, containing 20 L of media. The obtained bacterial cellulose pellicles, about 5 cm thick, were cut into small pieces (ca. 2 cm × 2 cm). Those pieces were boiled repeated times in distilled water and then boiled in an aqueous solution of 10% (v/v) NaOH (Ph. Eur., Panreac Quimica Sau) in order to remove bacterial cells and the absorbed culture media. Finally, the pH was lowered to 7 by boiling in distilled water several times.

2.2. Sulfuric acid hydrolysis

Once neutral pH was reached, bacterial cellulose pellicles were ground in a blender. One fraction of the gel-like material was then compressed in order to remove most of the absorbed water, and another fraction was freeze-dried and ground into powder. The dried or freeze-dried cellulosic material was then treated with 301 mL sulfuric acid (96% Panreac)/L water, in a cellulose/acid ratio of approximately 8–10 g/L, at 50 °C for a fixed period of time (cf. Table 1).

The cellulose nanowhiskers were obtained as a white precipitate after several centrifugation and washing cycles at 12,500 rpm and 15 °C for 20 min. The pH of the samples was measured after the washing-centrifugation cycles, being around 2 for all the samples.

2.3. BCNWs post-treatments

In order to study the influence of pH in thermal stability, all the samples were re-suspended in deionised water and neutralized with sodium hydroxide until neutral pH and subsequently centrifuged to obtain the final product as a partially hydrated precipitate (BCNW 2hN, BCNW 48hN and BCNW 69hN).

Furthermore, and additional step of dialysis was applied to sample 69hN in order to evaluate the effect of this process in the material. Thus, the precipitate obtained after neutralization and centrifugation was re-suspended in water and subsequently subjected to dialysis against deionised water during one week. The product inside the dialysis membrane was then freeze-dried (BCNW 69hND).

The humidity of the centrifuged nanowhiskers was determined in order to calculate the amount of whiskers per gram of centrifugation precipitate.

A fraction of each sample was freeze-dried for XRD, FT-IR and TGA analyses.

2.4. Transmission electron microscopy (TEM)

One drop (8 μL) of a 0.001% aqueous suspension of BCNW was allowed to dry on a carbon coated grid (200 mesh). The crystals were stained with uranyl acetate. TEM was performed using a JEOL 1010 equipped with a digital Bioscan (Gatan) image acquisition system at 80 kV.

2.5. X-ray diffraction (XRD)

X-ray diffraction was carried out on a D5005 Bruker diffractometer. The instrument was equipped with a Cu tube and a secondary monochromator. The configuration of the equipment was θ–2θ and the samples were examined over the angular range of 5° to 45° with a step size of 0.02° and a count time of 4 s per point.

Peak fitting was carried out using Igor software package (Wavemetrics, Lake Oswego, Oregon). Gaussian function was used to fit the experimental diffraction profiles obtained. For the fitting procedure, the reflections considered were (i) three at 14.8°, 16.4° and 22.5° (corresponding to 1 0 1, 1 0 $\bar{1}$ and 0 0 2 crystal planes, respectively) assigned to the cellulose I allomorph, and (ii) the amorphous halo centered at approximately 18.5° 2θ. The crystallinity index CI (XD) was determined by the method reported by Wang, Ding, and

Table 1
Conditions for the sulfuric acid hydrolysis of bacterial cellulose.

Sample code	Cellulose	Hydrolisys time (h)	H_2SO_4 concentration (%(w/v))	pH
BCNW 2h	Freeze-dried	2	55.4	2.16
BCNW 2hN	Freeze-dried	2	55.4	6.40
BCNW 48h	Dried	48	50.7	2.00
BCNW 48hN	Dried	48	50.7	6.83
BCNW 69hN	Freeze-dried	69	55.4	6.61
BCNW 69hND	Freeze-dried	69	55.4	5.28

Cheng (2007):

$$\mathrm{CI(XD)} = \frac{\sum A_{\mathrm{Crystal}}}{A_{\mathrm{Total}}} \times 100 \quad (1)$$

where A_{Total} is the sum of the areas under all the diffraction peaks and $\sum A_{\mathrm{Crystal}}$ is the sum of the areas corresponding to crystalline peaks. The crystallite sizes were estimated from the 1 0 1, 1 0 $\bar{1}$ and 0 0 2 lattice planes of cellulose I using the well-known Scherrer equation:

$$D_{(h\ k\ l)} = \frac{k \cdot \lambda}{B_{(h\,k\,l)} \cdot \cos\theta} \quad (2)$$

where $D_{(h\,k\,l)}$ is the size of the crystallite (nm), k is the Scherrer constant (0.94), λ is the X-ray wavelength, $B_{(h\,k\,l)}$ is the full-width at half-maximum of the reflection $h\,k\,l$ and 2θ is the corresponding Bragg angle.

2.6. FT-IR analysis

Bacterial cellulose before acid digestion and the obtained nanowhiskers samples of ca. 2 mg were ground and dispersed in 200 mg of spectroscopic grade KBr. A pellet was then formed by compressing the sample at ca. 150 MPa.

FT-IR experiments were recorded in transmission mode in a controlled chamber at 21 °C and 40%RH using a Bruker (Rheinstetten, Germany) FT-IR Tensor 37 equipment. The spectra were taken at 1 cm^{-1} resolution averaging a minimum of 10 scans. Analysis of the spectra was performed using Grams/AI 7.02 (Galactic Industries, Salem, NH, USA) software.

2.7. Thermogravimetric analysis (TGA)

Thermogravimetric (TG) curves were recorded with a TA Instruments model Q500 TGA. The samples (ca. 20 mg) were heated from 50 °C to 600 °C with a heating rate of 10 °C/min under nitrogen atmosphere. Derivative TG curves (DTG) express the weight loss rate as a function of temperature.

2.8. Statistical analysis

One-way analysis of the variance (ANOVA) was performed using XLSTAT-Pro (Win) 7.5.3 (Addinsoft, NY) software package. Comparisons between samples were evaluated using the Tukey test ($\alpha = 0.05$).

3. Results and discussion

BCNWs were obtained by sulfuric acid digestion of bacterial cellulose (BC) and the effects of hydrolysis time and post-treatments on the obtained nanoparticles's properties were studied. Initially, freeze-dried BC was subjected to a short hydrolysis process of two hours, which is the typical hydrolysis time applied to vegetal cellulosic materials. However, after two hours the hydrolysis solution was not completely homogeneous, indicating that this hydrolysis time may not be enough to digest the amorphous domains of the material. Therefore, longer hydrolysis treatments were applied to both dried and freeze-dried cellulose. The time needed to obtain a homogeneous solution was 48 h and 69 h, respectively.

As previous studies pointed out that sulfuric acid hydrolysis causes a decrease in the thermal stability of the cellulosic material, samples were additionally neutralized with sodium hydroxide and further subjected to dialysis to evaluate if the thermal properties of the BCNWs improved by these post-treatments.

3.1. Morphological characterization of BCNWs

The morphology of BCNW suspensions was studied by means of TEM and the cross-sections and lengths of each sample were estimated from several measurements on TEM micrographs. Since analyzed areas are very small compared to the total sample area, the results obtained from TEM should be considered as a rough estimation of the actual size of the nanowhiskers and the aim of these measurements is to compare between the different samples.

From observation of Fig. 1 and Table 2 it can be deduced that no major changes in the BCNWs' cross-sections were induced by varying the acid hydrolysis conditions or the subsequent treatments. However, it appears that by increasing the hydrolysis time up to 48 h, the cross-section decreases (with a 95% confidence level), which may indicate that only for long hydrolysis times, such as 48 h, the acid is able to start breaking down the fibrils' bundles thus decreasing the amount of nanofibrils which conform them. Nevertheless, the wide variability of the data precludes from drawing absolute conclusions about the cross-sections of the nanowhiskers obtained.

On the other hand, a trend of decreasing BCNWs' length is observed when increasing the hydrolysis time. This reduction in length has been previously reported in cellulose samples when treated with strong acids, which produce the preferential digestion of disordered regions along the cellulose fibrils, thus resulting in shorter nanocrystals (Azizi Samir, Alloin, & Dufresne, 2005). Therefore, this shortening of cellulose nanofibrils, which is more obvious when increasing the hydrolysis time from 2 h up to 48 h, indicates that longer acid treatments lead to more crystalline materials in which a greater amount of amorphous regions have been digested along the fibrils. In contrast, subsequent neutralization or dialysis of the samples did not lead to significant changes in the BCNW length.

It can also be deduced from the present results that, for long hydrolysis times, the fact that the native BC used is freeze-dried (vs. dried) does not lead to a morphology of more aggregated nanowhiskers and no significant effect on the nanowhiskers' dimensions is observed.

The aspect ratio of the BCNWs is an important parameter which conditions the reinforcing effect of the nanowhiskers when incorporated into a polymeric matrix. Materials with aspect ratios higher than 30, such as tunicin whiskers ($L/D \sim 67$) have been reported to provide a considerably higher reinforcement effect as compared to nanofillers having lower aspect ratios, such as Avicel whiskers ($L/D \sim 10$) (Azizi Samir et al., 2005). Nevertheless, it has also been reported that for aspect ratios larger than 100, the Young's modulus reaches a plateau corresponding to the maximum point of reinforcement (Eichhorn et al., 2010). As shown in Table 2, the greatest

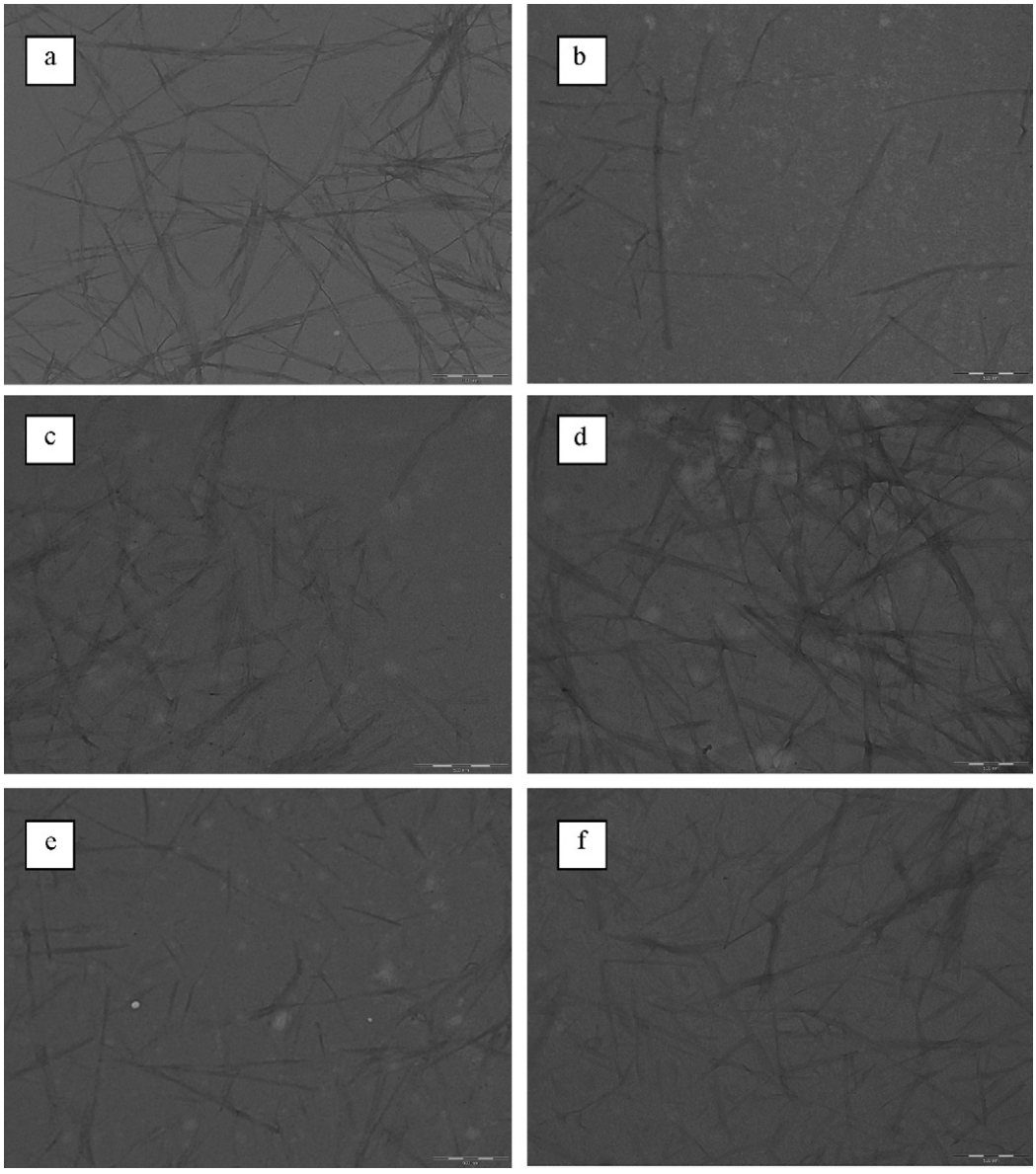

Fig. 1. TEM micrographs of: (a) BCNW hydrolyzed for 2 h; (b) same as (a) after neutralizing with NaOH; (c) BCNW hydrolyzed for 48 h; (d) same as (c) after neutralizing with NaOH; (e) BCNW hydrolyzed for 69 h and neutralized with NaOH; (f) same as (e) after dialysis. Scale markers correspond to 500 nm.

Table 2
Dimensions of BCNW prepared by different acid hydrolysis conditions. Measurements obtained from TEM micrographs.

Sample	Cross-section (nm)	Length (nm)	Aspect Ratio (*L*/*D*)
BCNW 2h	27.79[a] (8.71)	1449.51[a] (406.53)	52.16[a] (10.71)
BCNW 2hN	29.62[a] (10.37)	881.94[a] (543.89)	29.78[b] (16.80)
BCNW 48h	20.09[b] (5.45)	468.75[b] (250.57)	23.33[b] (6.28)
BCNW 48hN	18.49[b] (5.32)	567.11[b] (295.76)	30.67[b] (15.90)
BCNW 69hN	20.81[b] (7.66)	599.60[b] (325.26)	28.81[b] (16.38)
BCNW 69hND	19.34[b] (5.27)	470.29[b] (334.52)	24.32[b] (12.06)

Values between brackets correspond to standard deviations. The a and b letters correspond to the ANOVA statistical analysis and Tukey test of the data that indicate that with a 95% confidence level, the values are significantly different.

aspect ratio corresponds to a hydrolysis time of 2 h, decreasing down to 20–30 for longer treatments and, thus, from an aspect ratio viewpoint it would seem that the shortest treatment would provide the most adequate BCNWs. However, in order to determine the optimum acid hydrolysis conditions, other factors such as the crystallinity index and the thermal stability of the obtained BCNWs, which will be discussed in later sections of this study, have to be also taken into account. The effect of increasing the hydrolysis time and of applying post-treatments like neutralization or dialysis after the acid hydrolysis over the crystallinity and thermal stability of the material is discussed in what follows.

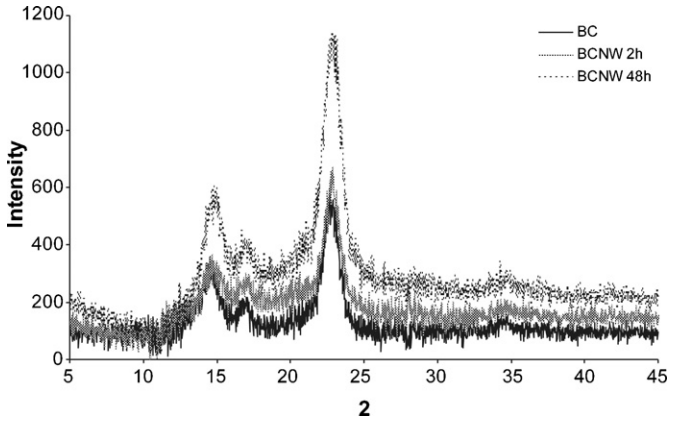

Fig. 2. X-ray diffraction patterns of native bacterial cellulose (BC) and the obtained nanowhiskers (BCNW) after 2 h and 48 h of sulfuric acid hydrolysis.

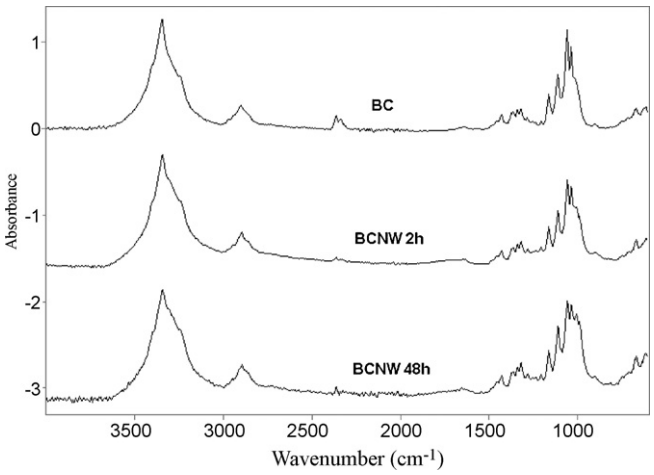

Fig. 3. FTIR spectra of native bacterial cellulose (BC) and the obtained nanowhiskers (BCNW) after 2 h and 48 h of sulfuric acid hydrolysis.

3.2. Effect of acid hydrolysis time on the crystallinity and thermal stability of BCNWs

Sulfuric acid treatment of cellulosic materials has been widely used as a way to extract cellulose nanocrystals since it causes a preferential hydrolysis of disordered or amorphous regions of the material through a surface reaction process, whereas crystalline domains have a higher resistance to acid attack and remain intact under controlled conditions (De Souza Lima & Borsali, 2004). By varying the hydrolysis time, it is possible to modify the crystallinity index of the obtained material and produce whiskers with controlled aspect ratio (Beck-Candanedo, Roman, & Gray, 2005; Yun, Cho, & Jin, 2010). Too short hydrolysis reactions will not lead to significant changes in the crystallinity of the material since, in the case of BC, the structure of ribbon-shaped crystalline bundles is not easily penetrated by acid molecules (Zhao et al., 2007). On the other hand, a too long reaction time will lead to the digestion of the crystalline domains of BC, thus leading to a decrease in the crystallinity. Previous studies revealed that in the case of BC, longer hydrolysis times than those used for plant cellulose are required to obtain a material with a crystallinity index of 82.2% (Martínez-Sanz et al., 2010).

In this work, in order to estimate the effect of hydrolysis time on the crystallinity and thermostability of the obtained BCNWs, two different acid digestion times were studied: a relatively short reaction time of 2 h and a longer hydrolysis of 48 h. Table 3 gathers the crystallinity index and the crystallite sizes calculated from the 1 0 1, 1 0 $\bar{1}$ and 0 0 2 lattice planes of cellulose I for untreated BC and its corresponding nanowhiskers obtained after different hydrolysis times, calculated from the X-ray diffraction patterns shown in Fig. 2.

As shown in Fig. 2, the untreated BC and the obtained BCNWs show three major diffraction peaks at 14.5°, 16.4° and 22.5° 2θ. According to the literature (Moharram & Mahmoud, 2007), these diffraction peaks are ascribed to the crystallographic planes 1 0 1, 1 0 $\bar{1}$ and 0 0 2 from the cellulose I allomorph. The crystallinity index calculated for the untreated BC (79.06%) is similar to that reported in a previous study for BC obtained through the same process (73.1%) (Martínez-Sanz et al., 2010). After 2 h of acid hydrolysis the crystallinity index of the material is not significantly altered, indicating that 2 h is not enough time for the acid to digest the amorphous material. Although 2 h is an hydrolysis time typically used for the extraction of cellulose nanowhiskers from vegetal resources, the structure of bacterial cellulose, consisting in the association of nanofibrils into highly crystalline ribbon-shaped bundles, hinders the penetration of the acid into those bundles and thus, it has been reported that acid hydrolysis of bacterial cellulose may be slower than for cotton or wood (Yun et al., 2010). On the other hand, the crystallinity increases up to 90.31% after 48 h. The increase of 11.25% in the crystallinity index of the material is similar to the increase of ~9% previously reported for a hydrolysis time of five days (Martínez-Sanz et al., 2010). This indicates that long hydrolysis times are required in order to cause a significant increase on the crystallinity of bacterial cellulose. Additionally, as showed in Table 3, an increase of the crystallite size in the 1 0 1 crystalline plane with increasing the hydrolysis time is observed, probably indicating that the smaller or more defective crystals are being digested by means of the sulfuric acid treatment as would be expected.

In addition to XRD, FT-IR analyses of native BC and the obtained nanowhiskers were developed in order to study the structural and chemical effects of the sulfuric acid hydrolysis. A qualitative analysis of the FT-IR spectra shown in Fig. 3 confirms that after the 2 h of hydrolysis, the crystallinity of the material has been slightly increased since sharpening of characteristic cellulose bands is observed. According to the literature (Oh, Yoo, Shin, & Seo, 2005), the bands at 4000–2995 cm^{-1}, 2900 cm^{-1}, 1430 cm^{-1}, 1375 cm^{-1} and 900 cm^{-1} are known to be especially sensitive to the cellulose molecular order. Broadening of these bands is related to greater disorder in the polysaccharide phase morphology and thus, the shape of these bands can be related to the amount of crystalline vs. amorphous fractions in cellulose. Even though the crystallinity index estimated by means of XRD is not significantly altered by the acid treatment, in the case of the 2 h hydrolysis a slight sharpening is observed especially in the broad band between 3000 and 3700 cm^{-1}, and the bands at 2900 cm^{-1} and 1430 cm^{-1}, corresponding to OH stretching intramolecular hydrogen bonds, CH stretching and CH_2 symmetric bending, respectively. In the case of the 48 h hydrolyzed sample, sharpening of the previous bands is more intense, hence confirming the increase in the crystallinity index of the material. A shoulder appearing at 1720 cm^{-1}, which is ascribed to carbonyl groups and has been previously related to the thermo-oxidative degradation of cellulose (Araki et al., 1998; Jain, Lal, & Bhatnagar, 1987) and that can be detected in cellulose with a degree of oxidation as low as 0.12 (Kim, Kuga, Wada, Okano, & Kondo, 2000), confirms that degradation is occurring to some extent.

As reported in previous studies, treatment with sulfuric acid has an effect on the thermal stability of cellulose crystals (Araki et al., 1998; Martínez-Sanz et al., 2010; Rosa et al., 2010). This effect results in lower degradation temperature and in broadening of the degradation range. The longer the hydrolysis time the stronger the break down of the mats into nanowhiskers but the lower the ther-

Table 3
Crystallinity index (CI) and crystallite sizes ($D_{(101)}$, $D_{(1\bar{0}1)}$ and $D_{(102)}$) determined from the XRD patterns from native bacterial cellulose (BC) and the obtained nanowhiskers (BCNW) with different hydrolysis times.

Sample	CI (%)	$D_{(101)}$ (nm)	$D_{(1\bar{0}1)}$(nm)	$D_{(102)}$ (nm)
BC	79.06[a] (0.44)	0.84[a] (0.04)	1.74[a] (0.07)	1.06[a] (0.01)
BCNW 2h	76.64[a] (0.62)	1.16[b] (0.01)	1.74[a] (0.03)	1.17[b] (0.00)
BCNW 48h	90.31[b] (0.79)	1.37[c] (0.02)	1.50[a] (0.27)	1.04[a] (0.02)

Values between brackets correspond to standard deviations. The a and b letters correspond to the ANOVA statistical analysis and Tukey test of the data that indicate that with a 95% confidence level, the values are significantly different.

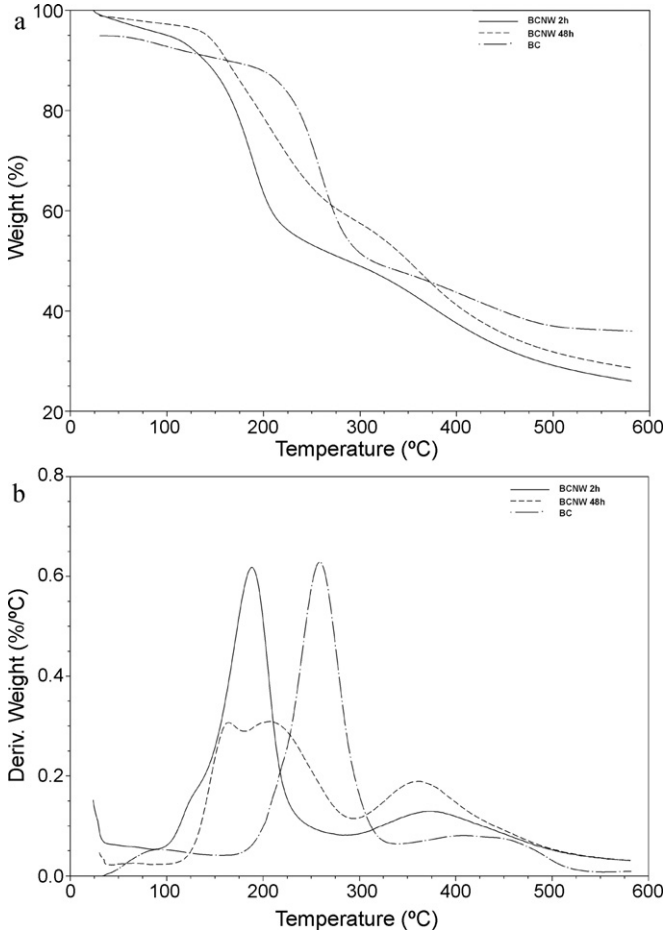

Fig. 4. TG (a) and DTG curves (b) of native BC, BCNW 2h and BCNW 48h.

mal stability attained due to increased sulfate incorporation into the BC.

Thermogravimetric analyses were carried out to confirm this effect on thermal stability of BCNW. Fig. 4 shows the TG and DTG curves of native BC and BCNW obtained after different hydrolysis times. It is observed that there is a significant change in the degradation profile of BC after applying acid hydrolysis. In all the samples, the mass loss below 100 °C is ascribed to water loss. The first degradation step, with an onset temperature of 175 °C, 92.26 °C and 98.23 °C for BC, BCNW 2h and BCNW 48h, respectively, corresponds to cellulose degradation processes such as depolymerisation, dehydration and decomposition of glycosyl units (Araki et al., 1998). In the case of the nanowhiskers obtained after 48 h of hydrolysis (BCNW 48 h), this degradation process is divided into two steps, as shown in the DTG curve. It has been previously suggested that degradation process of highly sulfated samples is best described in terms of two sub-processes (Araki et al., 1998; Julien, Chornet, & Overend, 1993). The first sub-process corresponds to the degradation of the more accessible regions, which are highly sulfated, and the second sub-process corresponds to the breakdown of the crystalline fraction which has not been attacked by sulfuric acid. In the case of the 2 h hydrolysis sample, the first sub-process is not observed in the DTG curve but a small shoulder appears instead, indicating that this hydrolysis time is not enough to yield a great amount of highly sulfated regions. Finally, the second degradation step, which takes place above 275 °C, corresponds to the oxidation and breakdown of the charred residue.

As listed in Table 4, there is a noticeable shift of the degradation to lower temperatures when applying the sulfuric acid treatment and degradation takes place over a broader temperature range when increasing the hydrolysis time. Nevertheless, there is no great difference in the degradation onset temperature between the 2 h and 48 h treated BCNW, which could be related to the fact that the pH of the material is very similar for both of them (see Table 1).

As a conclusion, a 2 h sulfuric acid hydrolysis treatment applied to BC led to materials with a crystallinity index similar to the native BC but with significantly lower thermal stability. On the other hand, when subjecting the material to a more extensive hydrolysis treatment, a significant increase in the crystallinity of the product was observed without further decreasing the degradation onset temperature as compared to the short hydrolysis time. The increase in the crystallites' size and the appearance of a shoulder in the FT-IR spectra corresponding to carbonyl groups, suggest that partial digestion of small or defective crystalline domains takes place with long hydrolysis times. In both cases, the obtained BCNW have a considerably lower thermal stability than that of BC. With the aim of increasing the thermal stability of the material, neutralization of BCNW suspensions obtained after hydrolysis was carried out.

3.3. *Effect of neutralization and dialysis on the crystallinity and thermal stability of BCNWs*

Previous studies pointed out that when neutralizing the acid sulfate groups of cellulose nanowhiskers suspensions with NaOH, the thermal stability of the material increased (Favier et al., 1995). BCNW suspensions of the previously analyzed samples were subjected to neutralization and the changes in the thermostability and crystallinity of the materials were studied. Furthermore, an additional sample was obtained after neutralizing a suspension of BCNWs subjected to 69 h of hydrolysis. Dialysis was subsequently applied to this sample to evaluate if additional improvements in thermal stability or crystallinity occurred.

Table 5 lists the crystallinity indexes of the different samples, obtained from the diffraction patterns shown in Fig. 5. It is observed that after neutralizing the material, a slight increase in the crystallinity index is observed for both the 2 h and 48 h hydrolyzed BCNWs, (cf. Table 3 vs. Table 5). Nevertheless, this crystallinity increase is significant only for the 48 h hydrolyzed sample (with a 95% confidence level). On the other hand, the crystallite sizes of the cellulose I in the 1 0 1 lattice plane are significantly decreased (with a 95% confidence level) after neutralizing the material, although these remain bigger for the samples subjected to longer hydrolysis times. This may indicate that the slight increase observed in the crystallinity of the material could be caused by the formation of new

Table 4
Onset temperature, degradation temperature of the first process (T_{D1}), degradation temperature of the second process (T_{D2}) and corresponding weight losses (WL_1 and WL_2) of native bacterial cellulose (BC) and the obtained nanowhiskers (BCNW) with different hydrolysis times.

Sample	Onset T (°C)	T_{D1} (°C)	WL_1 (%)	T_{D2} (°C)	WL_2 (%)
BC	169.1	258.7	32.02	408.0	24.91
BCNW 2h	93.1	188.3	29.6	371.7	29.26
BCNW 48h	98.8	163.4	10.47	361.1	28.61
		206.8	12.98		

Table 5
Crystallinity index (CI) and crystallite sizes ($D_{(1\,0\,1)}$, $D_{(1\,\bar{0}\,1)}$ and $D_{(1\,0\,2)}$) determined from the XRD patterns from neutralized or neutralized/dialyzed BCNW obtained after different hydrolysis times.

Sample	CI (%)	$D_{(1\,0\,1)}$(nm)	$D_{(1\,\bar{0}\,1)}$(nm)	$D_{(1\,0\,2)}$ (nm)
BCNW 2hN	80.41[a] (2.56)	0.89[a] (0.03)	1.60[b] (0.05)	1.05[a] (0.00)
BCNW 48hN	95.30[b] (0.26)	1.04[b] (0.00)	1.63[b] (0.01)	1.15[c] (0.00)
BCNW 69hN	94.92[b] (0.25)	1.13[c] (0.01)	1.21[a] (0.11)	1.11[bc] (0.02)
BCNW 69hND	77.26[a] (2.03)	0.89[a] (0.02)	1.53[b] (0.02)	1.07[ab] (0.00)

Values between brackets correspond to standard deviations. The a, b and c letters correspond to the ANOVA statistical analysis and Tukey test of the data that indicate that with a 95% confidence level, the values are significantly different.

but smaller crystals as a consequence of the NaOH addition. It was also confirmed that the observed increase in crystallinity was not due to the formation of sodium sulfate. This salt can be produced by the reaction of free sulfate groups with sodium and it is usually found in the material when the neutralized suspension consists in the turbid supernatant obtained after several centrifugation cycles. In this case, the material consists of the centrifugation precipitate, so that if any of this salt was formed, it was removed with the liquid supernatant. Furthermore, the XRD patterns of the materials do not show any evidence that the salt is present in the material. Therefore, the process of adding sodium hydroxide until neutral pH may have a nucleating effect on samples which have been subjected to an extensive hydrolysis time, leading to the formation of new cellulose I crystals and, thus, increasing the overall crystallinity index of the material. To the best of our knowledge, this effect has not been previously reported.

It is also deduced from the result that increasing the hydrolysis time from 48 h up to 69 h does not significantly alter the amount of crystalline fraction in the material but the crystallite size from the 1 0 1 plane is still increased.

As shown in Table 5, when subjecting the 69 h hydrolyzed sample to dialysis, the crystallinity index is significantly diminished. In addition, the crystallite sizes from the 1 0 1 and 1 0 $\bar{1}$ lattice planes are significantly altered, getting values close to the ones corresponding to the BCNW 2hN sample. It might be possible that when incorporating an additional step of dialysis, the smallest cellulose I crystals, i.e. those crystals which suffered a more intense acid digestion, are able to pass through the membrane pores and just the bigger ones remain inside the membrane. If this is the case, it would not be desirable to add a dialysis step to the BCNWs' extraction process.

Taking into account the crystallinity indexes, the optimum hydrolysis conditions would be 48 h and subsequent neutralization of the product.

The FT-IR spectra of the BCNW before and after being neutralized are shown in Fig. 6. After neutralization of the material, the shoulder present at 1730 cm^{-1} completely disappears indicating that the sulfate groups have been properly neutralized. Apart from a slight increase in the intensity of cellulose characteristic bands such as the ones appearing at 2896, 1430 and 1163 cm^{-1} (corresponding to CH stretching, CH_2 symmetric bending at C-6 and COC bending at β-glycosidic bond, respectively), there are no other significant changes in the spectra after neutralizing, confirming that no chemical modification of the material has taken place.

After dialysis the opposite effect is observed, i.e. a decrease in the intensity of most cellulose characteristic bands is observed, which is in accordance with the remarkable decrease in crystallinity observed by means of XRD.

Regarding the thermal stability of the material, neutralization clearly shifts the degradation of the material towards higher tem-

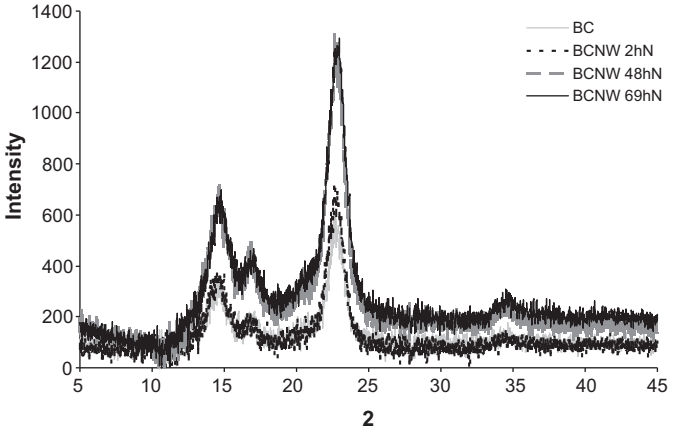

Fig. 5. X-ray diffraction patterns of native bacterial cellulose (BC) and the obtained nanowhiskers (BCNW) after 2 h and 48 h of sulfuric acid hydrolysis.

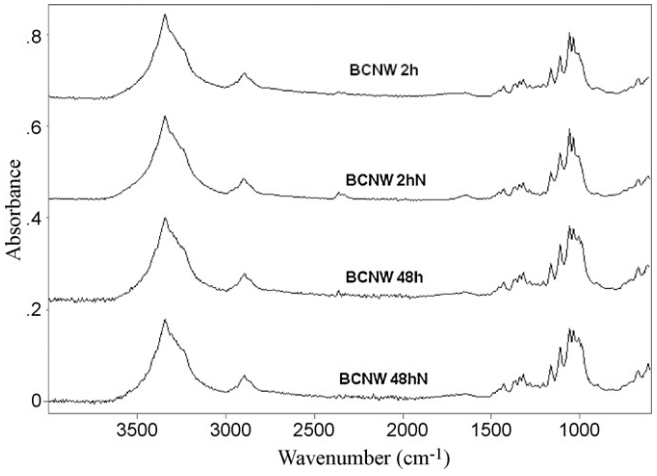

Fig. 6. FTIR spectra of BCNW before and after neutralization.

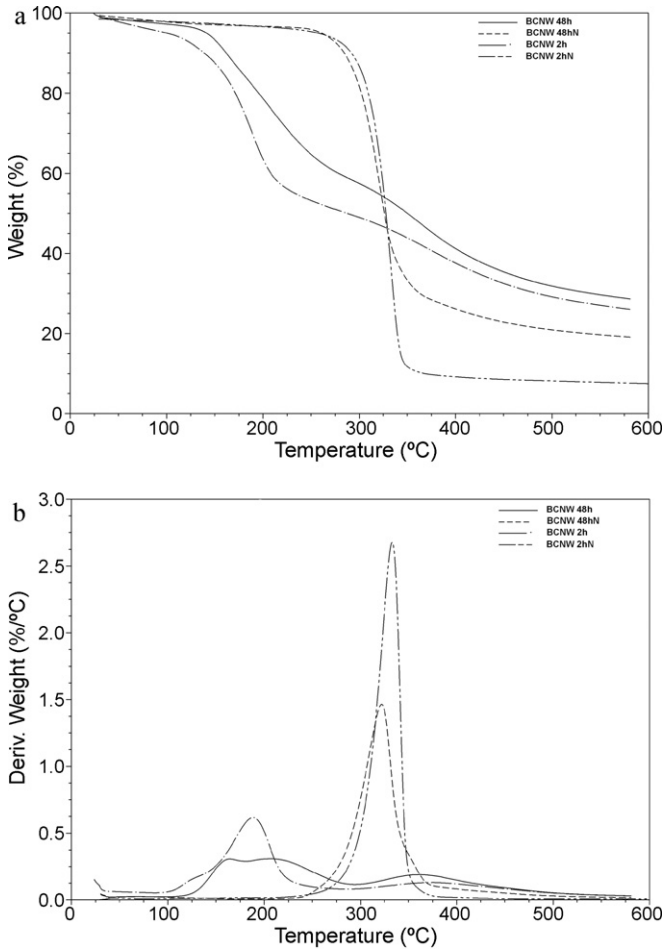

Fig. 7. TG (a) and DTG curves (b) of BCNW 2h and BCNW 48h before and after neutralization.

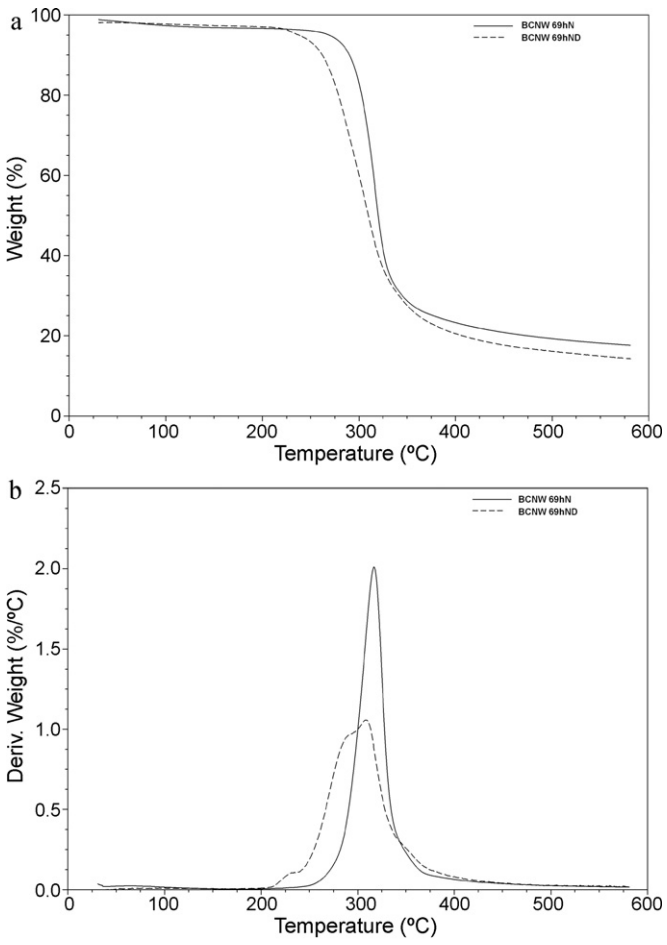

Fig. 8. TG (a) and DTG curves (b) of neutralized BCNW 69h before and after dialysis.

peratures, also leading to the occurrence of the process within a narrower temperature range due to the higher pH of the material, as shown in Fig. 7. It is also observed that neutralized samples present a different degradation profile consisting in just one pyrolysis process. This effect has been previously observed for cellulose nanocrystals (CNC) (Julien et al., 1993). It was suggested that the first degradation process is related to the primary pyrolysis of CNC catalyzed by sulfate groups present in the surface of the material. When comparing the neutralized samples, it is also observed that when the hydrolysis time applied is longer, the degradation range gets wider, which is probably due to the particles' size heterogeneity caused by the longer acid treatment, which is able to yield some smaller particles.

Since sulfuric acid is a well-known dehydration catalyst, it facilitates the formation of char residue (Kim et al., 2000). Indeed, it can be observed that in the case of BCNWs with sulfate groups the amount of char residue at 600 °C is remarkably larger than for neutralized samples.

On the other hand, if comparing the TGA profiles of the neutralized samples with the BC, it can be seen that the thermal stability of the neutralized BCNWs is even higher than the one of the native BC, displaying higher degradation temperatures and narrower degradation profiles but lower char residues at 600 °C. This highlights the convenience of neutralizing the samples after a long hydrolysis treatment since, in that way, amorphous domains which are thermally weaker than the crystalline fractions are digested by the sulfuric acid and besides, sulfate groups introduced during hydrolysis are removed. As a result, a material with a greater thermal stability than pure BC is obtained.

Table 6
Onset temperature, degradation temperature of the first process (T_{D1}), degradation temperature of the second process (T_{D2}) and corresponding weight losses (WL_1 and WL_2) of neutralized or neutralized/dialyzed BCNW obtained after different hydrolysis times.

Sample	Onset T (°C)	T_{D1} (°C)	WL_1 (%)
BC 2hN	212.3	332.5	61.98
BCNW 48hN	226.7	322.0	43.82
BCNW 69hN	222.5	317.0	43.77
BCNW 69hND	204.0	308.5	48.7

The effect of the dialysis process on the thermal stability is shown in Fig. 8. After dialysis the degradation is shifted to a lower onset temperature and takes place over a wider temperature range. The DTG curve presents three degradation steps, similar as it was observed for the sulfated samples. Nevertheless, the weight loss produced during the first and third steps is not significant if compared with the one produced during the second degradation step and, thus, only one degradation step was considered for the results listed in Table 6. This effect is probably due to the more acidic pH of the material after the dialysis process (see Table 1), i.e. some of the Na^+ alkaline ions which were interacting with the sulfate groups present in the surface of the material may have passed through the dialysis membrane and therefore there is a small fraction of cellulose chains which start degrading at a lower temperature due to the catalytic action of the sulfate groups. Again, these results confirm that a dialysis step is

unsuitable for the extraction of BCNWs using the method presented here.

According to the results, the most thermally stable material is obtained when neutralizing the BCNWs without applying any further dialysis step.

4. Conclusions

This study intends to characterize the effect of various acid hydrolysis treatments in the morphology and thermal properties of BCNWs. Sulfuric acid treatment of bacterial cellulose yields cellulose nanowhiskers, with a nanofibrillar crystalline morphology consisting of the cellulose I crystal allomorph and with a high aspect ratio ranging from 20 to 50 depending on the applied hydrolysis conditions. The length of BCNWs has been found to decrease when applying a relatively long hydrolysis time compared to a 2 h treatment. Furthermore, it was observed that short times (~2 h) which are typically applied for plant cellulose whiskers' extraction, are not enough to digest the amorphous domains of the material and, thus, the crystallinity index is not significantly altered. Nevertheless, when applying longer hydrolysis treatments, such as 48 h, a considerable increase in the crystallinity index of the BCNWs is observed, suggesting that the acid has been able to attack the amorphous regions which are holding together the nanofibrils' bundles. In general, the sulfuric acid treatment, even at short hydrolysis times, leads to a remarkable decrease in the thermostability of the cellulosic materials.

Neutralization after hydrolysis gives rise to an important increase in the thermostability of BCNWs, obtaining a material which can be processed at temperatures above 200 °C. It also causes a small increase in the crystallinity index, without modifying its morphology or chemical structure. On the other hand, an additional step of dialysis after neutralization does not lead to any additional improvement but to a decrease in the crystallinity and thermal stability of the material. From the results, it can be stated that long sulfuric acid hydrolysis times such as 48 h, followed by neutralization allow the production of highly crystalline BCNWs, which have a high aspect ratio and a thermal stability high enough to use them as reinforcing agent in melt-compoundable nanocomposites.

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