CEN

WORKSHOP

AGREEMENT

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Ultrasound-assisted production of lignin nanoparticles (BIOMAC)

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Foreword

This CEN Workshop Agreement (CWA 18187:2025) has been developed in accordance with the CEN-CENELEC Guide 29 “CEN/CENELEC Workshop Agreements – A rapid way to standardization” and with the relevant provisions of CEN/CENELEC Internal Regulations - Part 2. It was approved by the Workshop CEN “Ultrasound-assisted production of lignin nanoparticles”, the secretariat of which is held by “UNE” consisting of representatives of interested parties on 2025-02-10, the constitution of which was supported by CEN following the public call for participation made on 2025-01-09. However, this CEN Workshop Agreement does not necessarily include all relevant stakeholders.

The final text of this CEN Workshop Agreement was provided to CEN for publication on 2025-06-26.

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The European Committee for Standardization (CEN) draws attention to the fact that it is claimed that compliance with this document may involve the use of a patent concerning ultrasound-assisted process for the production of lignin nanoparticles given in Clause 5 and which is claimed to be relevant for the following clause of this document: Clause 5 Technical Requirements and Specifications.

CEN takes no position concerning the evidence, validity and scope of this patent right.

The holder of this patent right has assured CEN that it is willing to negotiate licences under non-discriminatory basis and on fair, reasonable terms and conditions with applicants throughout the world. In this respect, the statement of the holder of this patent right is registered with CEN. Information may be obtained from:

Creative Nano P.C

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Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights other than those identified above. CEN shall not be held responsible for identifying any or all such patent rights.

Although the Workshop parties have made every effort to ensure the reliability and accuracy of technical and non-technical descriptions, the Workshop is not able to guarantee, explicitly or implicitly, the correctness of this document. Anyone who applies this CEN Workshop Agreement shall be aware that neither the Workshop nor CEN, can be held liable for damages or losses of any kind whatsoever. The use of this CEN Workshop Agreement does not relieve users of their responsibility for their own actions, and they apply this document at their own risk. The CEN Workshop Agreement should not be construed as legal advice authoritatively endorsed by CEN.

Introduction

The transition to a sustainable bioeconomy requires the development and validation of innovative technologies that exploit renewable resources while minimizing environmental impact. Lignin, a primary byproduct of the pulp and paper industry, represents a significant yet underutilized renewable resource that is currently mostly burned to generate heat and power [1]. Recent advancements have enabled the production of lignin nanoparticles (LNPs), which have high added-value properties compared to bulk lignin for various applications, including bio-based composites [2-5].

Lignin NPs offer several advantages over conventional synthetic NPs. As a naturally derived biopolymer, lignin is abundant, cost-effective, biodegradable, and non-toxic, making it a sustainable alternative with minimal environmental impact. The versatile and eco-friendly potential of LNPs makes them suitable for applications across various sectors. For instance, in the food packaging industry, materials reinforced with LNPs demonstrate extended shelf life due to the antimicrobial and antioxidant properties of lignin, while also reducing dependence on petroleum-based plastics [6-8]. Furthermore, in agriculture, LNPs can be used for the controlled release of fertilizers and pesticides, or in the production of biodegradable mulch films and pots, thereby minimizing environmental burden [9-10]. In medicine, they can act as carriers for targeted drug delivery owing to their biocompatibility, while in cosmetics, they serve as antioxidants and UV-protective agents, enhancing product safety and efficacy [11-13].

Unlike synthetic NPs, the production of LNPs can often be achieved using greener, solvent-free or low-energy processes [14]. The production of LNPs using ultrasound-assisted processes in aqueous media has emerged as a promising, environmentally benign technology by virtue of its capability to control particle size, enhance material physicochemical properties, and reduce dependence on chemical compounds [15-16]. In this context, this document introduces the concept of producing LNPs at a pilot-line scale using ultrasonication. This document outlines the methodological procedures required to produce LNPs with controlled specifications. Furthermore, test methods for the physicochemical characterization of LNPs are also included.

The present process was developed by Creative Nano PC within the framework of the BIOMAC Horizon 2020 project “European Sustainable BIObased nanoMAterials Community” and falls within the patent EP4471093A1 rights [17].

This agreement is necessary due to the increasing interest in biopolymers and the potential for LNPs to significantly impact materials technology, offering eco-friendly alternatives to conventional nano additives. With a focus on environmental sustainability, this CEN Workshop Agreement (CWA) addresses the process parameters and quality control protocols essential for the consistent and reproducible production of LNPs.

1 Scope

This CEN Workshop Agreement (CWA) provides a set of requirements and guidelines and outlines the methodology for the pilot-scale production of LNPs with controlled particle size using an ultrasound-assisted process and water as the liquid medium. The methodology applies to lignin sourced from different types of biomass, such as hardwood, softwood, and non-wood biomass, processed through a custom-made ultrasound-assisted pilot line.

The following document provides:

- Guidelines for setting up and operating the ultrasonication (US) equipment to ensure consistency and repeatability of LNPs production. This is covered in Section 5.1.
- The technical parameters necessary for the ultrasonication treatment of lignin to acquire LNPs with specific properties, such as particle size, morphological and structural characteristics. This is covered in Section 5.2.
- Quality control measurements and testing protocols to assess the LNPs' physicochemical properties, ensuring they meet specifications suitable for various industrial applications. This is covered in Section 5.3.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at http://www.iso.org/obp/

— IEC Electropedia: available at http://www.electropedia.org/

3.1
lignin nanoparticles (LNPs)
LNPs in powder form with a particle size smaller than 200 nm or LNPs dispersed in an aqueous medium with a hydrodynamic diameter ($\bar{x}_{DLS}$) smaller than 700 nm

Note 1 to entry: We acknowledge that the strict ISO 80004-1:2023 definition of a nanoparticle is a particle with at least one dimension below 100 nm. However, due to inherent synthesis variability and sample preparation, a minor fraction of the particles may exceed this threshold. This exceptional presence of particles exceeding the size of 100 nm does not alter the overall nanoscale character of the material, which actually defines its functional properties.

3.2
suspension
liquid in which LNPs are dispersed

Note 1 to entry: In this CWA the liquid is reverse osmosis water.

3.3
pilot line
semi-industrial scale setup used to produce LNPs in a capacity of at least 50 g. per day, and which can be further upscaled to demonstrate full industrial production capabilities

4 Abbreviated terms

ATR	Attenuated Total Reflectance
DLS	Dynamic Light Scattering
DSC	Differential Scanning Calorimetry
FTIR	Fourier Transform Infrared
$\bar{x}_{DLS}$	Hydrodynamic diameter
LNPs	Lignin nanoparticles
NMR	Nuclear magnetic resonance
PDI	Polydispersity index
PL	Pilot line
PS	Particle size
RO	Reverse osmosis
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
Tg	Glass Transition Temperature
TGA	Thermogravimetric analysis
US	Ultrasonication

5 Technical Requirements and Specifications

The ultrasound assisted PL has been designed and manufactured to produce dispersions of LNPs in RO-water, employing a continuous stream process tailored specifically for this purpose. LNPs in powder form can be acquired dry using a semi-industrial freeze dryer. A detailed presentation of the PL setup and the operational steps required to produce LNPs at pilot scale is provided below.

5.1 Equipment Specifications

To run the procedure described in this text, the following equipment shall be made available:

- Ultrasound generator.
- Ultrasonication probe.
- Multi-walled Reactors with a circulatory system.
- Water circulators for controlling temperature in the Reactors.
- Temperature monitoring systems.

The features of the equipment listed above are provided in the sections below.

The temperature control should also be set according to the instructions in 5.1.4.

5.1.1 Ultrasound generator

The ultrasound generator shall have the following features:

- Frequency: 20-25 kHz.

- Power: 1500 W.
- Mechanical Amplitude: 50-100%
- Pulse duty cycle: from 50% to 90% US on.

5.1.2 Ultrasonication probe

The ultrasonication probe shall be made of the Titanium alloy Ti-6Al-4V.

5.1.3 Reactors

The reactors shall have the following features:

- Triple-walled reactor:
  - main tank with a volume of 50L, made from stainless steel (type AlSI316) and equipped with an overhead high-speed stirrer,
  - intermediate jacket for water recirculation to control temperature in the reactor space,
  - insulation layer.

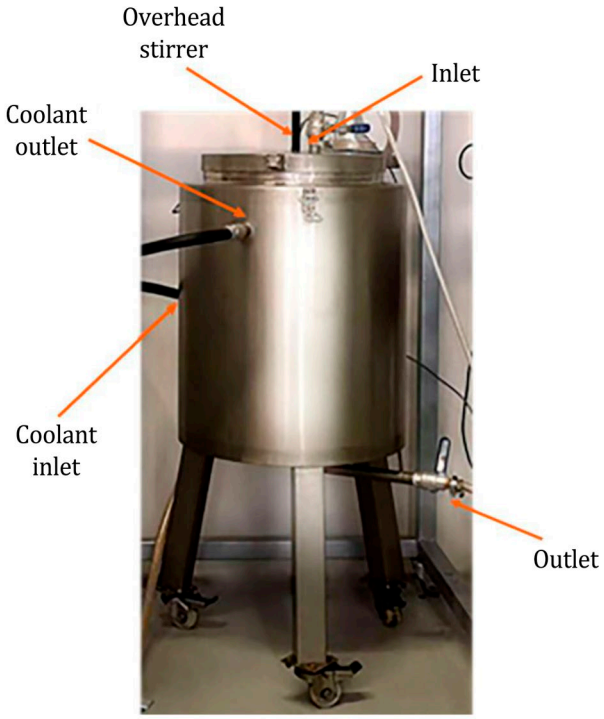


Figure 1 — Triple-walled reactor

- External double-walled flow-cell with a working volume of 500 mL made of stainless steel in which the ultrasonication probe is integrated.

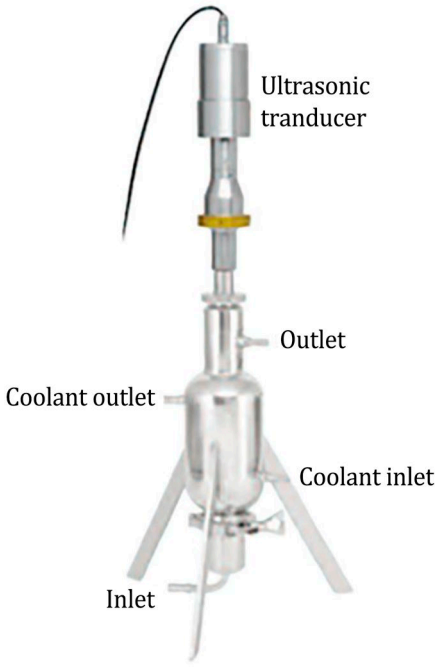


Figure 2 — External double-walled flow-cell with integrated ultrasonication probe

- The main tank shall be connected to the flow cell via silicone tubing. The liquid dispersion shall be recirculated with the aid of a peristaltic pump.

5.1.4 Temperature control system

The temperature control shall be implemented using two external cooling circulators. One circulator is connected to the triple-walled reactor while the second one to the double-walled external flow-cell reactor. The circulators are used to maintain a constant temperature of the LNPs dispersion in the reactors and prevent excessive heating during the US process. The temperature of the dispersion shall be constantly monitored using two PT100 sensors located at the inlet and the outlet of the flow cell. The temperature of the dispersion shall be controlled by adjusting the temperature of the fluid circulating within the multi-walled reactors.

5.2 Description of the process

5.2.1 Lignin pretreatment

Raw lignin is dried under vacuum at 80 °C overnight. The final moisture content of lignin shall be less than 10% by mass.

The dried lignin is ground in a semi-industrial grinder at room temperature to break down larger aggregates. The starting size of lignin particles shall be less than 10 μm (micrometre) prior to US.

5.2.2 Mixing

Ground lignin is weighed and then added to 10-30 L water under vigorous stirring in the main tank to achieve the targeted concentration. The final lignin concentration in the aqueous dispersion shall be between 0,5 % to 10 % m/v (mass/volume).

5.2.3 Dispersion Circulation

The lignin aqueous dispersion is continuously recirculated between the main tank and the flow cell, ensuring uniform exposure to ultrasonication with the aid of a peristaltic pump.

5.2.4 Temperature Control

The process described herein shall be conducted at a temperature range of the LNPs dispersion between 15 °C and 60 °C. The optimum temperature range shall be between 45 °C and 50 °C. The temperature shall be controlled with a deviation of 2 °C during the whole process.

5.2.5 Ultrasonication Treatment

Ultrasonication is applied for at least 1 hour in the flow cell using a titanium alloy probe integrated into the flow cell. The US energy settings (energy, mechanical amplitude and pulse duty cycle) are adjusted according to the specific requirements of the lignin type being processed. For 1h of US treatment the energy applied is 5 400 kJ with mechanical amplitude at 100 % and pulse duty cycle: t_{on} = 9s, t_{off} = 1s.

NOTE Under extreme or prolonged sonication conditions, there is a risk of contamination from the probe material. Users should regularly check probes for wear. In all the processes presented herein, contamination was not observed.

5.2.6 Freezing and Freeze-Drying

The ultrasound treated dispersion is first frozen at -45 °C and then freeze-dried until full sublimation of the water to yield dry LNPs in powder form. The ice condenser operates at -50 °C, with a vacuum pressure of 0,6 mbar to 0,8 mbar applied during the process. The ice condenser has a capacity of 10 kg per 24 hours.

Figure 3 — Semi-industrial freeze dryer

5.3 Quality Control and Assurance

5.3.1 Sampling during the process

Sampling must be performed at fixed time intervals during the US process. This is critical for monitoring the $\bar{x}_{DLS}$ and PDI of the LNPs.

- Sampling Frequency: Samples shall be taken every hour during the US treatment to assess the progressive reduction in particle size.

- Analysis Technique: The evaluation of lignin's particle size and PDI is measured using DLS. Samples shall be diluted to a concentration of approximately 0,01% m/v. After dilution, the samples shall be placed in an ultrasonic bath for 2 minutes.

For reference, Figure 4 shows the $\bar{x}_{DLS}$ measurements of the lignin dispersion over a 4-hour US process. Figure 4 demonstrates the gradual reduction of particle size over time, underlining the effectiveness of the US process in achieving the desired LNP dimensions. In this example, soda lignin was used at a concentration of 1,0 % w/v (with a total volume of 30 L) and a reaction temperature of approximately 45 °C. The energy applied was 5 400 kJ per hour with mechanical amplitude at 100 % and pulse duty cycle: t_{on} = 9s, t_{off} = 1s

This process resulted in a dispersion of LNPs with a final $\bar{x}_{DLS}$ of 503 nm after 4 h.

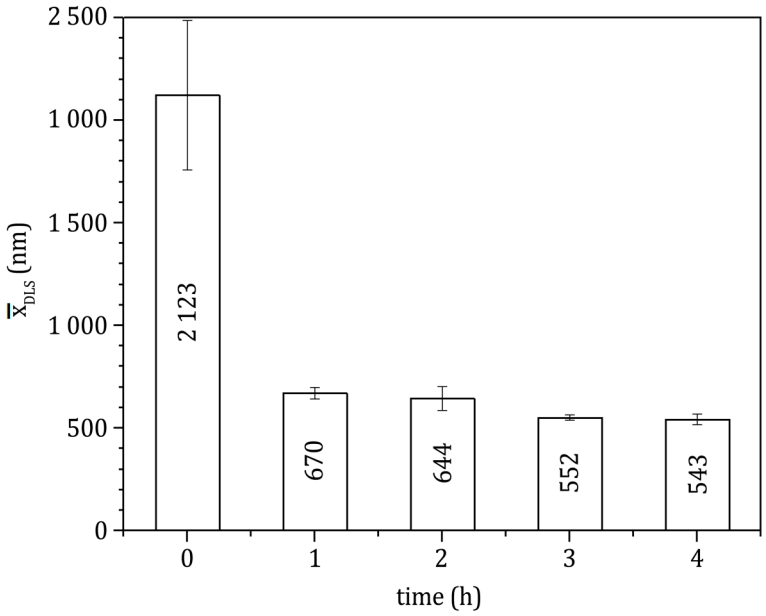


Figure 4 — $\bar{x}_{DLS}$ values measured by DLS at 1-hour intervals, during the 4-hour treatment in the ultrasound-assisted PL of soda lignin dispersed in water

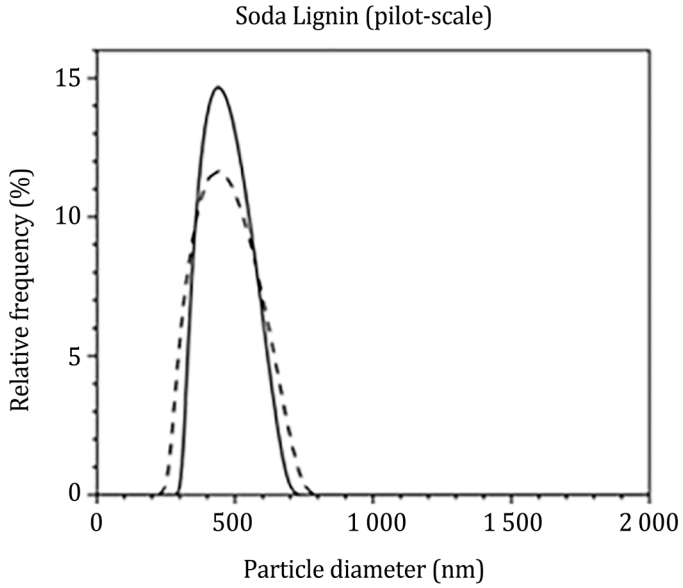
5.3.2 Post-Processing Quality Control

5.3.2.1 Dynamic Light Scattering (DLS)

Following the freeze-drying process, it is crucial to evaluate the $\bar{x}_{DLS}$ of the LNPs to determine if any changes occurred during the drying process. DLS shall be performed to measure the $\bar{x}_{DLS}$. The DLS results obtained before and after freeze-drying shall be compared to verify the stability of the particle size.

To prepare the lignin and LNPs samples for DLS analysis, the powders must be adequately dispersed. For this reason, they shall be dispersed in water at a concentration of approximately 100 ppm (parts per million). The dispersion process shall be performed by US treatment of the lignin and LNPs powders for 5 minutes using a US processor with a proper US probe. This step is crucial to achieving a homogeneous dispersion of lignin particles. As an example, Figure 5 shows the particle size distribution curves of LNPs

before and after the freeze-drying process, indicating that the freeze-drying process does not adversely affect the particle size of the nanoparticles.



Key

U/S: 447 ± 100 nm

after freeze drying: (456 ± 74) nm

Figure 5 — Particle size distribution curve measured by DLS of the LNPs produced by the ultrasound-assisted PL after 4 h (see section 5.3.1), followed by freeze-drying of soda lignin

5.3.2.2 Fourier Transform Infrared (FTIR) / Attenuated Total Reflectance (ATR) Spectroscopy

FTIR/ATR is a vital tool for evaluating the lignin's chemical structure before and after US. This analysis helps to identify any alterations in the functional groups of lignin that may result from the US treatment.

Spectra shall be recorded in transmission mode, at room temperature, in air, by accumulating at least 64 scans at a resolution of at least 4 cm^{-1} in the 4 000 to 500 cm^{-1} wavenumber range. The analysis shall be made on a pellet obtained by mixing KBr and LNPs in powder form or using a small amount of LNPs in the case of ATR.

A comparative analysis shall be performed by analysing the spectra to compare the pre- and post-ultrasonication samples. Any shifts in peak positions or changes in the intensity of absorption bands, shall be looked at, particularly those associated with aromatic skeletal vibrations (around 1 600 cm^{-1}), hydroxyl groups (around 3 400 cm^{-1}), and carbonyl stretching (around 1 730 cm^{-1}).

5.3.2.3 Scanning electron microscopy (SEM)

SEM is a complementary tool for evaluating lignin's morphology and size distribution at both the microscale and nanoscale. LNP samples shall be coated with a conductive layer by sputtering (e.g. gold) before SEM analysis to enhance image quality.

5.3.2.4 Transmission electron microscopy (TEM)

TEM is a supplementary technique for analysing the lignin particles. This analysis provides insights into the nanoparticle size and morphology at high resolutions. LNPs shall be first dispersed in water, avoiding the use of organic solvents, which may dissolve the lignin or affect its size. This dispersion shall then be

subjected to US in an ultrasonic bath for 30 minutes to ensure uniform particle distribution. After US, a drop of the lignin dispersion shall be carefully deposited onto a lacey carbon copper grid and left overnight at room temperature to evaporate.

As a reference, Figure 6 depicts the TEM image of soda LNPs produced by the ultrasound-assisted PL after 4 hours of US treatment, followed by freeze-drying. The image demonstrates that the LNPs exhibit a particle size ranging between 50 nm and 200 nm, as measured by TEM. This showcases the effective size reduction and uniformity achieved through the specified processing conditions.

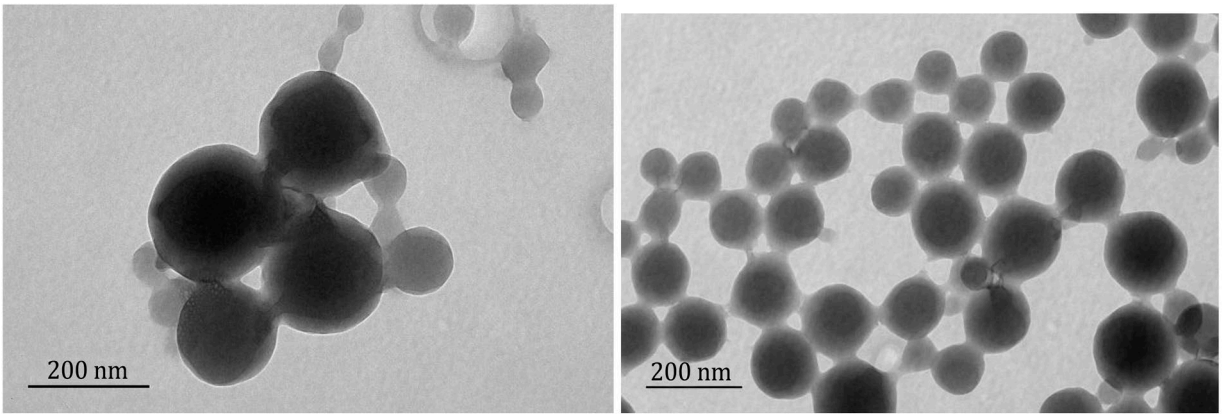


Figure 6 — TEM images at different magnification of the LNPs produced by the ultrasound-assisted PL after 4 h of US treatment, followed by freeze drying of a soda lignin dispersion (1 % m/v) in water at 45 °C

5.3.2.5 Differential scanning calorimetry (DSC)

DSC analyses are necessary to assess the thermal transitions in lignin before and after US. This characterization technique enables the identification of changes in the thermal behaviour of lignin that may result from the US treatment.

DSC analyses shall be carried out on lignin samples in powder form under a nitrogen flux. The heating/cooling scan rate shall be between 10 °C min^{-1} and 20 °C min^{-1}. The measurements shall consist of three runs (heating/cooling/heating) from room temperature to 200 °C.

The glass transition temperature (Tg) of the materials shall be evaluated as inflection point in the second heating run.

A comparative analysis shall be performed by analysing the DSC trace of pre- and post-ultrasonication lignin samples. Any changes in the value of Tg shall be recorded and noted.

5.3.2.6 Thermogravimetric analysis (TGA)

TGA measurements are needed to evaluate the thermal stability of lignin before and after US. In particular, this characterization technique enables the identification of changes in the thermolytic and thermo-oxidative behaviour of lignin that may result from the US treatment.

TGA measurements shall be carried out on lignin and LNPs samples in powder form. The samples shall be heated from ambient temperature to 800 °C at a scan rate of 10 °C to 20 °C min^{-1} in either air (thermo-oxidative response) or nitrogen (thermolytic response) atmosphere. The mass loss (%) and the mass loss derivative (%/ °C) traces shall be recorded.

A comparative analysis shall be performed by analysing the TGA trace of pre- and post-ultrasonication lignin and LNPs samples. Any changes in the 5 %, 10 % and 50 % mass loss, in the residual mass (%) at 800 °C and in the maximum mass-loss derivative temperature shall be recorded and noted.

5.3.2.7 ^{31}P-Nuclear magnetic resonance (NMR) spectroscopy

Quantitative ^{31}P-NMR spectroscopy shall be used to assess the type and concentration of hydroxyl and carboxyl functionalities in lignin and LNPs samples and how they change as a result of the US process.

Prior to analysis, the lignin and LNPs samples shall be dried overnight under vacuum at 40 °C to remove moisture. After this, lignin and LNPs samples in solution shall be prepared for analysis according to the procedure described [18-19].

After recording the spectra, the signal integration shall be performed in the following regions: 153,0 ppm −150,0 ppm (signal associated with the internal standard), 150,0 ppm to145,0 ppm (signal associated with aliphatic hydroxyl groups), 145,0 ppm to 136,0 ppm (signal associated with phenolic hydroxyl groups), and 136,0 ppm to 132,0 ppm (signal associated with carboxylic groups).

A comparative analysis shall be performed by analysing the ^{31}P-NMR spectra to compare the pre- and post-ultrasonication samples. Any change in the integration value for the target signals listed above shall be looked at and noted.

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