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High-throughput Soxhlet extraction method applied for analysis of leaf lignocellulose and non-structural substances

Alejandra Torres-Rodriguez[a,*], Roshanak Darvishzadeh[a], Andrew K. Skidmore[a], Erna Fränzel-Luiten[b], Benno Knaken[b], Boelo Schuur[b]

[a] Department of Natural Resources, Faculty of Geo-Information Science and Earth Observation (ITC), University of Twente, P.O. box 217, Enschede 7500 AE, the Netherlands
[b] Sustainable Process Technology Group, Faculty of Science and Technology, University of Twente, P.O. box 217, Enschede 7500 AE, the Netherlands

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ABSTRACT

The traditional Soxhlet extraction method is commonly employed to extract soluble components from non-soluble components in a solid matrix, for example, non-structural substances in biomass samples that can be separated from structural lignocellulosic compounds in biomass samples. Conventional laboratory procedures for such extractions typically involve a low sample throughput, with each run being performed individually, resulting in time-consuming and labour-intensive processes, making them impractical for analysing large sample sets. In research fields such as Earth Observation in Forest Ecosystems, extensive fieldwork sampling is required across large study areas, resulting in a substantial number of leaf samples, each with limited mass. In this study, an innovative adaptation of the conventional National Renewable Energy Laboratory (NREL) Soxhlet method is developed to create a high-throughput mini-Soxhlet apparatus that enables the simultaneous extraction of up to nineteen samples, each with a mass of 0.3 g per sample. With this adaptation, we measured the lignocellulose and extractive in 343 leaf samples collected from four temperate forest tree species. This modified approach enhances versatility and can be applied to all solid-liquid extractions and various types of vegetation tissues, such as tree leaves, shrubs, crops, feedstock, and other non-woody samples.

- The solid-liquid extraction method has been implemented in a heating block facilitating 19 small flasks to measure multiple samples simultaneously while requiring only a small sample mass.
- The apparatus set-up was constructed using an alumina heating block mounted on a standard laboratory heating plate. Boiling flask tubes were placed in the heating block and equipped with condenser caps and filters on glass rods on which the solid samples were placed.
- The adjustments made the method suitable for application to diverse vegetation tissues and non-woody sample types. It holds particular appeal for research areas that necessitate a high sample number.

* Corresponding author.
E-mail address: a.torresrodriguez@utwente.nl (A. Torres-Rodriguez).
Social media: @torresro_a (A. Torres-Rodriguez)

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Name and reference of original method:	Sluiter, A., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D. [1]. Determination of extractives in biomass. Laboratory analytical procedure (LAP). National Renewable Energy Laboratory. Golden, CO, USA, 1–9.
Resource availability:	Not applicable

Method details

Solvent extraction, also known as 'solid-liquid extraction' or leaching, is a crucial method for separating analytes (non-cell wall constituents) from lignocellulosic material and, it is often employed as a sample preparation step before analytical processes to enhance the accuracy of subsequent procedures aimed at quantifying individual chemical components of biomass samples, such as lignin and cellulose (i.e., [2–4]).

Soxhlet extraction, widely recognized as the standard technique for extracting analytes from solid samples, is favoured over alternative leaching techniques due to its superior effectiveness [5]. It still is labour-intensive. There have been some developments on the original Soxhlet extraction to reduce the extraction time and the amount of solvent consumption, as well as to automate or semi-automate the process. These enhancements include the application of higher temperatures and pressures, microwave irradiation, ultrasound, and others, each with its distinct advantages and disadvantages, such as cost [6].

The U.S. National Renewable Energy Laboratory (NREL) has developed laboratory analytical procedures (LAPs) that apply to both woody and non-woody biomass [1]. The NREL method outlines the use of an automated extraction process involving the Dionex Accelerated Solvent Extractor apparatus. The other alternative, which fundamentally parallels the principles of the original Soxhlet technique, is simpler to replicate and has been widely referenced in academic literature.

However, the NREL Soxhlet method necessitates processing one sample at a time, requiring a high sample mass - typically 2 to 10 g of dry sample - which may be relatively high in specific applications. This makes it time-consuming and impractical for a large number of samples The method is usually employed in applications with a relatively low sample throughput, with each sample being measured sequentially in laboratory procedures.

Throughout history, humans have extracted various plant chemicals for applications in medicine, food, cosmetics, and other industries [7–10]. A standardised technique employed in these extraction processes is Soxhlet extraction from different parts of the plant [11–13]. Notably, research related to the original method and the biomass components predominantly focused on woody materials, with limited studies on these constituents in bamboo, sugarcane, feedstock and senescent leaves [14–18].

Collecting large numbers of samples is essential in research fields such as remote sensing. A large number of tree leaves are needed to compare reflectance data from remote sensing instruments with the measurements of their biochemical content as ground truth. Remote sensing can be employed to estimate various biochemical constituents within vegetation canopies using multiple platforms and sensors from the field, including airborne or satellite levels. To do this, field samples are collected and subsequently analysed in the laboratory to measure the studied biochemicals. Field data measurements are pivotal in the parametrisation, calibration, validation, and upscaling of remote sensing models [19,20]. However, acquiring ground truth data through fieldwork poses a substantial challenge. Field data collection in research is typically characterised by significant labour, time, and resource costs. Moreover, these challenges are magnified in research domains such as remote sensing in forestry ecosystems. For instance, study areas often cover vast areas with high variability in tree traits; this requires a statistically representative sample size and results in a high sample count. Another challenge is the limited mass quantity that can be collected per sample, as leaves must be collected from the top of the canopy.

To facilitate the analysis of such large sample numbers, each with limited mass it was necessary to adapt the original Soxhlet method by NREL [1]. Several modifications were made to the original method to address the constraints, detailed further in the following sections.

Furthermore, it is important to highlight the following points:

- The extraction yield was determined through a gravimetric method, after the removal of extractives, the weight of the dried residue, was measured and compared to the initial weight. The values for lignocellulose (extractives-free) and the soluble non-structural components (extractives) were reported as percentages of the dry weight (DW%).
- To address the limited sample mass available, we used 0.3 g of sample dry mass and 6 ml of solvent, aiming for a 1:20 ratio between the grams of leaf sample and millilitres of solvent [21]. This differs from the original NREL protocol, which recommends using between 2 and 10 g of sample, with a "*minimum of 8 g of extracted sample required for complete compositional analysis*" [1].
- In determining the solvent to use, the original protocol suggests conducting two extraction steps, one with water and another with ethanol. However, in our case, we exclusively employed ethanol extraction since we aimed to determine the combined presence of all non-structural substances rather than focusing solely on water-extractable materials. Because of the diverse nature of the extractives, ethanol was chosen as a solvent. This is due to its high polarity making it an excellent solvent for extracting a broader type of compounds – and for its recognised safety.

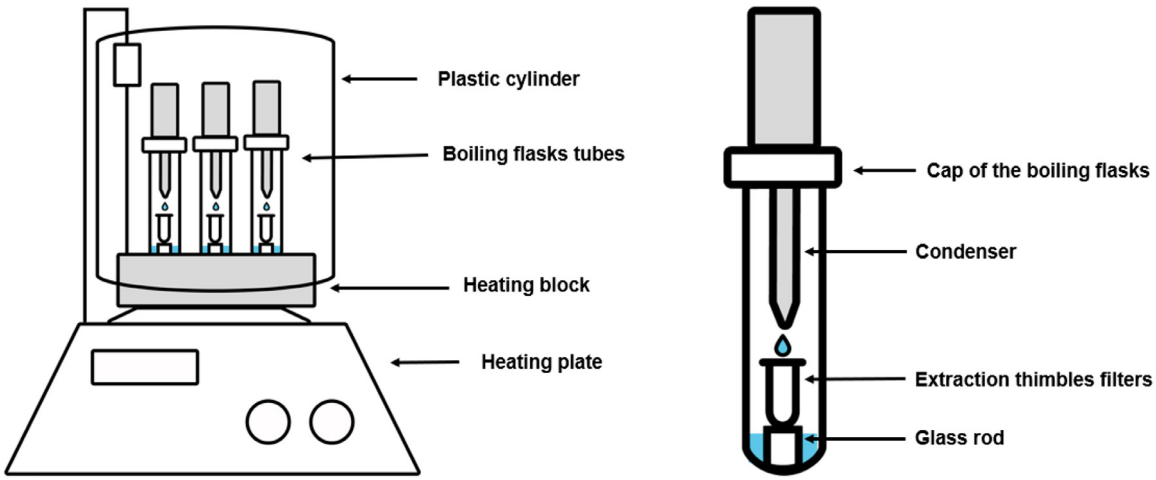

Fig. 1. High-throughput mini Soxhlet extraction apparatus.

- Ash content refers to the inorganic amount in biomass, such as minerals and salts. While refining the method adjustments, an ash correction was discarded [3,4] after testing a subset of samples, which revealed ash content below 2% of the total dry sample mass (data not shown).

Terminology

Sample: leaf powder sample

Extractives: also called non-structural substances, comprise a wide range of chemical components soluble in ethanol through extraction processes, such as non-structural carbohydrates and nitrogen-bearing compounds.

Lignocellulose: refers to lignin, cellulose, and hemicellulose together. In other words, it is determined as the content of the extractive-free biomass.

Reagents: Ethanol (96% EtOH)

Materials

a. Analytical balance
b. Oven set to 105 + 5 °C for glassware drying.
c. drying oven set to 65 °C for drying the leaf samples.
d. Tweezers
e. Disposable glass pipettes (150 mm leigh)
f. Materials necessary for extraction apparatus:
   - Heating plate.
   - The aluminium heating block station.
   - The condenser system is made on each boiling flask.
     - Metal sticks (heat pipe 0.7 K/W, 4 mm x 200 mm) attached to a base made of anodized aluminium (LED heat sink 6.3 K/W, 20 mm x 10 mm).
     - Boiling flask tubes with round-bottom and screw-cap. Dimensions: 20 mm diameter and 150 mm height (Wheaton brand).
     - Glass rods
     - Extraction thimbles filters of 10 × 50 mm, with wall thickness varying from 1 mm to 3 mm (Soxtherm brand).
   - Plastic cylinder protection made of Lexan and an aluminium ring as a base.

Apparatus description

The High-throughput mini Soxhlet extraction set-up consisted of an aluminium heating block designed and constructed in-house to be mounted on the heating plate. It has round holes drilled into its surface to allow a total of nineteen boiling flask tubes to be placed on top, with each tube containing a filter and a condensation system. Fig. 1 details the extraction apparatus set-up. Additionally, a plastic cylinder was added for extra protection.

A condenser system is set up for each boiling flask. A heat pipe stick was affixed to a base and then connected, to the screw cap of each boiling flask tube, thereby functioning as a condenser system (see Fig. 1). Inside each flask tube, a glass rod was placed at

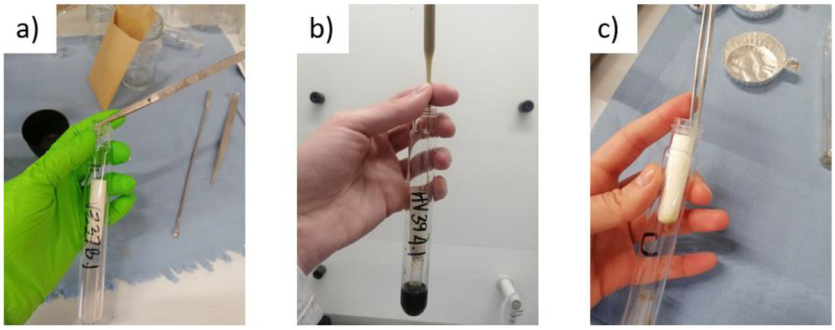

Fig. 2. (a) Thimble filter placed inside the flask tube. (b) After the extraction, the extractives were carefully removed from the flask tube. (c) After drying, the thimble filter was removed from the flask tube.

the base, with its length sufficient to be submerged in the boiling solvent but ensuring it did not come into contact with the thimble located on top.

Method

- As sample preparation, the samples were dried in the oven at 65 °C for 72 h to reach a stable dry weight content [22]. The samples were then ground into a homogenous powder and stored in a desiccator. Thimble filters were dried by leaving them the night before the experiment in the oven at 105 °C.
- The weight of the empty thimble filter was recorded after they were dried (Weight empty filter). Then, 0.3 +/- 0.01 g of sample mass was added to the thimble, and the weight was recorded again (Weight filter + sample). Inside flask tubes, glass rods were added and 6 mL of ethanol. Next, the thimble filters with the sample were placed carefully on the glass rods above the liquid level (Fig. 2.a). The cap of the flask tube was closed, ensuring that the condenser attached to the screw cap points inside the thimble filter. This procedure was repeated for all the nineteen flask tubes. Then, the flask tubes were mounted on the heating block.
- The system was set to run, and the extraction process ran for 22–24 h at a temperature slightly above the boiling point of ethanol (78 °C). The temperature of the heating plate differed from that within the flask tubes due to heat losses, thus the heating plate was set to 107 °C to maintain a temperature of approximately 88 °C within the flask tubes. After the extraction time was finalised, the flask tubes were let to cool down before removing the cap from each of them. The liquid (extractives + solvent) was carefully removed without touching the thimble filter using a pipette (Fig. 2b).
- Afterwards, the flask tubes without the cap were placed in a drying oven at 105 °C for a minimum of 5 h to ensure that the thimble filter was completely dry, and its weight constant. It is worth mentioning that removing the thimble filter from the flask tubes before they are completely dry caused them to break into pieces. Each dried thimble was extracted from the flask tubes with tweezers (Fig. 2c). The weight of the thimble filter and the remaining extractives free sample were recorded (Weight after extraction filter + sample).

Calculations

- The total sample weight (~0.3 g) was calculated as follows:

$$\textbf{Sample weight } [\mathbf{g}] = \text{Weight}_{filter\ +\ sample} - \text{Weight}_{empty\ filter} \tag{1}$$

- The lignocellulose weight was calculated as Eq. (2) and determined in dry weight percentage as showed in the following equation Eq. (3):

$$\textbf{Lignocellulose weight } [\mathbf{g}] = \text{Weight after extraction}_{filter\ +\ sample} - \text{Weight}_{empty\ filter} \tag{2}$$

$$\textbf{Lignocellulose } [\mathbf{DW\%}] = \left(\frac{\text{Lignocellulose weight } [\text{g}]}{\text{Sample weight } [\text{g}]} \right) * 100 \tag{3}$$

- The extractive's weight in mass units was calculated following Eq. (4) and determined in dry weight percentage as showed in the subsequent equation Eq. (5):

$$\textbf{Extractives weight } [\mathbf{g}] = \text{Weight}_{filter\ +\ sample} - \text{Weight after extraction}_{\ filter\ +\ sample} \tag{4}$$

$$\textbf{Extractives } [\mathbf{DW\%}] = \left(\frac{\text{Extractives weight} [\text{g}]}{\text{Sample weight } [\text{g}]} \right) * 100 \tag{5}$$

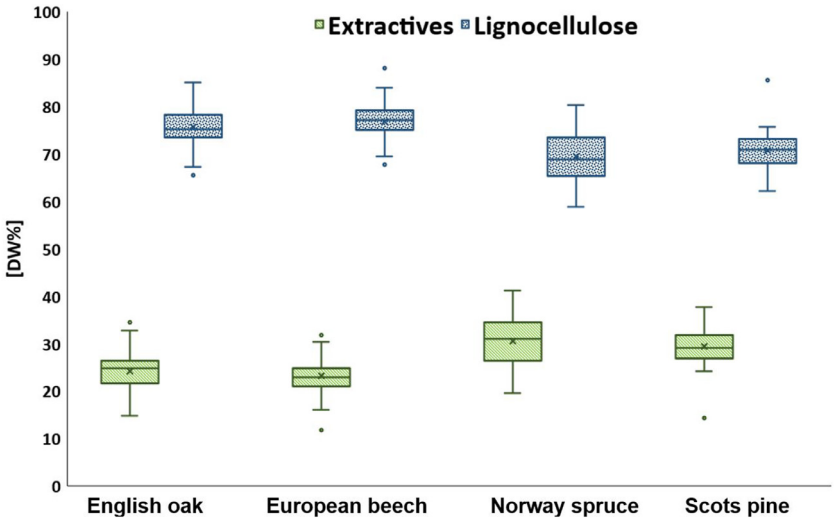

Fig. 3. Variation in extractives and lignocellulose content in leaves across tree species.

Method validation and conclusion

The lignocellulose and extractives content of 343 leaf samples were measured. To ensure precision, each sample was analysed in at least two duplicates and the following criteria were met: (a) The difference in sample weight [g] between duplicates did not exceed 0.01 g. (b) The Coefficient of Variation (CV) was calculated and occasionally repeated until the CV% fell below the 10% threshold. The results were reported as the average of the two duplicates and expressed as a percentage of the total dry-weight biomass (rounded to two decimal places).

The results of the measured lignocellulose and extractives are presented in Fig. 3 and notably among the four tree species, English oak, and European beech (broadleaved) exhibited higher lignocellulose content and lower extractives content when compared to Scots pine and Norway spruce (needle leaf conifers). The measurement ranges obtained generally align with existing literature for various plant tissue species [18,23–28], fortifying the robustness and broader relevance of our findings.

In conclusion, the high-throughput mini-Soxhlet apparatus introduces a versatile modification to the traditional Soxhlet method, innovatively enabling the parallel operation to handle a large number of samples with reduced sample mass. This refined approach expands the overall utility of the Soxhlet method, providing researchers with a valuable tool across diverse research fields.

Recommendations for future studies

Future studies should explore the method's sensitivity across various concentrations using a Reference Material (CRM) and compare the high-throughput mini-Soxhlet apparatus with the conventional Soxhlet. In addition, it is recommended that the method should be investigated on different sample types and, depending on the sample type and desired application, compliance with FDA and EC (US Food and Drug Administration and European Commission, respectively) requirements should be considered.

Ethics statements

The authors declare that no human subjects were involved in the data for this study, no animal experiments were conducted, and no data were collected from social media platforms. Consequently, informed consent, adherence to animal experimentation guidelines, and data redistribution policies were not applicable to this research.

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Declaration of Generative A.I. and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT in order to improve readability of the document. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Alejandra Torres-Rodriguez: Conceptualization, Investigation, Resources, Methodology, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Roshanak Darvishzadeh:** Conceptualization, Supervision, Writing – review & editing. **Andrew K. Skidmore:** Supervision, Writing – review & editing, Funding acquisition. **Erna Fränzel-Luiten:** Methodology. **Benno Knaken:** Methodology. **Boelo Schuur:** Conceptualization, Methodology, Writing – review & editing.

Data availability

The authors do not have permission to share data.

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