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Corresponding Author: Dr. Sergio Rosselli,

Corresponding Author's Institution:

First Author: Aurora Modica

Order of Authors: Aurora Modica; Sergio Rosselli; Giorgia Catinella; Francesco Sottile; Anna C Catania; Giuseppe Cavallaro; Giuseppe Lazzara; Luigi Botta; Alberto Spinella; Maurizio Bruno

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Suggested Reviewers: Giuliana Gorrasi
Department of Industrial Engineering, University of Salerno
ggorrasi@unisa.it
expert on biopolymers

Rawil Fahkrullin
Institute of Fundamental Biology, Kazan State University
kazanbio@gmail.com
expert on nanomaterials and physical chemical characterization

Magdalena Broda
Department of Wood Science, Poznań University of Life Sciences
magdalena.broda@up.poznan.pl
expert on lignocellulosic materials

Highlights

- Estimation of lignin/holocellulose ratio using ^{13}C CPMAS NMR spectroscopy
- Cellulose extraction from almond shells
- Employment of almond waste for cardboard obtainment

Solid state ^{13}C-NMR studies of the shells of *Prunus dulcis* and their derived materials

Aurora Modica[a], Sergio Rosselli[b,g*], Giorgia Catinella[a], Francesco Sottile[c,g], C. Anna Catania[c,g], Giuseppe Cavallaro[d], Giuseppe Lazzara[d,g], Luigi Botta[e,g], Alberto Spinella[f], Maurizio Bruno[a,g]

[a] *Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche (STEBICEF), Università degli Studi di Palermo, Viale delle Scienze,Ed. 16, Ed.4, I-90128 Palermo, Italy*

[b] *Dipartimento di Scienze Agrarie, Alimentari e Forestali (SAAF), Università degli Studi di Palermo, Viale delle Scienze, I-90128 Palermo, Italy*

[c] *Dipartimento di Architettura, Università degli Studi di Palermo, Viale delle Scienze, I-90128 Palermo, Italy*

[d] *Dipartimento di Chimica e Fisica, Università degli Studi di Palermo, Ed. 17, Viale delle Scienze, I-90128 Palermo, Italy*

[e] *Dipartimento di Ingegneria, Viale delle Scienze, Ed. 8, I-90128 Palermo, Italy*

[f] *Advanced Technologies Network Center, Viale delle Scienze, Ed. 18, I-90128 Palermo, Italy*

[g] *Centro Interdipartimentale di Ricerca "Riutilizzo bio-based degli scarti da matrici agroalimentari" (RIVIVE), Università degli Sudi di Palermo*

* Corresponding author. E-mail: sergio.rosselli@unipa.it

Abstract

Lignocellulosic fibres and microcrystalline cellulose have been obtained by simple alkaline treatment from softwood almond (*Prunus dulcis* Miller (D.A.) Webb.) shells constituting a large agro industrial waste in our territory. The materials have been characterized for the relative composition of lignin and holocellulose, applying a ^{13}C CPMAS NMR spectroscopy methodology. The fibrous material allowed the manufacture of a handmade cardboard obtaining an ecological material suitable for packaging purposes. These fibers along with the obtained microcrystalline cellulose can represent a new fate for the almond shells that are mainly used as firewood.

Keywords: Almond shell; Cellulose; Fibers; Handmade Cardboard; Microcellulose; ^{13}C CP MAS NMR

1. Introduction

The almond (*Prunus dulcis* Miller (D.A.) Webb.) cultivation in Sicily is very traditional and historically rooted (Barbera, Monte, & Sottile, 2005; Sottile, Barone, Barbera, & Palasciano, 2015; Distefano et al., 2013). In Italy, the almond production is based on hard or semi-hard shell cultivars, with rarely shelled rates exceeding 38-40% in the best growing conditions. On the contrary, the Californian and Australian productions are based on soft shell cultivars, sometimes papery, with shelling rate that can reach even 75-80%. The different variety type also determines a series of differences in terms of analytical composition of the seeds (Drogoudi et al., 2012; Yada, Huang, & Lapsley, 2013) as well as the consistency of the shell is due to a variable concentration of the main components and to a modification of its porosity and structure (Ledbetter, 2008).

Currently, many academic and industrial studies are focused on biodegradable materials in order to replace fossil oil based products, decrease plastic pollution and more broadly safeguard the environment and pay close attention to a sustainable development. (Maaloul, Arfi, Rendueles, Ghorbal, & Diaz, 2017; Nechyporchuk, Belgacem, & Bras, 2016; Lins, Bugatti, Livi, & Gorrasi, 2018; Zhang, Xiao, Chen, Wei, Liu, 2020). The use of agricultural by-products coming from nuts and almonds has been deeply studied (Demirbaş, 2002; Pirayesh & Khazaeian, 2012): besides of the pruning and other residuals coming from the fields, shells and hulls are the main by-products of the processing of almonds. Hulls are usually utilized in the animal feeding, shells highlight a high calorific power, similar to that of other wood matrices with reduced emissions of chemicals or residues in the ashes (Atkas et al., 2015; Chen et al., 2010).

More recently, European legislation has broadly proposed the use of biomass for sustainable energy production and, among these, a great deal of interest has been developed for residues from agricultural production (EU Commission, 2013). Italy produces about 40.000 tons of almond shell (AS) per year from the ordinary processing of the product. AS has not a real commercial use; it finds application only in a domestic or semi-industrial level for house heating or oven cooking and the effects of combustion has been widely studied (González et al., 2005; Martin-Lara, Ortuno, & Conesa, 2018). Also in this case, the AS showed a high quality not very different from that of pruning residues (González et al., 2005).

Increasingly, feedstock and agriculture wastes are interesting renewable natural resources to take advantage of the natural biodegradable polymers, lignin, hemicellulose and cellulose that are the main components of the AS (Esfahlan, Jamei, & Esfahlan, 2010). Some studies have evaluated the possibility of using AS as feedstock in order to obtain derivatives through specific applications (Essabir et al., 2013), such as particleboard (Gürü, Tekeli, & Bilici, 2006), bioethanol (Kacem et al., 2016), or as a functional component of novel food (Kacem et al., 2017). All these applications usually need minor or major modifications able to strongly modify the texture and the structure of the feedstock.

Cellulose and the other lignocellulosic materials are arisen in the cell walls of all plants (Stelte & Sanadi, 2009) and are extracted with different methods (Radotić, & Mićić, 2016) from the common sources as hardwood, softwood and cotton (Nechyporchuk et al., 2016) and recently also from vegetable pomace (pulp, peel, seeds, and stem) (Szymańska-Chargot, Chylińska, Gdula, Kozioł, & Zdunek, 2017). In particular, cellulose, a linear macromolecular chain of 1-4 linked β-glucopiranose units, is the most abundant polymer in nature, indeed the production of cellulose by photosynthesis is estimated to be 10^{11}–10^{12} t/year (Klemm, Schmauder, & Heinze, 2004; Zugenmaier, 2008).

Lignin is a highly complex polymer mainly biosynthesized from three monomers: 4-coumaryl, coniferyl and sinapyl alcohols, representing the p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units respectively. The composition of lignin is influenced by the origin and growth conditions of the plant and can even differ among cell types within the

same plant (Moura, Bonine, Viana, Dornelas, & Mazzafera, 2010). In general, softwood (gymnosperm) lignins are mainly composed of G-units with minor amounts of H-units, whereas hardwood (angiosperm - dicots) lignins are composed of G- and S-units in approximately equal ratios (Vanholme, Demedts, Morreel, Ralph, & Boerjan, 2010).
Viability of cellulose isn't depended only on its characteristics of biodegradability, renewability, strength and stiffness, but also on the different dimensions in which can be extracted and so on own versatility as a commercial product (Afra, Yousefi, Hadilam, & Nishino, 2013; Mokhothu, & John, 2015). Cellulose can be distinguished in elementary fibrils, fibrillar cellulose (FC) and crystalline cellulose (CC) (Osong, Norgren, & Engstrand, 2015).
Considering the recent attention toward cellulose-based fibers reinforcements as renewable alternative to their synthetic equivalents (Scaffaro, Botta, Lopresti, Maio, & Sutera, 2017), its use in pharmaceuticals, (Thoorens, Krier, Leclercq, Carlin, & Evrard, 2014) and food industry (Nsor-Atindana et al., 2017) we explored the possibility of obtaining cellulose from the agricultural waste shell almond as added value product.

2. Experimental

2.1. Plant material

Almond shells were supplied by a local farmer and originated from almond trees (*P. dulcis*) cultivated in Agrigento (Sicily, Italy) belonging to the variety Casteltermini.

2.2. Treatment of almond shells

Before the extraction of cellulose, dried almond shells were milled. The crushed material (400 g) were treated with 200 mL of a 7.5 wt% NaOH solution under reflux for 24h to remove hemicelluloses, starch, pectin and part of lignin. The shells and the clear fibres were filtrated and washed with distill water several times to decrease pH until 7. An aliquot (30 g) of the obtained fibers (**CF**) were treated with 50 mL of a 2.5 wt% NaClO solution at 60-70 °C for 1h, filtered, washed with distill water and air dried and then used for fabrication of paper. The remaining material (200 g) was treated again with 100 mL of a 7.5 wt% NaOH solution under reflux for 24h twice, filtered, washed until neutrality and bleached with a sodium hypochlorite solution 2.5 wt% at 70 °C for 1h under mechanical stirring. After these treatments a light powder (50 g) and shell fragments have been recovered.

2.3. ^{13}C CPMAS NMR

All ^{13}C CPMAS NMR spectra were acquired under the same instrumental parameters. All samples (~50mg) were compressed in 4 mm zirconia rotor with KEL-F caps. ^{13}C CPMAS NMR spectra were obtained at room temperature using a Bruker Avance II 400 MHz (9.4 T) spectrometer operating at 100.63 MHz for the ^{13}C nucleus with MAS rate of 13 kHz, a 90° pulse on the ^{1}H of 4.5 μs, a contact time of 1.5 ms, a repetition delay of 3 s and 4096 scans. The optimization of the Hartmann–Hahn condition was obtained by an adamantane sample standard. This compound was used also as external chemical shift reference.
The VCT (variable contact time) experiments were performed using the parameters described in the previous section The contact times used were 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0, 1.2, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, and 7.0 ms. A line broadening of 75 Hz was applied in all the free induction decays transformations. The integral of the signals was obtained through the TOPSPIN 2.0 available spectrometer software.

2.4. Thermogravimetry

Thermogravimetric (TG) analyses were carried out through a Q5000 IR apparatus (TA Instruments) under nitrogen atmosphere (gas flows of 25 and 10 cm^3 min^{-1} were employed for the sample and the balance, respectively). The experiments were carried out by heating the sample (ca. 5 mg) from room temperature to 600 °C. The heating rate (β) was set at 20 °C min^{-1}. According to literature (Blanco, Abate, Bottino, & Bottino, 2014), the temperature calibration was conducted by using the Curie temperatures of proper standards (nickel, cobalt, and their alloys).

2.5. Scanning Electron Microscopy (SEM)

The morphology of cellulose samples was analyzed by scanning electron microscopy (SEM; Quanta 200 ESEM, FEI, Hillsboro, OR, USA). In particular, the cellulose powder was directly glued onto a sample holder. All the samples were sputter coated with a thin layer of gold under argon atmosphere for 90 s (Scancoat Six Edwards, Crawley, UK) in order to avoid electrostatic charging under the electron beam. SEM micrographs were taken in secondary electron imaging mode by using an accelerating voltage of 15.0 kV, spot size 3.5 at a working distance of about 10 mm.

2.6. Paper making

In order to obtain a paper sheet from almond shells fibres, a suspension of cellulosic fibres (**CF**) was prepared. Fibres were saturated with water and screened with a wire mesh made from synthetic material, non-corroding and inert.
A thin layer of interwoven fibres was formed. The mesh was vertically dipped into the water suspension with fibres, and then lifted out horizontally, forming a uniform sheet. The open area of the frame ruled the size of the cardboard. The sheet was then pressed to remove the excess of water, and left to dry at open air.
A sheet of paper composed only of cellulosic fibres is water absorbent, so water-based inks or other aqueous medium would penetrate and spread in it. Impregnation of the paper with various substances that retard such wetting and penetration is called sizing. In this case the paper sheet was sized by impregnation with rise starch glue.
The paper sample obtained with this method is 100 mm x 30 mm and it has a thickness of 1.407 mm, measured using a Mitutoyo Digimatic Micrometer, IP65, 0-1".
A pH of 7.0 was measured with a HI-1413 Glass Bodied Flat pH Electrode (Hanna) on the paper surface.

2.7. Mechanical properties

Tensile properties on paper samples were determined by means of DMA Q800 instrument (TA Instruments). Tensile tests were performed on rectangular paper samples (10 mm × 3 mm) under a stress ramp of 1 MPa min^{-1} at 25.0 ± 0.5 °C.

3. Result and discussion

The characterization of the lignocellulosic material obtained from almond shell was recently reported (Li, Liu, Hao, & Wang, 2018). The relative amounts of cellulose, hemicellulose and lignin have been estimated to be 38.47%, 28.82% and 29.54, respectively. This result seems to be in agreement with previously investigations on the composition of lignocellulose from almond shells (Saura-Calixto, Cañellas, & Garcia-Raso, 1983; Martínez et al., 1997). The aim of this work is to investigate the possibility of using the agricultural waste of AS as source of cellulose. Our starting material is peculiar, in fact the cultivar we examined is a *P. dulcis* species that produces a softer shell with a stratified

architecture (Fig. 1) and with presumably a lesser extent of the lignin component in the lignocellulosic material. In order to have a rapid quantification of the ratio of lignin/holocellulose of our materials, we decide to base our study on solid state NMR spectroscopy. The ^{13}C CP MAS NMR spectrum of this material is reported in Fig. 2 together with a SEM micrography that shows the great thickness of the lignified secondary walls of the cells composing the shell. In this spectrum, the signals at 173 ppm (carbonyl) and at 21 ppm (methyl) are relative of acetyl group occurring in the monosaccharides of hemicellulose disclosing a high grade of acetylation inducing an increasing of flexibility and water resistance of the fibers.

Fig. 1. *P. dulcis* soft-shells.

Furthermore, the signal at 153 ppm is correlated to aromatic carbon atoms carrying methoxy group (OCH_3) such as in coniferyl and sinapyl alcohol, monomeric units of the lignin. Also the signal at 56 ppm is relative at the methylic carbon atom of the methoxy group. The signals relative to anomeric carbons of carbohydrates of the cellulose and hemicellulose, as well as carbon atoms of syringyl moiety of lignin, occurring at 105 ppm. The rest of the signals (89, 83, 75, 72, 63 ppm) are representative of the remaining monosaccharide carbons. Generally, no reliable information of the relative abundance of lignin and cellulosic polymers can be drawn by this spectrum. In fact, the signals of a ^{13}C CPMAS NMR spectrum are generated by the magnetization transfer from the abundant spin ^{1}H to the rare spin ^{13}C nuclei. This transfer takes place in a time (tcp) which is optimal and different for each signal present in the spectrum due to the difference in proton density surrounding the various ^{13}C nuclei and the different mobility of the molecules. This implies that generally the integrals of a CPMAS NMR spectrum provide semiquantitative information that is affected by error. To overcome this problem, variable contact time (VCT) experiments can be performed and, according to the method reported by Haw, Maciel, & Schroeder (1984), it is possible to calculate the percentage of lignin in a lignocellulosic sample as long as the well-known model of the conifer lignin is used with the empirical formulas of $C_9H_{7.15}O_2(H_2O)_{0.4}(OCH_3)_{0.92}$ for repeating units. Applying this method, a composition of 22% (wt) of lignin and of 78% for the holocellulose has been found for our sample of *P. dulcis* shells.

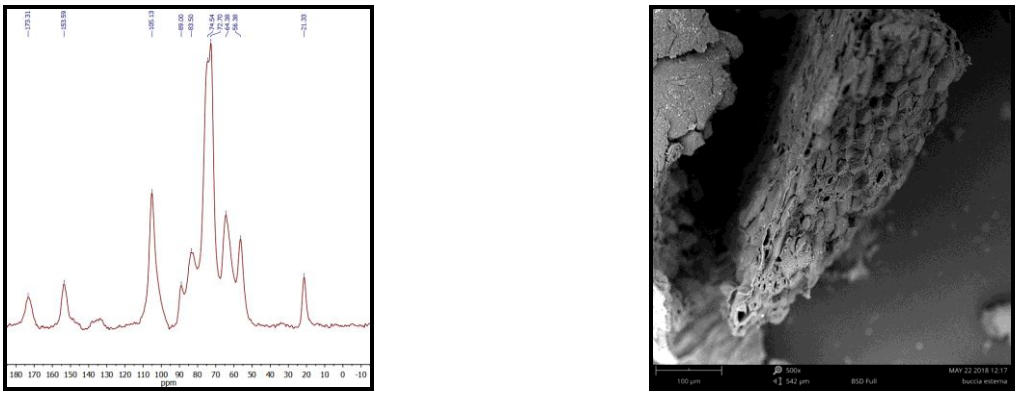

Fig. 2. ^{13}C CPMAS NMR (400 MHz) and SEM of powdered *P. dulcis* shells.

The evaluation of the acid insoluble fraction of lignin, using an improved method (Ioelovich, 2015), gave a very different result. An estimation of 41.5 % of Klason lignin has been found. So we argued that the composition of lignin of our sample should be very different from the conifer lignin. Since the CPMAS NMR is a very rapid and not manipulative method, we decided to use this technique in order to analyse lignin and cellulose mixtures, with a known composition, and comparing them with our samples. To do this, we needed to use the almond lignin of our *P. dulcis* shells. The lignin from almond shell was isolated by solvent extraction (Saha et al., 2019), obtaining a lignin with a structure more similar to the natural one without any structural modification, due to acid catalyzed reactions, in pure form. The material remaining from lignin extraction was refluxed with NaOCl (3% w/v) solution for 3 hours yielding pure cellulose. The ^{13}C CPMAS NMR spectra of the extracted lignin and cellulose are respectively showed in Fig. 3.

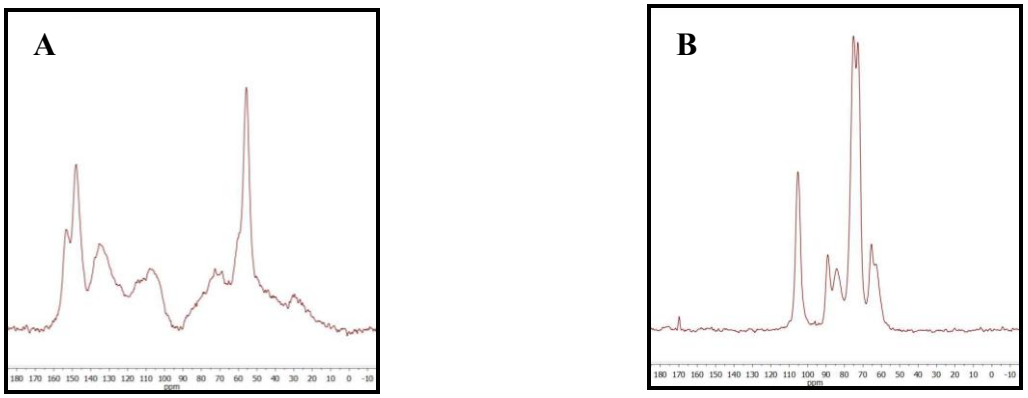

Fig. 3. ^{13}C CPMAS spectra of extracted lignin (**A**) and cellulose (**B**) from almond shell

As observed from Fig. 3, the signal occurring at 56 ppm, relative to metoxy groups of G and S units of lignin, has poor overlap with the signals at 63 ppm of cellulose. On the contrary, the signal of acetalic carbon occurring at 105 ppm of the cellulose has a certain overlap with aromatic signals of lignin. However, the latter should be proportional to the signal at 56 ppm. Starting from these pure materials we created a homogenous mixtures of cellulose with increasing amounts of lignin (10%-50%). The ^{13}C CPMAS spectra of these mixtures were registered (Fig. 4) with the same parameters and the ratio of the integral (I_{OMe}) of the signals in the range 58-51 ppm (normalized to 1) to the integral (I_{C1}) of the signals in the range 111-100 ppm was plotted *versus* lignin/holocellulose ratio, obtaining the graphic in Fig. 5.

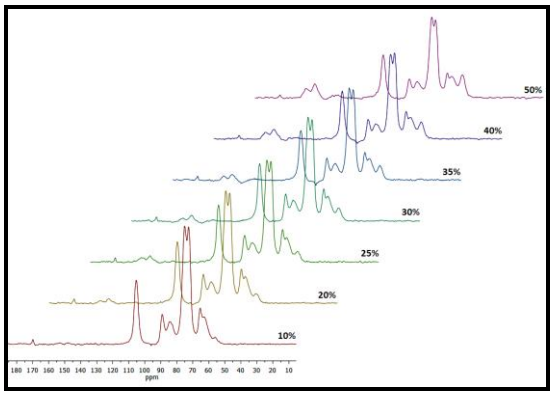

Fig. 4. ^{13}C CPMAS spectra of mixtures of cellulose with increasing lignin amounts

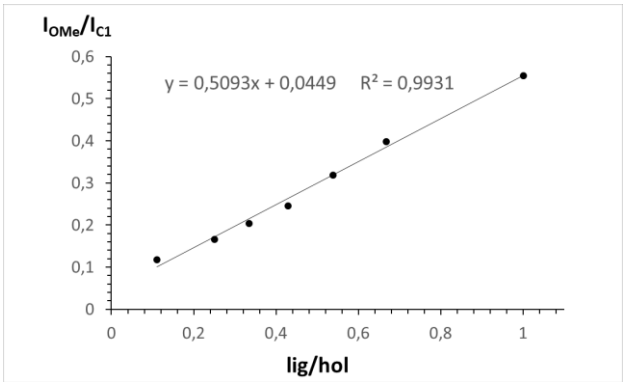

Fig. 5. Fitting of integral ratio I_{OMe}/I_{C1} *versus* lignin/holocellulose ratio

A good linear correlation was obtained from these data and this calibration curve allowed us to estimate the lignin/holocellulose ratio in the our cultivar of *P. dulcis* shells. A value of 0.81 was obtained corresponding to a 44.8% of lignin and 55.2% of holocellulose. This value for lignin is compatible with the 41.5% of Klason lignin found, considering that our sample could contain minor components not detected by CPMAS NMR.

Using the pure lignin and cellulose isolated, further investigations have been made on these samples by the thermogravimetric analysis (TGA) and data are shown in Fig. 6.

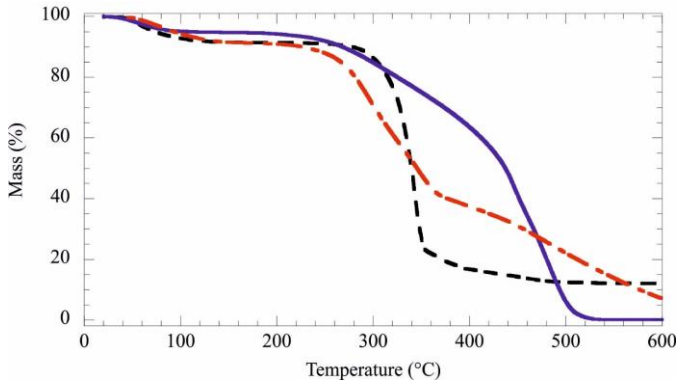

Fig. 6. Thermogravimetric curves for almond shell (red), cellulose (black) and lignin (purple).

As reported in literature, the mass loss at 200-400 °C is related to the depolymerization of hemicellulose and the cleavage of the glycosidic linkage of cellulose (Barneto, Carmona. Alfonso, & Serrano, 2010; Barneto, Vila, Ariza, & Vidal, 2011), while the mass loss at 420-600 °C can be ascribed to the decomposition of the lignin portion (Mannai, Ammar, Yanez, Elaloui, & Moussaoui, 2016).

The pure lignin showed a first mass loss (5.25%) occurring between room temperature to 150 °C (ML_{150}) attributed to the expulsion of water molecules physically adsorbed onto the samples (Cavallaro, Lazzara, Milioto, Parisi, & Ruisi, 2017; Lisuzzo, Cavallaro, Pasbakhsh, Milioto, & Lazzara, 2019) and two mass losses of 30.6% and 57.4% at temperature ranges of 200-400 °C and 420-600 °C, respectively. No residual mass at 600 °C was detected.

The pure cellulose showed a mass loss of 8.52% between 25-150 °C also, a great mass loss of 75.2% in the range of 200-400 °C, a 2.82% mass loss between 420-600 °C and a residual mass of 10.4% at 600 °C.

From these data it is evident that in the temperature range of carbohydrate polymers decomposition, a consistent grade of lignin depolymerization occurs, and a residue at 600 °C accounting of about 10% of the sample weight is present only for cellulose.

The TGA analysis of almond shell (Table 1) was elaborated on the base of these considerations: the mass loss of 53.4% was found at 200-400 °C temperature range for the almond shell, but the 30.6% of this loss is due to the quantity of lignin occurring in this sample, similarly the 75.2% of the mass loss is produced by the occurring amount of holocellulose. The same amounts of lignin and holocellulose are responsible for the mass loss (27.9%) occurring at the temperature range of 420-600 °C and in particular, the 57.4% of this loss is due to lignin and the 2.82% to holocellulose. Solving this equations system, a value of 46.06% and 52.27% of lignin and holocellulose were found. To the amount of holocellulose a 10.4% of the residual mass at 600°C (7.16%) must be added, giving a 53.24% of holocellulose. From this values a lignin/holocellulose ratio of 0.87 was calculated.

Table 1. Thermogravimetric parameters.

Sample	ML_{150} (wt%)	$ML_{200-400}$ (wt%)	$ML_{420-600}$ (wt%)	MR_{600} (wt%)
Almond Shell	8.46 ± 0.07	53.4 ± 2	27.9 ± 1.7	7.16 ± 0.07
Cellulose	8.52 ± 0.07	75.2 ± 2	2.82 ± 0.04	10.4 ± 1.1
Lignin	5.25 ± 0.05	30.6 ± 1.7	57.4 ± 2	0

This lignin/holocellulose ratio is close to the value obtained by CPMAS NMR confirming once again, that this method is quite reliable to detect the relative composition of lignocellulosic material in a easily and fast way.

With the aim to remove lignin, in order to obtain a material suitable for the paper fabrication, an alkaline hydrolysis has been carried out on the crushed almond shells. A 24 hours treatment, refluxing the material with a 7.5% NaOH solution, produced a material composed by fragment of shells and fibers arising from the reticulum of fibers between the two layers of the shell. These fibers were separated and blanched with NaOCl solution to give fibrous material with a yield of 7.5% (wt). This substance was examined through ^{13}C CP MAS NMR in order to determinate the lignin/holocellulose ratio. From the spectrum analysis it is evident that after this treatment signals for acetoxy groups disappeared, the basic hydrolysis of acetic esters of hemicellulose moiety occurred, however the most of hemicellulose should have been removed with this treatment as previously reported in literature (Maaloul et al., 2017) consequently it is very likely the ratio lignin/cellulose is obtained. The aromatic signals of lignin decrease their intensities, and the lignin/holocellulose (probably almost all cellulose) ratio of 0.58 was observed (Fig. 7). Furthermore, the relative intensities of the peaks at 89 and 83 ppm (C-4 of the crystalline and amorphous cellulose respectively) changes with respect to the not treated shells, showing a more polysaccharide ordinate structure. The SEM micrography of this sample (Fig. 7) shows peculiar helical spring shape fibers. These formations should arise from the cellulose fibers of secondary cell wall of spiralated tracheas constituting the vascular bundle.

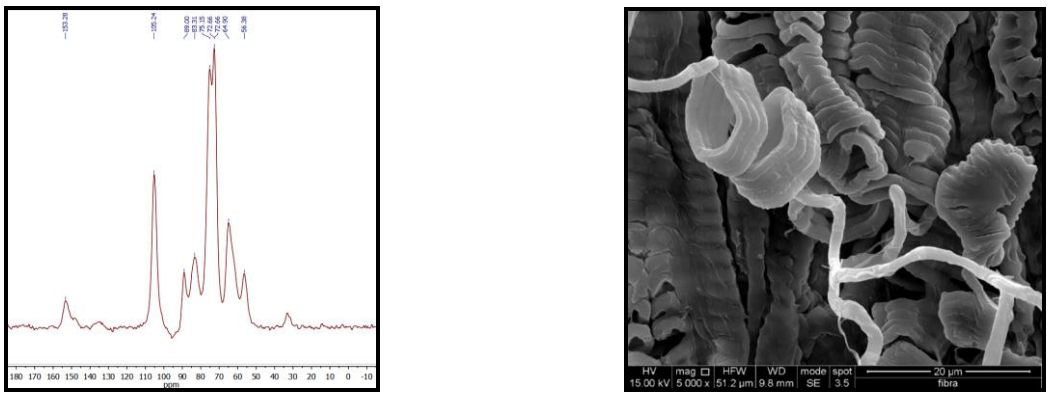

Fig. 7. ^{13}C CPMAS NMR (400 MHz) and SEM of *P. dulcis* treated vascular bundle.

The process used to obtain the paper sample (Fig. 8) is quite similar to the original ones first elaborated in Asia and Europe, before automated machinery prevailed. A little handmade cardboard was prepared using this fibrous material. Tensile measurements provided information on the mechanical performance of paper sample. The stress vs strain curve shows a trend that is consistent with cellulose paper (Cavallaro, Lazzara, Milioto, & Parisi, 2014; Cavallaro et al., 2017). The elastic modulus of ca. 180 MPa indicates that a material virtually suitable for packaging application has been obtained (Fig. 8).

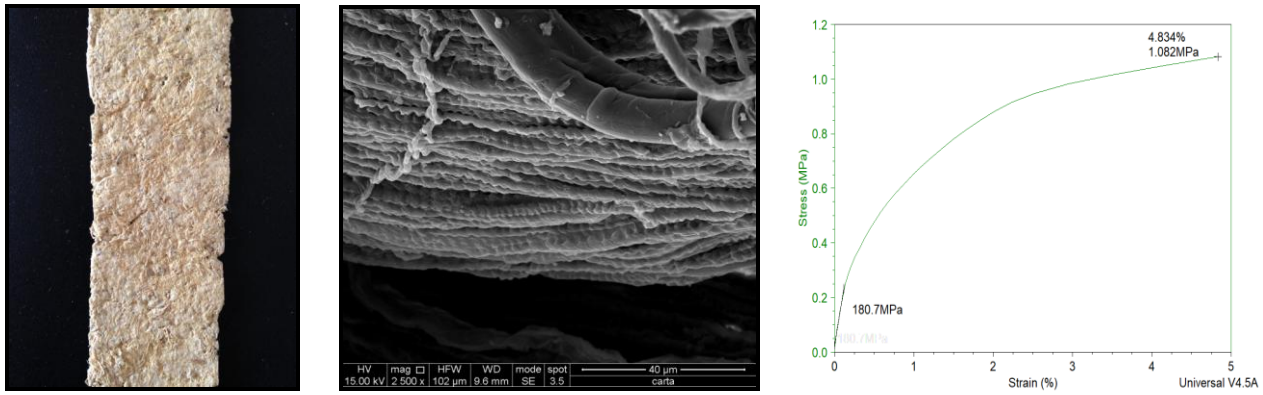

Fig. 8. The almond fibers cardboard and the SEM of the fibers of the thickness and tensile stress–strain curve

The treated shell fragments were subjected to a further alkanine treatment of overall 48 hours. After blanching of the material, a white powder and shell fragments were recovered. The SEM analysis of the powder showed a micrometric composition of particles (Fig.9). The CPMAS NMR spectrum for this material showed typical signals for cellulose and no detectable signals for lignin. The NMR spectrum shows an increasing of the crystalline C2 cellulose peak with respect to the starting material in fact, the SEM at 10000X shows the fibrillar nature of the cellulose (Fig. 9).

Fig. 9. ^{13}C CPMAS NMR (400 MHz) and SEM of cellulose particles obtained from *P. dulcis*

4. Conclusions

In the perspective of a recycling of agricultural waste, *P. dulcis* soft-shell was investigated. Despite the easy of cracking, this cultivar produces a high lignin content shell. It is the stratified structure of the shell, rather than the lignin amount that makes the shell soft. A quantitative approach, using the quick ^{13}C CP MAS NMR technique, has been developed comparing the results with a calibration curve obtained using lignin and cellulose extracted from the same matrix has been analysed. Alkaline treatment do not reduces much the lignin content in the material, but produces fibers, deriving from the vascular bundle, suitable to realize a handmade cardboard with appropriate tensile characteristics and, after a more intense alkaline treatment, pure microcrystalline cellulose can be obtained findin various applications i.e. in the cultural heritage field for artwork conservation and consolidation and as excipient in the pharmaceutical industry.

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