

# **Lignin isolation from kraft and organosolv process using *Cynara cardunculus* L.**



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## 1. Introduction

*Cynara cardunculus* L., cardoon is a non-woody thistle plant that has received attention as a biomass crop with a promising value as feedstock within a biorefinery context. *Cynara cardunculus* is an herbaceous perennial plant, with annual cycles, well adapted to the Mediterranean climate. The annual biomass production may attain 14 to 20 t ha<sup>-1</sup> under experimental conditions [1,2]. Cardoon has been used traditionally for different purposes: inulin can be extracted from the roots [3], cardosins for cheese making, from the capitula [4] and polyphenols, with pharmacological properties, from the leaves [5]. The green leaves may be used as food and fodder in winter [6]. Oil can be extracted from the seeds, with good quality for transesterification into biodiesel [7]. Cardoon biomass has also been the focus of research for bioenergy production by combustion, gasification or pyrolysis [8]. Incorporation as fibers for pulp and paper production was also tested after delignification [9]. This versatility of the cardoon biomass as a feedstock for different purposes potentiates this crop as a promising raw-material for biorefineries.

In this context, lignin is an important chemical component that requires an adequate valorization within the full resource use concept. Lignin influences the biomass heating value and may be used for the production of bio-based products e.g. antioxidants, resins, polymers, while its content and composition influence fractionation and recovery processes [10]. Lignin characterization is therefore background information for its better use and product design. The lignin composition in *Cynara* biomass has been studied by our group [11] and a recent manuscript was submitted for publication [12]. The isolation of the lignin using different methodologies is the next step of our goal. In this way, the work proposed for this STSM was developed in three tasks: task 1) pre-treatment of cardoon stalks with steam explosion (C<sub>SE</sub>); task 2) pulp production with cardoon and C<sub>SE</sub> by kraft and organosolv processes; task 3) isolation of the lignin present in the correspondent liquors. Simultaneously, the raw materials (cardoon, C<sub>SE</sub> and the pulps) were treated with a mixture of enzymes to compare the potential of saccharification of these samples, having in mind the global use of the raw material in the context of biorefinery.

The work was developed in collaboration with Dr. Juan Carlos Villar, in the Instituto Nacional de Investigación y Tecnología Agraria Alimentaria (INIA), in Centro de Investigación Forestal (Madrid, Spain) during the period of 7 of April to 8 of May 2015. The works are still developing, and is our goal to compile then to be published in a peer-review publication, as briefly as possible.

## **2. Material and Methods**

### **2.1. Sampling and material characterization**

*Cynara cardunculus* L. also known as cardoon, was obtained from the experimental field of Politechnical University of Madrid.

The works regarding chemical characterization of cardoon are being developed, applying NREL standards; the total extractives are being determined as sum of ethanol and water extracts. Total lignin by sum of Klason and soluble lignin and sugars determine according to NREL/TP-510-42618. The sugars will be determined by HPLC using an Agilent column Hi-Plex-H 300 x 7.7 mm. A 5mM solution of sulphuric acid was used as mobile phase, with a flux of 0.6 mL/min, at a 65 °C of temperature and a run total time of 30 min.

### **2.2. Pre-treatment with steam explosion**

A sample of 2.05 Kg was weighted and inserted in the steam explosion apparatus (Fig. 1 a). The conditions established for the pre-treatment were: 5 min at a maximum pressure of 8.0 Kg/cm<sup>2</sup>. From this experiment were recovered, a liquid and a solid (called C<sub>SE</sub>) fractions (Fig 1 b, c). The liquid was collected for sugars determination by HPLC and the solid was dried at air to be used for pulping and enzymatic hydrolysis experiments, as explained further.

Posteriorly, two samples of cardoon after steam explosion (C<sub>SE</sub>) were collected for chemical characterization (total extractives, total lignin and sugars content, applying the procedures mentioned in 2.1), and a sample taken for water content determination.

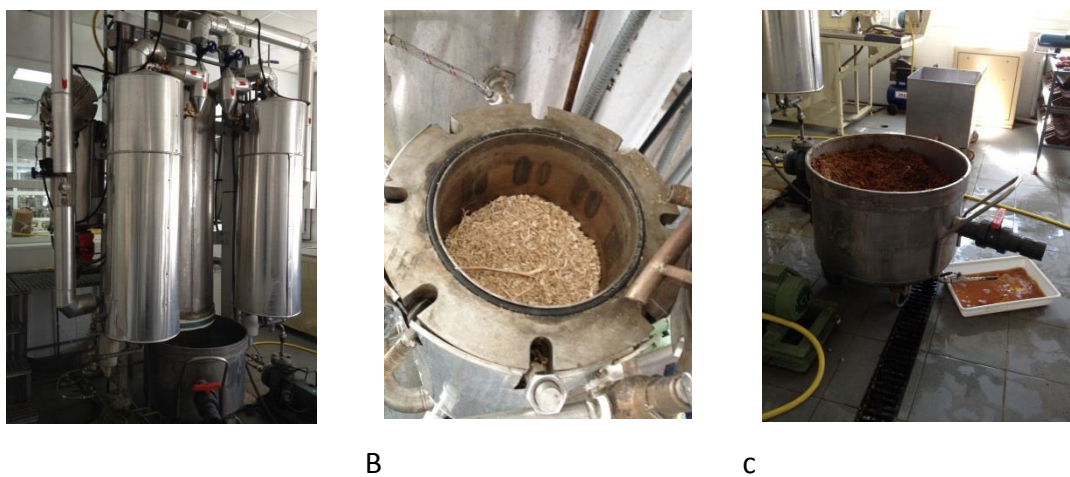


Figure 1. Steam explosion apparatus (a); cardoon before steam explosion (b); solid and liquid collected (c).

### 2.3. Delignification experiments

Two delignification processes were tested: kraft and organosolv. The conditions for kraft process were as follows: 18% of active alkali, 25% sulfidity and a liquor-to-cardoon ratio of 11. For the organosolv process a solution of 33% ethanol:water (v/v), and 1% of sulfuric acid was added to cardoon (control) and cardoon after steam explosion ( $C_{SE}$ ) also to obtain a liquor-to-cardoon ratio of 11. In Table 1 is presented the designation attributed to the pulping experiments. Two batches were made and a total of eight pulps obtained, each pulp were washed and disintegrated in a disintegrator MK.IIIC from Messmer Instruments, and maintained for two days at room temperature to dry. After that, a sample was taken to determined water content, and total yield was determined.

Table 1. Experimental design of the pulping experiments.

Designation	Sample	Pulping process
1	Cardoon steam explosion, $C_{SE}$	Kraft
2	Cardoon steam explosion, $C_{SE}$	Organosolv
3	Cardoon control, C	Kraft
4	Cardoon control, C	Organosolv

#### *Determination of alkali in the white and black liquors*

The content in alkali present in sodium hydroxide (NaOH), and sodium sulfide (Na<sub>2</sub>S) solutions and posteriorly in the white and black liquors were determined by titration with hydrochloric acid (HCl) 1 N and formaldehyde (CH<sub>3</sub>) 38% stabilized with methanol, as a procedure adapted from TAPPI [13]. The procedure to determine the alkali for example in NaOH was as follows: 5 mL of NaOH was mixt with 150 mL of distilled water, and 25 mL of barium chlorate (BaCl<sub>2</sub>) saturated solution. This solution is maintained under stirring while a volume of hydrochloric acid added until pH 9.3. After that, 3 mL of formaldehyde was added producing a change in the pH, and more hydrochloric acid was added until pH 9.3, and the new volume registered for calculation. The same procedure was made for the sodium sulfide solution and to the white and black liquors.

#### 2.4. Enzymatic hydrolysis

##### *Determination of enzyme activity*

The activity of the enzymes Novozymes and Celluclast was determined in separate, but the activity of the mixture was determined. The procedure was made in duplicate, and a control teste was made for comparison.

For the determination of each enzyme activity, 10 µL of each enzyme was measured and added to a 10 mL tube, then was added 990 µL of citrate buffer and a sample of filter paper. The tube was maintained at 50 °C for 1 hour. Posteriorly, 1 mL of 3,5-dinitrosalicylic acid (DNS) was added and the absorbance at 575 nm measured.

The same procedure was applied for the determination of mixture activity but in this case a 5 µL of each was taken for the mixture.

A calibration equation of glucose concentration was used for the determination of the enzymes activity.

$$Abs = 0.98 \times gluc - 0.01$$

Where Abs is the absorbance measured at 575 nm by UV spectrometer; gluc is the glucose concentration. The equation was obtained in previous experiments.

### *Enzymatic experiments*

Two commercial enzymes: Novozymes and Celluclast were used for the enzymatic experiments. Three experiments were made (Table 2): two applying the mixture of enzyme and one control. The raw material used was: i) using cardoon (C), cardoon after steam explosion (C<sub>SE</sub>), pulp 1, 2, 3 and 4 from the first pulping experiment; ii) the same but the pulps were from the second pulping experiment; for the control were used C, CSE and pulp 1, 2, 3 and 4 from the two pulping experiments.

Around 4 g (oven dry) of each sample was weighted, 40 mL of buffer (prepared with citric acid and sodium hydroxide) was added, and maintained under agitation for 18 hours. After this time, 1.35 mL of the mixture of two commercial enzymes Novozymes (0.6 mL) and Celluclast (0.75 mL) was added to each sample. A 1 mL of the liquid was taken (point 0) and the samples placed in an incubator (from P.Selecta) at 50 °C, under 149 rpm of agitation (Fig. 2). In the first day of experiment, each two hours 1 mL of the liquid was taken, in a total of six collections, designated from 0 to 5. The liquid collected was filtrated using a nylon syringe filter of 23 mm x 0.45 µm, and inserted in a 2 mL vial for sugar analysis by HPLC as described earlier. In the next two days, two samples were collected per day, named 6, 7, 8 and 9, respectively. In the fourth day, only one sample was taken, referred to point 10 corresponding to 72 hours of experiment as described in Table 2. For the control experiment only buffer was added to the samples.

Table 2. Experimental design of the sample collection in the enzymatic hydrolysis.

Sample	Experiment 1	Experiment 2	Control
	Time (hours)		
0	0	0	0
1	2	2	2
2	4	4	4
3	6	6	6
4	8	8	8
5	10	10	10
6	24	24	24
7	26	26	-
8	36	36	-
9	38	38	-
10	72	72	72

During the control experiment, the pulps 2 and 4 used absorbed much of the buffer and therefore, only the points 0 to 6, and 10 were collected for all samples (Table 2).



Figure 2. Samples in incubator at 50 °C.

## 2.5. Lignin isolation experiments

### *Black liquors*

For the lignin isolation from the black liquor, a solution of calcium chlorate (aprox. 1.4 g  $\text{Ca}^{+2}$ ) dissolved in ethanol was prepared. A 100 mL of this solution was added to 100 mL of black liquor, forming instantaneously a precipitated (Fig. 3 a, b) that was filtrated and washed with 50 mL of the solution.

The filtrated was recovered for further analysis, for instance to measure the absorbance at 280 nm [14] to evaluate the presence of soluble lignin. The residue (mainly lignin) was dried in a vacuum oven at 50 °C. Afterwards, it will be characterized by pyrolysis linked to gas chromatography and mass detector (Py-GC/MS), to evaluate the type of lignin present.

### *Organosolv liquid*

After the production of organosolv pulps, the remained liquid was recovered; the ethanol present was recuperated and distilled. The remained liquid was used for the isolation of the lignin using the calcium chlorate in ethanol solution, as mentioned before.

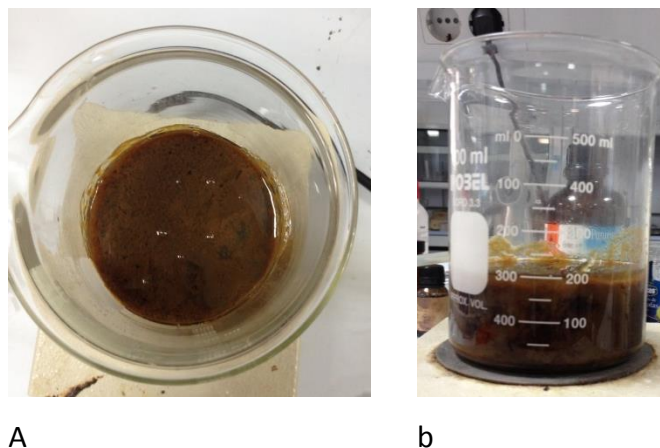


Figure 3. Lignin isolation from kraft liquor: formation of the precipitated after adding calcium chlorate to black liquor (a, b).

### 3. Expected results and further analysis

#### 3.1. Chemical characterization

The chemical characterization of cardoon, cardoon after steam explosion and their respective pulps (kraft and organosolv) are being characterized. In Figure 4 are shown a sample of cardoon (a) and cardoon after steam explosion (b). It can be seen that after this pre-treatment, the raw material maintain its structure, but presents a darker colour comparatively to the starting material.



Figure 4. Cardoon before (a) and after steam explosion (b).

### 3.2. Pulping experiments

Total yield of the pulps produced by kraft and organosolv processes using cardoon and cardoon after steam explosion ( $C_{SE}$ ) was determined and the values presented in Table 3. In Figure 5 are shown the pulps with correspondence with Table 1.

The yield of the kraft pulps varied from 44.8% to 45.1%, when using respectively cardoon after steam explosion ( $C_{SE}$ ) and cardoon. This was more or less expected, because during the pre-treatment of steam explosion there are some compounds, mainly extractives that are removed from the raw material. It is expected to confirm this hypothesis after HPLC analysis of the liquid collected after steam explosion, and these analysis are being performed.



Figure 5. Pulps produced from cardoon control and after steam explosion.

Contrarily to the yield of kraft pulps, the organosolv pulps yields where higher varying from 61.2% to 64.5%. This can be explained by less aggressive conditions used during the production of organosolv pulps, comparatively to those for the kraft pulps.

When we compare the behavior of cardoon and  $C_{SE}$ , we can see that the pulps from  $C_{SE}$  present higher yields when reported to the initial cardoon (64.5% vs 61.2%), which is somehow unexpected. Therefore, it has to be find in literature some explanation of this phenomena.

**Table 3.** Mean values of total yield (% of initial cardoon) of the pulps produced by kraft and organosolv processes using cardoon (C) and cardoon after steam explosion (C<sub>SE</sub>).

sample			Total yield (%)
1	C <sub>SE</sub>	kraft	44.8
2		Organosolv	64.5
3	Cardoon	Kraft	45.1
4		Organosolv	61.2

### 3.3. Enzymatic hydrolysis

The results from the enzymatic hydrolysis are still being acquired by HPLC and data that is already collected is being treated. Therefore, what can be said is that during the treatment, the enzymes actuated more efficiently in the pulps comparatively to the raw material (cardoon and C<sub>SE</sub>). In fact, either cardoon or C<sub>SE</sub> were not visibly attacked by the enzymes. On the other hand, the kraft pulps were more attacked than the organosolvs pulps. In Figure 6 are presented the samples after the enzymatic treatment (Fig. 6.a) and the samples from the control experiments where no enzymes were applied (Fig. 6.b). During the control, the samples presented the same structure, as it was expected. Therefore, it is expected that almost no sugars will be degraded comparatively to the samples that were submitted to enzymes hydrolysis, but this is still under evaluation as already mentioned.

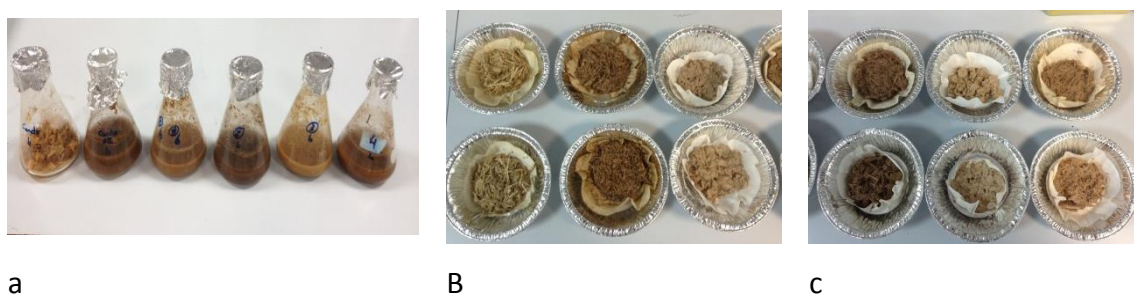


Figure 6. Samples after the 72h of enzymatic treatment for the experiment 1 (a); and for the control (b, c).

### 3.4. Lignin isolation

The procedure used for the lignin isolation from black liquor was well succeed, as can be seen in Figure 7. This Figure shows a considerable amount of solid recovered after the addition of the calcium chlorate dissolved in ethanol, as mentioned in literature [15]. The yield of lignin recovery is under evaluation, and its characterization will be made by Py-GC/MS(FID), and the results will help to explain what type of lignin was recovered and then isolated by this methodology. Nevertheless, since the lignin is being isolated using a combination of calcium salt, and as described in the literature the results from pyrolysis analysis can be influenced by salts, before this analysis, the extraction of calcium have to be made.



Figure 7. Solid residue obtained by filtration, after adding calcium chlorate to black liquor.

In the case of the organosolv liquor, the addition of the calcium chlorate solution was not successful, as can be seen in Figure 8, where no solid was obtained after filtration. Therefore, more experiments involving other types of solutions have to be tested.



a



B

Figure 8. Solid residue obtained after adding calcium chlorate to organosolv liquor.

#### 4. Further collaboration with the host Institution

The results of this collaboration are still been obtained by the host Institution (INIA-CIFOR), but also be ISA-CEF. Therefore, it is expected to maintain the collaborative work with the host institution. This collaboration can be carried through the development of the works related with the lignin isolation from liquors, and/or through international projects with collaboration of both Institutions Instituto Nacional de Investigación y Tecnología Agraria Alimentaria, Centro de Investigación Forestal (INIA-CIFOR) and Instituto Superior de Agronomia, Centro de Estudos Florestais (ISA-CEF).

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