## Determination of Silica in Wheat Leaves with ATR-FTIR-Chemometrics

## The beneficial effects of silicon for plants have been noticed for more than a century, but only during the last two decades its role in high productivity agriculture systems has been thoroughly studied. The presence of silicon in plants contributes to the response of stress-relief mechanisms for environmental events such as drought and pathogen attack. Because of the importance of crops such as wheat, barley, rice, and other grasses that accumulate Si, the understanding of the relationship between this element and plant science is the focus of numerous scientific efforts. Quantification of this element is a difficult and costly task and destructive wet chemistry methods are commonly used. With the recent development of chemometric tools, analysis of silicon in complex matrices have been proved feasible.

## In this work, we developed a method for silicon determination in wheat leaves using infrared spectroscopy and chemometrics. Dried leaves grown in a greenhouse were analyzed by means of attenuated total reflection infrared spectroscopy (ATR-FTIR) and inductively coupled plasma-optical emission spectroscopy elemental analysis (ICP-OES).

## A series of models based on multivariate ordinary least squares regression using varying sets of wave numbers selected by a genetic algorithm, was built using baseline corrected ATR-FTIR spectra from wheat leaves samples. Models built with these sets showed a powerful correlation with the silicon content determined by elemental analysis. The performance in prediction of each model was assessed using repeated k-fold cross validation, showing a maximum error of prediction (RMSEP) of 0.1% wt. with minimum model complexity of 4 selected variables. However a strong dependence on the matrix was noted when compared with other plant tissues

## Background

The presence of silicon (Si) in plants contributes to the response of stress-relief mechanisms for environmental events such as drought and pathogen attack. Because of the importance of crops such as wheat, barley, rice, and other grasses that accumulate Si, understanding the relationship between this element and plant polymers has been the focus of numerous scientific efforts.

Silicon is a soil constituent; therefore, plants invariably grow in Si-rich-environments(Epstein, 1994). In soil, Si is available in the form of silicic acid solutions. Some plants accumulate enough silicic acid that Si precipitates as silica, e.g., phytoliths. The accumulation occurs mainly in root endodermis, epidermal cells of both leaf and inflorescence bracts(Kumar et al., 2017), as well as awns and leaf macrohairs(Głazowska et al., 2018).

The molecular forms of Si that are transported through the xylem are known: only monomers and dimers of silicic acid have been found in the shoots of wheat and rice, and no organo-silicon structures have been identified(Casey et al., 2004). Remarkably, the hemicellulose callose may be templating Si deposition in horsetails (*Equisetum*)(Law and Exley, 2011), and may also play a role in Si deposition of algae and plants in general (Brugiére and Exley, 2017).

Si accumulation seems to be an ancient mechanism because it is present in early divergent plant lineages and was lost in the course of evolution as silica is less present in later-evolving plants (Trembath-Reichert et al., 2015). In ancient silica - accumulators such as *Equisetum* species, silica may play a major structural role (Yamanaka et al., 2012). This structural role was taken later in evolution by lignin. As a general tendency, later-evolving plants have more lignin and less silica (Trembath-Reichert et al., 2015). Lignin may fulfill a stronger mechanical role because, unlike silica, it can crosslink with cell wall polysaccharides (Yamanaka et al., 2012).

However, the presence of Si and lignin are not mutually exclusive, and the relationship (if any) unknown. For example, in rice Si deposition starts when the plant cell synthesize and accumulate lignin in the secondary wall (Fang and Ma, 2006; Zhang et al., 2013), while Si surplus is positively correlated with lignin content and mechanical strength (Zhang et al., 2015). In *Brachypodium*, Si surplus induces changes in lignin composition (Głazowska et al., 2018).

In this work, the relationship between the presence, quantity, or chemical structure of lignin and the capacity -or need- of plants to accumulate silica. Particularly, we seek to explore both Si interactions with lignin in wheat (*Triticum aestivum*) and basic Si structures *in-plant* using by laballing the plants with 29Si isotope and study the chemical structures with solid state NMR. Two experiments were established in greenhouse conditions as follows:

## Greenhouse experiment

The experimental design was inspired by an article published in 1999(Rafi and Epstein, 1999). In that experiment, four wheat plants were grown in a 100 ml tank containing 0.5 mM Na2SiO3. Si was absorbed by the plants until it was depleted in ca. 80 days. Another set of plants was grown in a Si-free environment with the same conditions; as there were no stressors, the Si-free plants grew normally. On day 80, the mature Si-free plants were transferred into a solution containing 0.5 mM Na2SiO3. The mature plants absorbed the same amount of Si in three days, as did plants grown in solutions to which Si had been added during their whole life. The experiment was set up both in a greenhouse and in a controlled growing chamber yielding the same result: plants deprived of Si avidly absorbed the element at maturity (when all lignin was in place).

We wondered if there was any difference in lignin content, quantity, and quality between these treatments. Thus, in our experimental design, we included the same treatments: (1) A group grown for 90 days in a solution containing 1.5 mM Na2SiO3 -- this group was called Si+; (2) a group (Si++) grown without Si until day 85, then 1.5 mM Na2SiO3 added to the tank; (3) a control group without silicon (Si-).

**Nutrient solution**: The nutrient solution was prepared according to a Standard Operational Procedure of the greenhouse at the University of Copenhagen in Rolighedsvej. In this procedure, commercial fertilizers are mixed in three different 250 L tanks with the formulations in Table 1. Then, an automatized pumping system mixes different ratios of the solutions in the tanks. The solutions are mixed with ozonized water which contained Si levels of 0.24±0.08 mM SiO2. For the experiment, ca.150 L of the nutrient solution was used weekly. The Si content was measured on every occasion with the method described below. Then 1.5 mM Na2SiO3 was added to 50 or 100 L of solution (depending on the timeline) from a 30 mM stock solution diluted in dm-water, while the same amount of dm-water was added to the controls. The measured Si content after dilution was between 1.5±0.16 mM. At the end of each week, another Si sampling was performed, and the concentrations were always above 0.6 mM, which ensures Si surplus.

pH was adjusted to 6 using HCl or NaOH solutions.

Table 1. Nutrient solution



**Silicon measurements:** Dissolved silica was measured by heteropoly blue method using the Hach protocol 8186 and a DR/1900 spectrophotometer (HACH Company, Loveland, USA) as described by the manufacturer. A Si standard solution of 1.0 mg/L also from Hach (cat 110649) was used for calibration. Polypropylene volumetric flasks were used for dilution of samples to avoid Si contamination.

**Plant production:** Wheat seeds (JB Asano) were surface sterilized as follows. The seeds were immersed in 2.7% sodium hypochlorite solution (v/v) and a drop of Tween 80 for 30 minutes with constant stirring, then rinsed with MilliQ water five times (10 min each). The seeds were vernalized in the dark at 5°C in MilliQ water for four days. After that, the seeds were placed in Si-free oasis horticubes (Smithers-Oasis, Kent, Ohio). The cubes were soaked in 1/10 strength nutrient solution (adjusted to pH 5.5), and excess water drained. Each seed was placed directly into a wet cube. Once seeds started rooted and grew actively (4-7 days), they were left in a cold room for a vernalization period of 50 days.

After vernalization, the plants were cultured using the RainForest 72 aeroponic culture system (GHE, Fleurance, France), and placed in a greenhouse, mean temperature 20°C, under 9 h/15 h light (80–100 μE m−2 s−1)/dark regime. (Figure 1).



Figure 1. RainForest 72 aeroponic culture system (GHE, Fleurance, France) used to produce wheat plants in a greenhouse.

## Greenhouse experiment to test basic Si structures *in-plant* with 29Si isotopic labeling

A replicate of the previous experiment was set up in low-density polyethylene laboratory bottles (Vitlab, Seeheim, Germany) (Figure 2). Random seedlings were selected and carefully washed with the nutrient solution described above. The plants were put in 1 L bottles containing 600 mL of nutrient solution and aerated with an air pump fitted with an air stone (Oxyboost APR300, AQUAEL, Dubowo, Poland). Hydroponic containers were placed in the same greenhouse as described above. The bottles were wrapped in black plastic and aluminum foil to avoid algae proliferation in the nutrient solution.



Figure 2. Greenhouse experiment to test basic Si structures in-plant with 29Si isotopic labeling

The nutrient solution was the same as in the previous experiment except for the Si source. A 29Si-enriched solution was prepared using 99.7% 29SiO2 (CortecNet, France). The solutions were made from Milli-Q water. Solutions were kept in polyethylene containers which were kept sealed and refrigerated. Adjustments to pH were made with HCl or NaOH solutions.

The 29Si-enriched solution was prepared in a stainless steel reactor: 0.3 mg 29SiO2 was mixed with 4 g of molten NaOH. NaOH was melted in the reactor, when the temperature reached 500°C, the SiO2 was added and heated 1 h. After cooling to ambient temperature, the solution was diluted to ca. 9 mM Si, the pH was lowered by dropwise addition of HCl to pH ≈ 8 for 2 days (to allow for depolymerization) and then the pH was further lowered to pH 4 and the solution degassed to produce a stable stock solution of monomeric silicic acid (Swedlund et al., 2009) . Si concentration was measured using a standard Hach kit (Hach Company, Loveland, CO, USA). A non-enriched SiO2 was also prepared for control plants.

600 mL of nutrient solution was added to each bottle, and the Si content adjusted to 1.5 mM SiO2. In the course of one week, ca. 400 mL of water evaporated and Si absorbed by the plants. Then, the solution was again brought to 600 mL and 1.5 mM SiO2. As the availability of the solution containing 29SiO2 was only 3 liters, it was never changed completely but “regenerated” weekly.

**Sample preparation for further analysis:** Periodically, sample plants were harvested and divided between roots, pods, leaves, flag leaves, and inflorescence bracts according to the day of harvesting (Table 2). In 2016, we made the first attempt to perform this experiment. However, the plants did not grow as expected and appeared yellow and not fit. Despite this, we collect the samples to perform some of the analysis and have a secondary data set to test some of the models.

Table 2. Plant parts harvested

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Plant Part**  **Day** | **Unlabelled plants** | | | | **29Si** |
| **60** | **70** | **80** | **90** | **90** |
| Stem | X | X | X | X | X |
| Pods |  |  |  | X | X |
| Flag leaves |  |  |  | X | X |
| Leaves | X | X | X | X | X |
| Inflorescences | X | X | X | X | X |
| Roots | X | X | X | X | X |
| Grain |  |  |  | X | X |

The biomass was dried, ground, and extracted, using the option of *extensive solvent extraction method* published in (Mansfield et al., 2012).

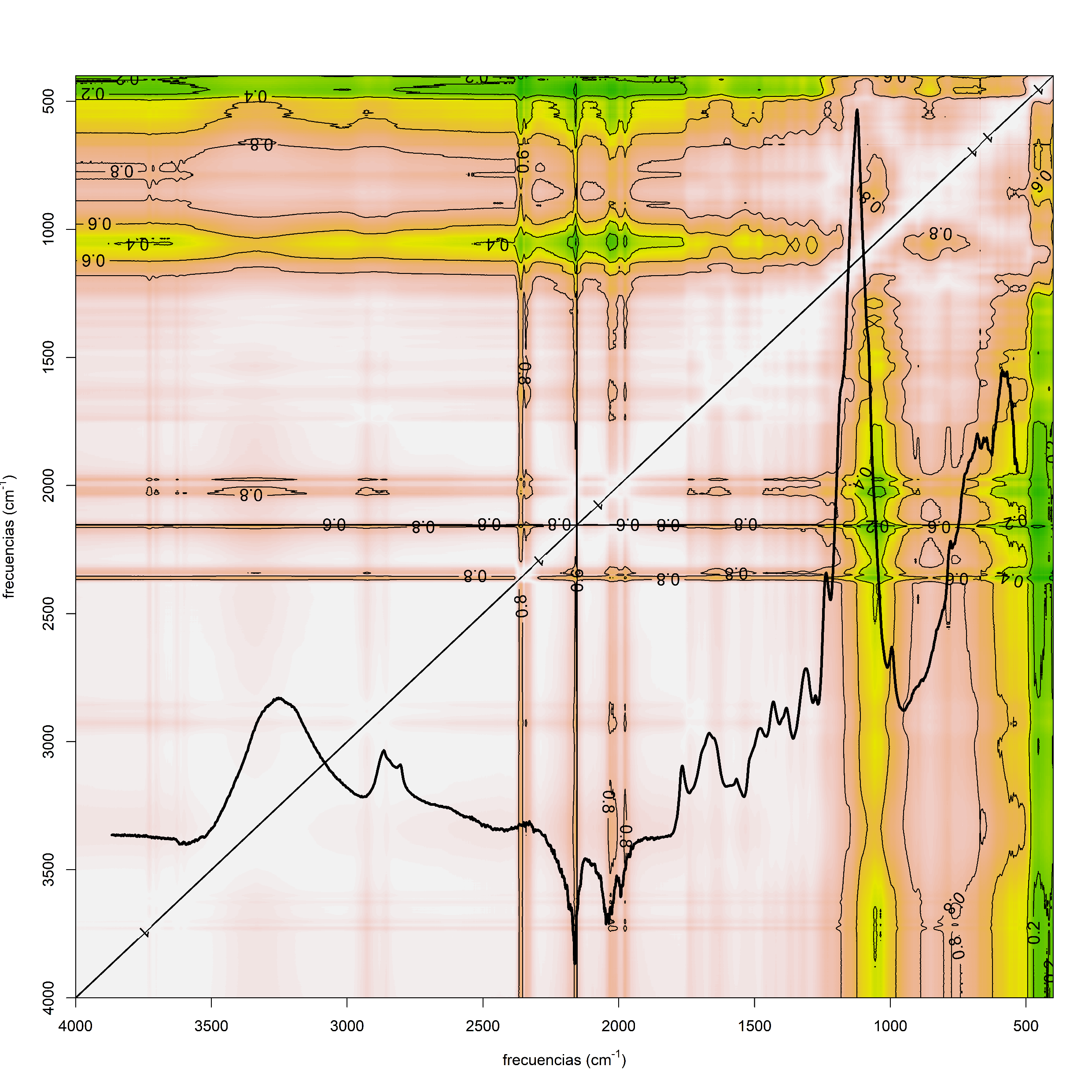
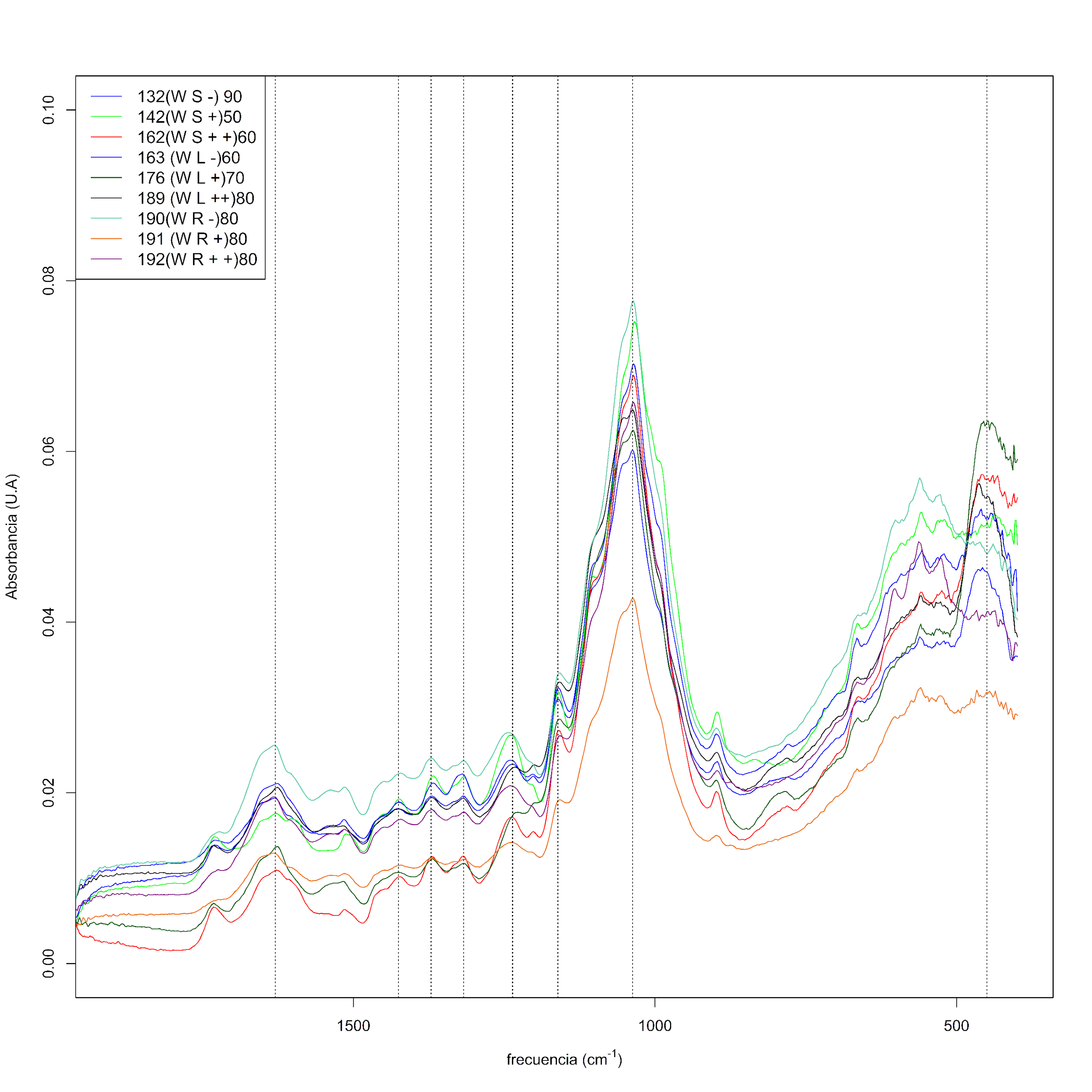
ATR-FTIR

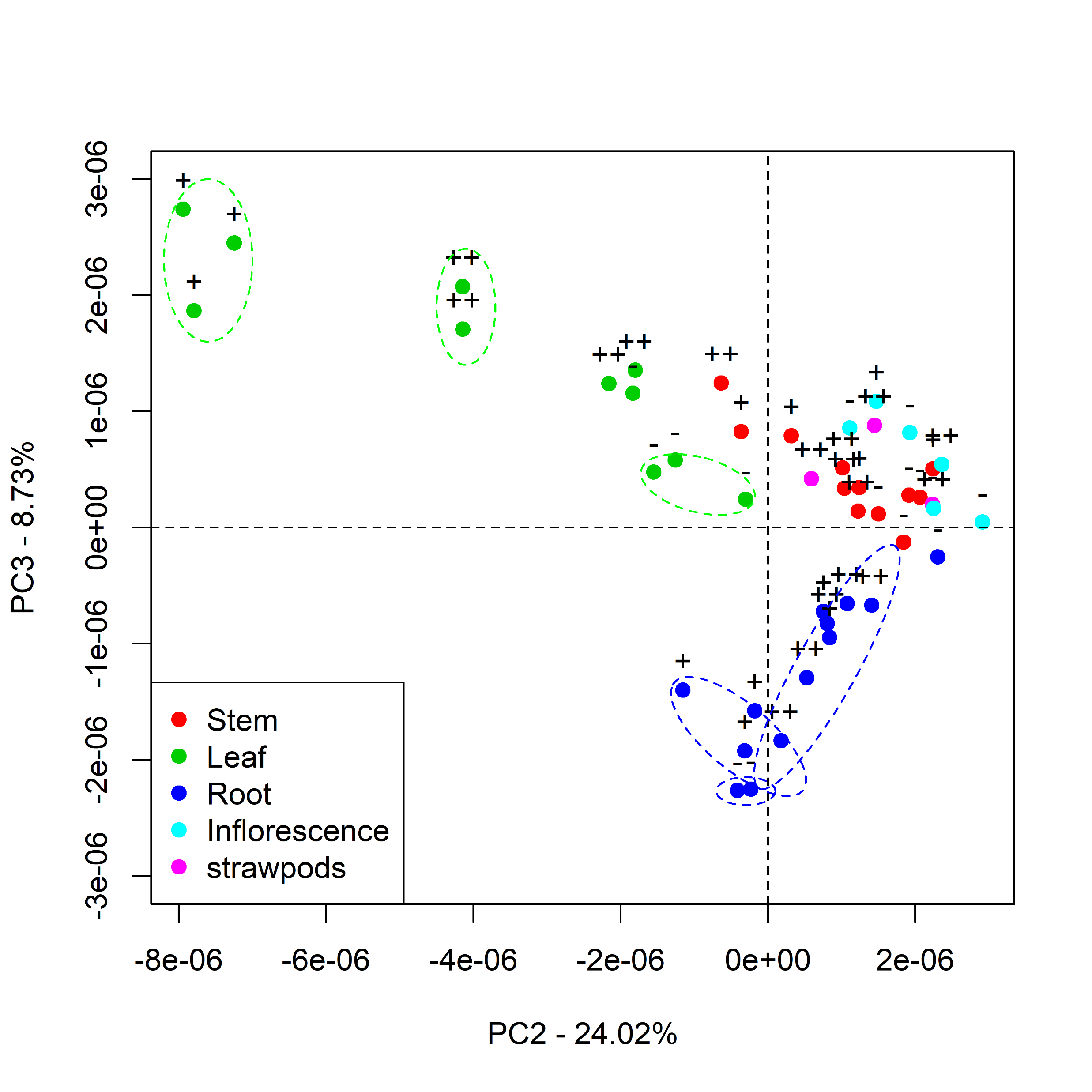
**29Si and 13C NMR Spectroscopy:** Solid-state Nuclear Magnetic Resonance (NMR) spectra were recorded using a Bruker Avance 400 spectrometer (9.4 T) operating at 100.62, 79.49 MHz for 13C, and 29Si respectively, employing a double tuned solid-state probe equipped with 4 mm (o.d.) spinners. The single-pulse (SP) MAS and cross-polarization (CP) MAS spectra were recorded using a spin-rate of 9 kHz, a temperature of 296 K, ramped CP 16 with contact times of 2 ms for 13C and 8 ms for 29Si in acquisition of CP/MAS spectra and 1H TPPM decoupling (80 kHz rf-field strength) 17 during acquisition (49.3 ms for 13C and 42.6 ms for 29Si). Recycle delays and number of scans were 8 s and 1024 or the 13C CP/MAS spectra, whereas these values were 2 s and 8192 and 256 s and 256 for the 29Si CP/MAS and SP/MAS NMR spectra, respectively. For 13C CP/MAS spectra rf-field strengths of 80 kHz were employed for 1H and 13C during CP whereas 1H and 29Si rf-field strengths of 55.6 kHz were utilized during CP for the 29Si CP/MAS spectra. The 29Si SP/MAS spectra were recorded using a pulse with a flip-angle of 54.7 degrees (rf-field strength 55.6 kHz). All 13C MAS spectra were referenced (externally) to the carbonyl resonance in α-glycine at 176.5 ppm, whereas the 29Si MAS spectra were referenced to (externally) to the resonance of 3(-methylsilyl)-1-propanesulfonic acid Na salt at 1.4 ppm. All spectra were apodized by a Lorentzian linebroadening of 10 Hz. The 29Si CP/MAS and SP/MAS spectra were deconvoluted using OriginPro 9.1

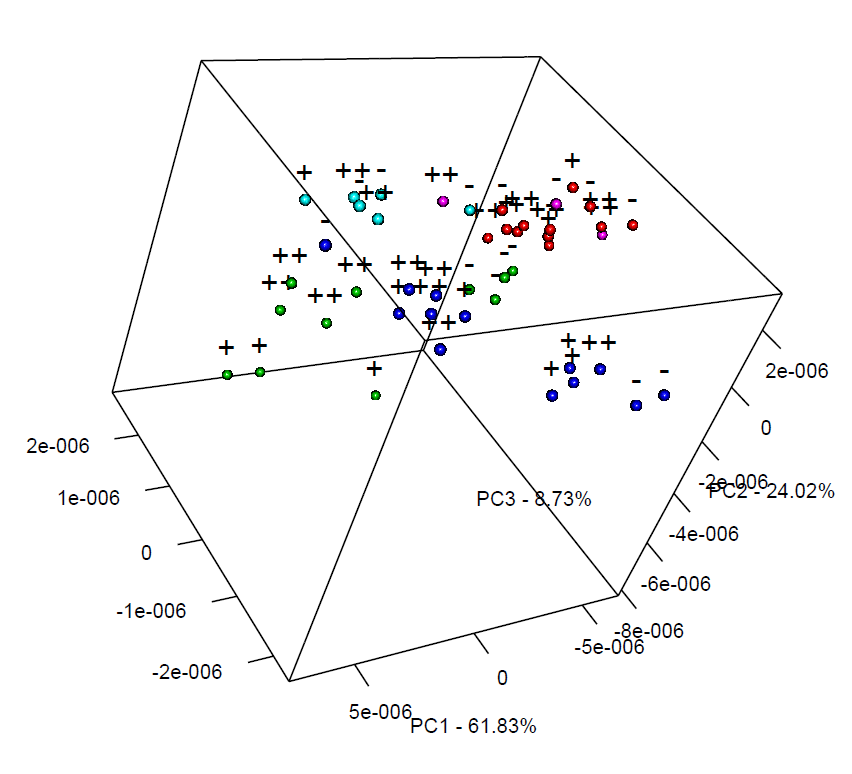
**Lignin quantification:** Lignin was quantified using the double hydrolysis method described by The National Renewable Energy Laboratory in the USA (Sluiter et al., 2008).

**Elemental analysis:** Multi-elemental analyses of the solid samples was performed using inductively coupled plasma-optical emission spectroscopy (ICP-OES). A sample (10–50 mg) was mixed with 500–2500 μL 70% HNO3, 250–1000 μL 15% H2O2 and 40–200 μL 49% HF, and then digested in a pressurized microwave oven for 10 minutes with a starting pressure of 40 bar and a temperature of 240°C. After digestion, samples were diluted to a final 3.5% acid concentration with Milli-Q water before measurement on an ICP-OES (Model Optima 5300 DV, PerkinElmer) equipped with a HF-resistant sample introduction kit. For quantification, an external 10-point calibration standard P/N 4400-132565 and P/N 4400-ICP-MSCS (CPI International, Amsterdam) was used. A certified reference material (CRM) NCS 73013 Spinach leaf was analyzed together with the samples to evaluate the accuracy and precision of the analysis.

**Carbon and nitrogen analysis:** Total C and N concentrations were analyzed by combustion at 1150 °C using a vario Macro cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). Dry samples (20-50 mg) were weighed into tin capsules. Data quality was evaluated by analyzing standard reference materials (141d acetanilide, National Institute of Standards and Technology, Gaithersburg, MD, USA).



Results



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