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Effects of successive microwave and enzymatic treatments on the release of p-hydroxycinnamic acids from two types of grass biomass

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Abstract

Biomass recalcitrance is one of the main bottlenecks in lignocellulosic biorefinery deployment. Physico-chemical pretreatments and enzymatic hydrolysis are two procedures that can be combined to overcome this recalcitrance. In this study microwave pretreatment has been selected for its relevant conditions that allow for biomass recalcitrance to be reduced, along with the maintenance of a low consumption of energy and reactants. A xylanolytic enzymatic cocktail, Rovabio® Advance, was investigated for its ability to hydrolyze maize stalks and *Miscanthus* leaves after pressurized, chemical-free microwave pretreatment. This combination was implemented to increase the breakage of ester bonds and thus facilitate the release of *p*-hydroxycinnamic acids. This study demonstrates how, in comparison with *Miscanthus*, both pretreatments are more effective in releasing *p*-hydroxycinnamic acids from maize stalks, due to their lower parietal content. The successive free-chemical process seems to be particularly promising on maize stalks, since it led to a ferulic acid release yield of 18.2%, compared to 5.5% for microwave pretreatment only or 7.6% when performing enzymatic hydrolysis without a microwave pretreatment step.

Key words: Enzymatic hydrolysis, grass biomass, *p*-hydroxycinnamic acids, microwave pretreatment

I. Introduction

EU dedicated policies and measures to meet the energy and climate targets for 2030 [1] have greatly aroused interest in lignocellulosic biomass (LCB) utilization for bioenergy and green chemistry applications since the last two decades. Nonedible LCB could as a matter of fact successfully substitute at least partially fossil-based raw materials for the chemical, material and energy sectors and even bring new functionalities to the market [2, 3]. Together with abundancy, low price and durability, nonedible LCB is interesting because of its polymeric carbohydrates content, as well as secondary metabolites content. LCB is a versatile feedstock that can be converted in intermediate platforms, building blocks, secondary chemicals, among other products. However, the recalcitrance of LCB – i.e. its natural ability to counteract parietal polymer degradation due to external attacks – is one of the main bottleneck that limits its successful use as raw material for bio-based products. LCB is essentially made up of the carbohydrate polymer cellulose and hemicellulose and aromatic polymer lignin, which strongly interact with one another through covalent and hydrogen bonds.

In order to be efficiently transformed into energy and/or chemical intermediates, LCB should first be pretreated so that polymeric components become more accessible for further chemical conversion [4, 7]. This pretreatment phase is only essential in soft physico-chemical conversions, *i.e.*, under low pressure and temperature conditions, or with low reagent concentrations. Indeed, under high pressure, high temperature or in very concentrated acids or bases, the biomass is completely broken down and pretreatment is no longer necessary. Extensive worldwide research is ongoing to address this problem. Numerous pretreatment technologies, such as mechanical, thermal, chemical, biological, etc., or a combination of several have been studied [4, 6, 8]; however, to date, the “ideal” technology has not yet been identified.

In nature, LCB is mainly broken down by the depolymerization activity of enzymes (ligninolytic, hemicellulolytic and cellulolytic). These enzymes are produced and secreted by bacteria and fungi, which are capable of degrading the highly resistant lignocellulosic cell walls of plants [9, 10]. Enzymatic hydrolysis of cellulose, the first and unavoidable step in biological conversion, is essentially limited by the structure and porosity of cell plant walls, which reduce the accessibility of enzymes to their substrate [5, 11]. In addition, hemicellulose and lignin that interact and protect cellulose, are substituted by *p*-hydroxycinnamic acids (*p*HCA): *para*-coumaric acid (*p*CA), ferulic acid (FA) and their derivatives, which play an important role in the structural cohesion of the parietal network, in the protection against predatory attacks and in limiting cellulolytic enzyme accessibility to cellulose [12, 13].

The presence of FA ester-bound to polysaccharides (arabino- and glucurono-arabino-xylans) in the cell wall is a characteristic feature of monocotyledons. It represents about 0.5 %DM (dry matter) to 1 %DM of wheat or maize straw, 1 %DM of wheat bran and up to 3 %DM of the pericarp of maize grains [14-16]. However, FA is a noteworthy molecule for its potential in green chemistry, particularly in cosmetics or pharma applications, or as a precursor of vanillin. The latter molecule has a high added value when produced naturally [17-20]. FA can also be connected to lignin via ether covalent links, forming a bond between lignin and hemicellulose which further complicates the structure of the biomass. In all cases, FA (as well as *p*CA) percentages remain low in comparison with the parietal polymer contents (cellulose, hemicellulose and lignin) that represent up to 90 %DM in *Miscanthus* leaves and 65 %DM in maize stalks [21].

The advantage of physical pretreatments versus chemical pretreatments is based on the fact that they do not involve the use of chemicals and thus are regarded as more environmentally friendly. However, certain can be very energy demanding, such as grinding. In an industrial process, often, the grinding stage is not economically feasible [22]. Other physical pre-treatments that consume less energy are therefore being optimized. Extrusion and microwave technology are known processes for uses other than biomass pretreatments: indeed the extrusion process is applied to plastic and food industries [23] while microwave technology is applied to wood drying [24] among other applications. In both cases, the processes should be adapted to the type of biomass pretreatment and optimized in order to become economically competitive.

In this study, focus was put on developing a chemical-free microwave pretreatment followed by enzymatic hydrolysis. In previous studies, microwave pretreatment alone was tested and optimized in order to free *p*-hydroxycinnamic acids: the resulting yields did not exceed 5 % in the case of ferulic acid extracted from maize stalks [21, 25]. A recent study on microwave pretreatment applied to lignocellulosic biomass has shown that microwaves are a promising pretreatment method [26], but works concerning phenolic acids release are scarce (10% of the mentioned works). The objective of our study is therefore to conduct successive chemical-free microwave and a biological treatment in order to increase the extraction yields of *p*-hydroxycinnamic acids while preserving their properties.

Following microwave pretreatment, enzymatic hydrolysis should release a larger amount of *p*-hydroxycinnamic acids. Enzymes operate primarily in synergy with other enzymes produced by microorganisms in order to significantly attack the complex heterologous biomass structure. This is particularly true for hemicellulose-active enzymes, not only because of the structural complexity of hemicellulose, but also because they are interconnected with the other LCB constituents *via* covalent bonds [27]. Feruloyl esterases (FAE) catalyze the hydrolysis of the ester bond between a monomeric or dimeric *p*-hydroxycinnamic acid moiety and a sugar moiety, part of the pectin or arabinoxylan within the plant’s secondary cell wall [28]. Most FAEs have been observed to be more effective on feruloylated oligosaccharides generated by the action of a xylanase [29].

Recent studies pointed out that integrated approaches combining two or more pretreatment technologies can enhance the conversion yield and product selectivity, while the production of inhibitors is reduced [17, 30-32]. In the present study, a successive chemical-free microwave - enzymatic pretreatment was tested with the aim of performing efficient disruption of grass lignocellulosic biomass to then increase the enzymatic release of *p*-hydroxycinnamic acids. *Miscanthus* leaves and maize stalks were chosen for their high *p*-hydroxycinnamic acid content [28] and for their noteworthy properties: sustainable resource, good productivity, possibility of cultivation on marginal lands with a relatively low demand for inputs [2]. Microwave technology was selected as a physical pretreatment because of its numerous advantages including volumetric and non-contact heating, low reactant consumption, fast reaction time [24, 33, 34] among others, according to recent studies [21, 25]. Microwaves are electromagnetic waves that move in the propagation medium at a speed close to that of light ($3.108 m.s^{-1}$). They are composed of an electric field and a magnetic field perpendicular to each other and oscillating at the same frequency, between 300MHz and 30GHz, with a wavelength between 1m and 1cm [33]. Microwave heating occurs via two main mechanisms; dielectric heating via the orientation of polar molecules (dipoles) and heating by ionic conduction via charged chemical species (cations and anions), following the electric field. As the field oscillates, the molecules also oscillate to orient themselves according to the electric field. Their rapid movement in the material dissipates energy which is translated into a heating of the material. Contrary to the classical modes of heating by conduction or convection, microwave heating is extremely fast and concerns the global volume of the material (and not only the surface) [35].

Moreover, separation and purification processes after microwave pretreatment will be easier compared to ultrasounds or extrusion, because with microwave pretreatment no colloidal (or very thin) particles are produced.
The innovative approach of this work is to compare *p*-hydroxycinnamic acid yields obtained after applying (i) only microwave pretreatment, (ii) only enzymatic hydrolysis or (iii) microwave pretreatment followed by enzymatic hydrolysis. Another novelty in this study was to understand how biomass composition and organization affect the *p*-hydroxycinnamic acid release yields.

II. Material and methods

II.1 Raw Biomass and Reagents

Maize stalks genotypes (F 98902 and F 7025) were supplied by INRAE IJPB (Versailles-Grignon unit, Versailles Cedex, 78026, France) and were harvested in September 2016.
Miscanthus leaves *(M. x giganteus Britannique* noted GIB, *M. x giganteus Floridulus* noted FLO and *M. sinensis Rotsilber* noted ROT) were supplied by INRAE AgroImpact (Estrées Mons experimental unit, Péronne, 80203, France) and were harvested in February 2017.
Air-dried samples of biomass were coarsely crushed (Viking, model GE 220, STIHL, Stuttgart, Germany) before being finely ground to 1 mm (using a Fritsch Pulverisette 19) and sieved to retain only particles between 200 µm and 1000 µm (Figure 1). Ground and sieved samples were stored in closed boxes at ambient temperature until use. Biomass compositions were compared between 2016/2017 and 2018 (date of the study) in order to ascertain whether storage had an impact on composition. As no significant differences were detected (results not shown), biomass compositions were considered to be identical between the harvest date and 2018.

The commercial enzymatic cocktail, Rovabio®, was provided by Adisseo CINABio (Toulouse, France) and consisted of an enzymatic mixture of endo-1,4-xylanase, feruloyl and acetyl esterases, and endo-1,3,(4)-glucanase.

All chemicals were purchased from Merck. Ultrapure water (Merck Millipore Quantum TEX) was used for all pretreatments and analyses.

II.2 Microwave pretreatments

Microwave pretreatment was performed using a Minilabotron 2000 (SAIREM, FRANCE), operating at 2.45 GHz with a maximum power of 2 kW. This equipment was used to pretreat biomass using a PTFE reactor, which does not absorb microwaves (PTFE/TFM.BOLA (T18) hydrolyzing digestion vessel with liners, Cat. No. A250-08), can be tightly closed and supports pressures up to 20 bar (Figure 2). The pressure reactor (G) consists of a reaction vessel (E) and a cover composed of a part that screws onto the lower part (A) and a plug (B) that serves as a safety valve. Two safety membranes (C and D) placed between the tank and the cover ensure the tightness of the pressure reactor. These membranes can withstand a maximum pressure of 20 bar.

In a previous study [21], optimal conditions were determined to reach temperatures higher than 100 °C without exceeding a pressure of 4 bar, so as to ensure that the temperature does not rise above 150 °C, which could damage some biomass components, *i.e.,* the *p*-hydroxycinnamic acids [36]. 2 gDM of biomass were added to 40 ml ultrapure water in the reactor corresponding to a 4.7 %DM S:L ratio. Neither magnetic nor mechanical stirring were implemented in the vessel, since the reaction medium self-mixes during boiling. Samples underwent one hour of pre-soaking at ambient temperature before microwave treatment, which lasted 180 seconds at 300 W. The final pressure did not exceed 4 bar, corresponding to a peak temperature of 150 °C, thus preventing the degradation of *p*-hydroxycinnamic acids. Pressure and temperature could not be directly monitored during tests but were calculated [21]. In order to have sufficient pretreated biomass to perform further analyses and due to the size limitation of the pressurized microwave vessel, the treatment was carried out on five samples which were pooled. Microwave pretreatments under these specific conditions were identified as PMW (pressurized microwave).

Folowing the treatment, the vessel was air-cooled for 15 min before weighing and opening. The reaction medium was filtered through a 200 µm sieve; solids were washed with 300 mL of ultrapure water to remove by-products. The solid fraction was dried in an oven for 72 hours at 40 °C. The dry matter content (DM) was then measured in order to assess the amount of dissolved matter during processing. The amount of remaining solid matter ($g_{\text{pretreated solid biomass}}/g_{\text{raw matter}}$) was an indicator of the effectiveness of the treatment, as it indicates the extent of solubilization of the parietal content after microwave pre-treatment. Finally, the liquid fraction was filtered through a cellulose filter (2.7 µm) and stored at -20°C until the quantification of *p*-hydroxycinnamic acids.

II.3 Enzymatic hydrolysis

To access cell wall biodegradability and to release *p*-hydroxycinnamic acids, raw and PMW treated biomass were subjected to enzymatic hydrolysis using the commercial enzyme cocktail, Rovabio®, primarily designed for the modification of *p*-hydroxycinnamic acids in maize tissues to enhance animal digestion; therefore this cocktail was selected because maize stalk was the studied biomass. Hence, although improved efficiency of the cocktail on maize stalks was expected compared to its effect on *Miscanthus* leaves, this cocktail was also applied to *Miscanthus* leaves for sakes of comparing results.

According to the method of utilization supplied by Adisséo, this is an enzymatic mixture of xylanase-1,4, ENDO-, Glucanase-1,3,(4), ENDO-β between 10,000 and 12,000 U/g (assayed by DNS method on birch xylan) and phenolic acid esterases.

Biomass (62 mg) was placed in hemolysis tubes with 2.5 ml ultrapure water. After one hour of soaking at ambient temperature (20°C) the Rovabio® cocktail (50U xylanase-equivalent/gDM) was added, and the medium was incubated at 50 °C, 110 rpm for 24 h. Subsequently, the suspension was centrifuged (12000 g, 5 min) and the supernatent was harvested and stored at 4°C and -20°C for futher analysis. Reactions with and without enzymes were performed in triplicate.

The aim of this study was not to optimise the enzymatic conversion, but to highlight the beneficial effect, absence of effect, or negative effect of chemical-free microwave pretreatment (at atmospheric pressure or under 4 bar) on the release of *p*-hydroxycinnamic acids in a successive physical - enzyme treatment. Thus, the enzymatic hydrolysis operating conditions were implemented in such a way that the effect of the hydrolysis was visible without completely erasing the effect of the microwave pretreatment. In order to better understand the study, Figure 3 summarises the three processes tested:

II.4 Biochemical analysis

The composition in parietal polymers of the biomass was determined before and after treatment using the Van Soest protocol [37]. This protocol is based on mass sequential partitioning of cell walls, from most extractible to less extractible, with successive extractions using different solvents (ultrapure water, neutral detergent solution, acid detergent solution and sulfuric acid 72 %).

Ferulic acid (FA) and *para*-coumaric acid (*p*CA) were quantified by HPLC in duplicate using an HPLC-DAD Waters system: autosampler 717, multisolvent delivery system 600, Diode Array Detector 2996. *p*-hydroxycinnamic acids (*p*HCA) were detected at 320 nm and peak areas calculated using Empower3 software (Waters). The mobile phases consisted of ultrapure water/formic acid - 95/5 (v/v, Solvent A) and acetonitrile/ultrapure water/formic acid - 80/15/5 (v/v/v/, Solvent B). The flow rate was 1 ml/min and the sample injection volume was 10 µl. Separation was performed at 30°C on a Waters Atlantis T3 Column, 100 Å, 5 µm, 4.6 mm X 250 mm (C18) equipped with a C18 - 4x3 mm Security Guard Cartridge (Phenomenex, France). Results were expressed in mg_{pHCA}/g_{DM} (equation [1]).

$$pHCA\left(\frac{mg}{g}\right)=\frac{pHCA\ (mg/l)\times \text{Total}\ Volume\ sample\ (l)}{initial\ biomass\ mass\ (g)} \qquad [1]$$

FA and *p*CA released after physco-chemical pretreatment or enzymatic hydrolysis were quantified in the liquid phase. Initial amounts of ester bonded FA and *p*CA were quantified after a mild alkaline extraction. Briefly, 20 mg raw matter were soaked during 15 hours in 2 ml NaOH 2 N to allow the release of the esterified *p*-hydroxycinnamic acids [38-40]. *p*HCA release yields were calculated (equation [2]):

- after microwave pretreatment (*p*HCA release exclusively due to microwave action);
- after enzymatic hydrolysis (*p*HCA release due to the action of enzymes.).

$$pHCA\ yield\ (\%)=\frac{\text{released}\ pHCA\ \left(\frac{mg}{g}\right)}{initial\ pHCA\ \left(\frac{mg}{g}\right)} \qquad [2]$$

In this study, cell wall biodegradability was used to determine the effect of enzymatic hydrolysis on biomass structure [41]. Cell wall biodegradability was defined by the reducing sugar content that could be quantified in enzymatic hydrolysis supernatants using the 3,5 Dinitrosalycilic acid method (DNS) [42]. One volume of supernatant was mixed with two volumes of DNS and heated to 95 °C for 10 min. The optical density was then measured at 540 nm using a TECAN SPARK spectrophotometer. Results were expressed in mg of xylose equivalent reducing ends per gDM of biomass. The natural release of reducing ends occurring in ultrapure water alone, in the same operational conditions as for enzymatic hydrolysis, was consistently deducted in the results.

II.5 Statistical analysis

All statistical analyses were performed using R (3.4.0) software. Anova and Tukey tests were used to determine the effect of biomass on *p*HCA release. An effect was considered significant if the *p*-value was less than 5%.

III. Results and Discussion

III.1 Raw and pretreated biomass biochemical composition

Biomass composition was analyzed for the distribution of parietal polymers before and after microwave treatment (Table 1).

Table 1: Raw and pressurized microwave pretreateds (PMW) biomass parietal composition analyzed by the Van Soest method

		Raw biomass					**PMW biomass**				
		Maize 98902	**Maize 7025**	***Miscanthus* GIB**	***Miscanthus* FLO**	***Miscanthus* ROT**	**Maize 98902**	**Maize 7025**	***Miscanthus* GIB**	***Miscanthus* FLO**	***Miscanthus* ROT**
Soluble content	**%DM**	**37.30 ± 1.9**	**35.70 ±1.3**	**8.29 ± 0.2**	**5.77 ± 2.4**	**6.63 ± 4.5**	**43.05 ± 3.8**	**39.95 ± 4.2**	**15.43 ± 4.6**	**13.41 ± 3.8**	**13.98 ± 2.6**
PMW soluble content	%DM	/	/	/	/	/	34.61 ± 3.8	34.45 ± 4.2	15.27 ± 4.6	13.01 ± 3.8	9.56 ± 2.6
VS[a] soluble content	%DM	37.30 ± 1.9	35.70 ±1.3	8.29 ± 0.2	5.77 ± 2.4	6.63 ± 4.5	8.44 ± 0.4	5.50 ± 0.4	0.16 ± 0.1	0.40 ± 0.3	4.42 ± 1.0
Cell wall	**%DM**	**62.70 ± 1.9**	**64.30 ± 1.3**	**91.71 ± 0.2**	**94.23 ± 2.4**	**93.37 ± 4.5**	**56.96 ± 0.4**	**60.05 ± 0.4**	**83.56 ± 0.1**	**86.59 ± 0.3**	**86.02 ± 1.0**
Hemicellulose	%DM	26.00 ± 0.9	30.09 ± 1.5	22.91 ± 3.6	25.86 ± 0.4	35.68 ± 1.2	16.59 ± 2.2	27.19 ± 0.3	22.13 ± 0.4	24.58 ± 0.8	32.85 ± 0.7
Cellulose	%DM	28.51 ± 0.9	27.36 ± 1.5	52.78 ± 3.5	51.78 ± 1.6	47.33 ± 2.4	28.96 ± 2.1	25.17 ± 1.8	40.62 ± 1.5	34.58 ± 1.2	43.48 ± 1.2
ADL[b]	%DM	6.85 ± 1.5	5.30 ± 1.0	15.46 ± 0.4	16.21 ± 0.8	10.14 ± 2.1	10.13 ± 0.5	6.51 ± 1.2	19.95 ± 1.4	26.42 ± 0.8	9.16 ± 0.9
Ash	%DM	1.18 ± 0.4	1.55 ± 0.6	0.56 ± 0.4	0.38 ± 0.38	0.22 ± 1.9	1.27 ± 0.2	1.18 ± 0.1	0.86 ± 0.1	1.00 ± 0.1	0.53 ± 0.3

Data are expressed as g of parietal component per 100 g of initial dry biomass matter (%DM) ± standard deviation

[a]: Van Soest

[b]: Acid Detergent Lignin

As indicated in Table 1, raw maize stalks and raw *Miscanthus* leaves were very different in terms of parietal composition. Raw *Miscanthus* leaves had a low soluble content (from 5.77 %DM for *Miscanthus* FLO to 8.29 %DM for *Miscanthus* GIB) in comparison to raw maize stalks which contained 35.70 %DM for maize 7025 and 37.30 %DM for maize 98902. The high parietal content of *Miscanthus* leaves was mainly due to their high cellulose (from 47.33 %DM for *Miscanthus* ROT to 52.78 %DM for *Miscanthus* GIB) and lignin (from 10.14 %DM for *Miscanthus* ROT to 16.21 %DM for *Miscanthus* FLO) contents, which were about two fold higher than for maize 98902 stalks (28.51 %DM for cellulose and 6.85 %DM for lignin). These results from maize stalks and *Miscanthus* leaves are consistent with those provided by Van der Weijde *et al.* [43] and Pang *et al.* [44].

After PMW (pressurized microwave pretreatment), PMW-treated biomass composition was modified compared to both raw biomasses. For all pretreated biomass samples, the total soluble content (sum of treatment-solubilized soluble fraction and Van Soest soluble fraction), was higher than the raw biomass Van Soest soluble content, thus pointing out cell wall solubilization. For *Miscanthus*, the soluble fractions doubled following microwave treatment: they increased from 8.29 %DM to 15.43 %DM for *Miscanthus* GIB, from 5.77 %DM to 13.41 %DM for *Miscanthus* FLO and from 6.63 %DM to 13.98 %DM for *Miscanthus* ROT. This increase in the *Miscanthus* soluble fraction results from the solubilization of the parietal cellulose component during microwave pretreatment, as highlighted in Table 1. Moreover, apart from *Miscanthus* ROT (which has a higher hemicellulose content than the GIB and FLO), the lignin content increased, indicating that chemical-free pressurized microwave pretreatment did not induce delignification, or very slightly (for *Miscanthus* ROT). VS soluble contents of the pretreated samples were very low, in particular for *Miscanthus* GIB (0.16 %DM) and *Miscanthus* FLO (0.40%DM), compared to PMW treatment-solubilized soluble contents (15.27 %DM for *Miscanthus* GIB, equivalent to 99% of the total soluble content and 13%DM for *Miscanthus* FLO, equivalent to 97% of the total soluble content). For *Miscanthus* ROT, the VS soluble content after pretreatment was 4.42 %DM, while the treatment-solubilized soluble content was 9.56% DM, equivalent to 68.4% of the total soluble content. As a result of the solubilization, PMW-treated *Miscanthus* cell walls (DM) decreased respectively 8.1% for *Miscanthus* GIB, 7.64% for *Miscanthus* FLO and 7.35% for *Miscanthus* ROT.

Thus, pressurized microwave pretreatment enhanced the solubilization of cellulose from *Miscanthus* cell walls more than hemicellulose, in contradiction to Boonmanumsin *et al.* [45]. These authors studied *Miscanthus sinensis* pressurized microwave pretreatment (120 °C for 15 min) and also observed an alteration in the composition of the biomass after the treatment. Nevertheless, according to the study, the changes in cell wall composition, which included hemicellulose solubilization and delignification, could result from the use of alkaline agents (ammonium hydroxide, NH_4OH) during the first steps of pretreatment. Indeed, alkali-based treatments promote the saponification of intermolecular ester bonds cross-linking xylan hemicellulose and lignin, resulting in biomass delignification [46]. In the present study, cellulose solubilization was mostly observed, as the decrease in cellulose content in the solid parietal fraction following liquid-solid phase separation was stronger than for the other fractions. The cellulose decrease in parietal residues was higher for *Miscanthus* GIB and *Miscanthus* FLO (-12.16 % and -17,2% respectively, as % of parietal component of initial biomass dry matter) and lower for *Miscanthus* ROT (-3.85%).

In the case of maize stalks, as for *Miscanthus* leaves, a partial parietal solubilization was observed following microwave pretreatment: soluble fractions increased from 37.30 %DM to 43.05 %DM for maize 98902 and from 35.70 %DM to 39.95 %DM for maize 7025. This parietal solubilization can mostly be explained by hemicellulose solubilization: hemicellulose decreased in cell walls from 26.00 %DM to 16.59 %DM for maize 98902 and from 30.09 %DM to 27.19 %DM for maize 7025. As for wheat straw, Fan *et al.* [47] found that 50 %DM of the biomass could be solubilized after pressurized microwave pretreatment at 190 °C, thus confirming the effect of pressurized microwave on biomass composition.

For both maize stalks and *Miscanthus* leaves (except for *Miscanthus* ROT), the lignin content increased from 6.85 %DM to 10.13 %DM for maize 98902, from 5.30 %DM to 6.51 %DM for maize 7025, from 15.46 %DM to19.95 %DM for *Miscanthus* GIB and from 16.21 %DM to 26.42 %DM for *Miscanthus* FLO. An increase in the proportion of lignin (0.6 % – 3.9 %) was also observed by Pang *et al*. [44] who worked on a maize stover and combined pressurized microwave and steam explosion at 190 °C for 5 min. These strong increases in ADL proportions could be explained by a variation in the lignin chemical bonds. Indeed, the lignin component solubilized in the ADS fraction and thus not accounted for in the Acid Detergent Lignin (ADL), is correlated to β-O-4 lignin bonds [48]. If the ADL fraction should increase, this could imply that the chemical bonds within the lignin have been reorganized due to the microwave pretreatment [25]. The chemical links between lignin units could be analyzed by conducting a thioacidolysis.

III.2 Release of *p*-hydroxycinnamic acids after microwave pretreatment

After MW pretreatment, the liquid phase was analyzed by HPLC to measure the amounts of solubilized *p*-hydroxycinnamic acid (*p*HCA). In Table 2, initial *p*HCA in raw biomass is presented and expressed as mg_{pHCA}/gDM. The content of each *p*-hydroxycinnamic acid (FA or *p*CA) in the liquid phase following treatments was expressed in mg_{pHCA}/l. As closed vessel was used, no evaporation occurred and the swelling volume was considered to remain equal to 1 ml/gDM for all tests. Using equation [3], the liquid collected after treatment was estimated to be 38 ml for all tests.

$$Collected\ liquid\ (\mathrm{l}) = Initial\ V - swelling\ V = 40 - 2 = 38\ ml \qquad [3]$$

The collected volume was used for calculating the extraction yields, expressed in $mg_{p\mathrm{HCA\ released}}/g_{p\mathrm{HCA\ initial}}$ (equation [2]) using the pretreated solid mass.

Table 2: p-Hydroxycinnamic acids (pHCA) release after microwave pretreatment

	Raw Biomass (mg_{pHCA}/gDM)		***p*HCA release in pretreated liquid (mg/l)**		**Extraction yield (% $mg_{pHCA\ released}/g_{pHCA\ initial}$)**	
	FA	***p*CA**	**FA**	***p*CA**	**FA**	***p*CA**
Maize 98902	4.2 ± 0.1	13.1 ± 2.6	12.22 ± 0.0	29.32 ± 0.1	5.48	4.21
Maize 7025	3.5 ± 0.0	8.4 ± 0.4	0.61 ± 0.0	3.10 ± 0.0	0.33	0.7
***Miscanthus* GIB**	2.1 ± 0.5	6.5 ± 2.4	0.73 ± 0.0	16.84 ± 0.2	0.65	4.9
***Miscanthus* FLO**	2.8 ± 0.3	5.8 ± 2.1	0.79 ± 0.0	13.81 ± 0.6	0.45	3.05
***Miscanthus* ROT**	3.1 ± 0.8	7.4 ± 1.6	2.37 ± 0.2	21.96 ± 2.1	1.53	5.56

According to Table 2, observed *p*CA concentrations (in mg/l) in the liquid phase were higher than FA concentrations for all tests. This could result from the covalent links within the structure of the biomass: FA is esterified and etherified to cell wall polymers whereas *p*CA is almost exclusively esterified to cell wall polymers with a small proportion (less than 10%) etherified to cell walls [27, 49, 50]. Ester links can be broken by mild alkaline hydrolysis (5 mL of 2 M NaOH, room temperature, 20 h), while the hydrolysis of ether links requires more severe conditions (5 mL of 4 M NaOH, 170 °C for 2 h) [51, 52]. This confirms that, inside the biomass structure, *p*CA was more easily accessible than FA, even though no alkaline hydrolysis was performed in the present study. Moreover, microwave treatment was widely studied during the nineties, when it seemed relevant for the determination of total *p*HCA, in various types of biomass, to break covalent bonds, whether ester or ether bonds [49].

Therefore, for all biomass samples, excepting maize 98902, *p*CA extraction yields were higher than FA extraction yields, thus confirming higher *p*CA accessibility subsequent to thermal pretreatment.

FA extraction yields were higher but similar to those obtained by Moreira *et al*. [53] who worked with brewer's spent grains and reached a 0.953 % FA yield after 20 min at 80 °C. In the present study, pressurized treatment might explain the yields that are higher than those obtained by Moreira *et al*. [53]. However, the characteristics of the raw biomass are undoubtedly the main explanatory factor for this difference.

To understand the biomass composition effects on *p*HCA solubilization, Anova tests were performed. In the tested conditions, FA and *p*CA releases (expressed in mg/l) were significantly dependent on the biomass, with *p*-values equal respectively to $6.33.10^{-10}$ and $1.05.10^{-5}$. Was the difference due to a species (maize or miscanthus) or to a genotype or a clone itself? A post-hoc statistical test (Tukey test) was conducted to identify the specific biomass responsible for this difference. When compared to maize 7025 or all *Miscanthus* leaves (*Miscanthus* GIB, ROT or FLO), maize 98902 was the biomass sample that allowed for the highest amount of *p*HCA to be directly released into the liquid phase. The *p*HCA release differences between biomass were not coincidental, but rather depend on the characteristics of the raw biomass.

Microwave pretreatment could produce a greater impact on maize 98902 than on other types of biomass due to its parietal organization. Indeed, as demonstrated in the "biomass biochemical composition" item, maize 98902 had 30 % less parietal components than

Miscanthus leaves. Since parietal components are responsible, to a large extent, for plant recalcitrance, (resistance to degradation and therefore to pretreatment effectiveness), the higher *p*HCA yield obtained from maize stalks could be expected, or at least understood [11].

III.3 Cell wall biodegradability and release of *p*-hydroxycinnamic acids after enzymatic hydrolysis

The pretreated biomass was subjected to enzymatic treatment in order to determine whether successive treatments (microwave pretreatment followed by enzymatic hydrolysis) could enhance *p*-hydroxycinnamic acid release. The enzymatic effects were quantified by analyzing two parameters: the cell wall biodegradability and the release of *p*-hydroxycinnamic acids.

III.3.1 Cell wall biodegradability

Figure 4 represents the amount of xylose-equivalent reducing ends released into the liquid after enzymatic hydrolysis for the different biomass samples. The higher the release of xylose equivalents, the more parietal polymers become accessible and the more easily *p*-hydroxycinnamic acids are extracted. For each biomass, results were compared between raw and microwave pretreated biomass.

As errors bars overlapped for maize 7025 (Figure 4), it is impossible to conclude about xylose equivalent ends released into the liquid phase between raw and PMW biomass.
Nevertheless, this result must be considered with caution. Indeed, during microwave pretreatment, soluble sugars – hence reducing ends – in monomer or oligomer forms were extracted and then solubilized in the liquid phase. These “easily degradable” compounds were therefore no longer present in the biomass during enzymatic hydrolysis and were not accounted for in the DNS (3,5 Dinitrosalycilic acid) test after enzymatic hydrolysis on pretreated biomass. On the contrary, they could be accounted for in the DNS test following enzymatic hydrolysis on raw biomass if enzymatic hydrolysis could impact them.
For maize 98902, the pressurized microwave pretreatment enabled a significantly higher release of xylose equivalent ends release than without pretreatment: indeed, at least 15 mg/g more reducing ends, equivalent to +57 %, were released compared to the same maize stalks that had not been pretreated. This result was consistent with Aguilar-Reynosa *et al.* [33] who demonstrated the positive impact of microwave pretreatment before enzymatic hydrolysis: a 95.1 % solids conversion was reached when maize stover was pretreated by microwave prior to hydrolysis, for 10 to 50 minutes at 160 °C to 200 °C. Similar results were obtained by Fan *et al.* [47]: *Laminaria saccharina*, a brown seaweed, could be converted into hydrolysate with a yield of 65 % when pressurized microwave was used as pretreatment. This could be expected, considering the lack of lignin in the biomass.
Results were contrary for *Miscanthus* leaves (Figure 4B): xylose equivalent ends were more than three-fold higher for raw biomass than for pretreated biomass. Raw biomass released on average 1.5 g xylose equivalent ends, which corresponds to a 25 % soluble fraction in *Miscanthus* leaves (Table 1). PMW and Rovabio® cocktail were not as efficient on *Miscanthus*

leaves as on maize 98902. PMW did not increase the biodegradability of *Miscanthus* leaves for enzymatic hydrolysis, probably due to the initial parietal composition of *Miscanthus* leaves that are rich in lignin and cellulose (respectively 13.9 % and 50.6 % in average) that are not easily degradable polymers [46].

Finally, the release of xylose equivalent ends was more than ten-fold higher for maize stalks than for *Miscanthus* leaves, thus highlighting the high resistance of *Miscanthus* leave walls to microwave and biological pretreatments as well as the efficiency of Rovabio® on maize residues. The enhanced release of xylose equivalent reducing ends using maize stalks should improve the action of the FAEs on the *p*-hydroxycinnamic acids present within the biomass and the *p*-hydroxycinnamic acid release yield should therefore increase.

III.3.2 p-hydroxycinnamic acids release after enzymatic hydrolysis

Following enzymatic hydrolysis, *p*-hydroxycinnamic acids (*p*HCA) were quantified and results are presented on Figure 5.

According to Figure 5, FA release (mg_{FA}/gDM) in maize stalks was more than four-fold higher than the release of *p*CA (mg_{pCA}/gDM), hence partly demonstrating the efficiency of the Rovabio® cocktail on FA release and thus the enrichment of FAE in the cocktail.

On the contrary, the release of FA in *Miscanthus* leaves was low (less than 0.02 g_{FA}/gDM), and even lower than the release of *p*CA in *Miscanthus* GIB (0.053 g_{pCA}/gDM).

Since Rovabio® was designed for maize residues to produce animal feed, as explained in I.3, the structure of maize stalks is particularly sensitive to the cocktail unlike *Miscanthus* leaves, which appear to be much more resistant towards these specific FAE. *Miscanthus* leaves also had a more parietal structure than maize stalks, which give them stronger resistance to external attacks, such as enzymatic hydrolysis, despite microwave pretreatment. Furthermore, it is likely that the *p*HCA organization within the *Miscanthus* structure made them less accessible to enzymes if they were bonded to lignin rather than to hemicellulose and by both ether and ester covalent bonds.

The release of FA from maize 98902 was two-fold the FA release of maize 7025, due to the higher initial FA content in raw maize 98902, respectively 4.2 mg_{FA}/gDM and 3.5 mg_{FA}/gDM. Enzymatic treatment therefore seems to be more appropriate for FA release in maize 98902.

III.4 Release of total *p*-hydroxycinnamic acids by pretreatment and enzymatic hydrolysis

According to the two previous sections, the global yields in FA and CA were calculated using equation [4]:

$$pHCA_{\,yield}\,(\%) = p\mathrm{HCA}_{microwave\ yield}\,(\%) + pHCA_{\,enzymatic\ hydrolysis\ yield}\,(\%) \qquad [4]$$

For enzymatic hydrolysis *p*HCA yield, it was necessary to subtract the mass solubilized during treatment from the initial pretreated mass. Results are presented for maize stalks and *Miscanthus* leaves in Figure 6.

The global FA yield for maize 98902 reached 18 %; 1/3 resulted from microwave released FA and 2/3 from enzymatic released FA. The part of enzymatic FA release was more important in maize 7025, representing 90 % of the total FA extraction. The *p*HCA and more particularly *p*CA in *Miscanthus* leaves were essentially released after the microwave pretreatment. These results highlight the substantial role of the nature of the biomass in the release of *p*HCA: for maize stalks, the enzymatic hydrolysis step clearly released more ferulic acid, whereas for *Miscanthus* leaves, the microwave pretreatment rather released more *p*-coumaric acid. Nevertheless, Figure 5 and 6 clearly illustrate esterases inefficiency on coumaric acid release either from *Miscanthus* or maize.

According to these results, it is doubtful whether microwave pretreatment of maize stalks had any effect on *p*HCA release. For maize stalks, if the conversion process had begun directly at the enzymatic hydrolysis stage (when the most PA had been released), the cost of microwave pretreatment could have been avoided. Consequently, enzymatic hydrolysis was performed directly on raw biomass maize stalks and *Miscanthus* leaves in order to highlight the benefits of starting the process with microwave pretreatment, under the conditions described in I.3. The results are summarized according to the three different treatment options (calculation details are available in Supplementary Table 1):

1. **Process 1**: Successive microwave pretreatment - enzymatic hydrolysis: total *p*HCA yield corresponded to the sum of *p*HCA released during microwave pretreatment and *p*HCA released during enzymatic hydrolysis.
2. **Process 2**: Microwave pretreatment alone: *p*HCA yield corresponded to *p*HCA released during microwave pretreatment.
3. **Process 3**: Enzymatic hydrolysis alone: *p*HCA yield corresponded to *p*HCA released during enzymatic hydrolysis on raw biomass

Table 3: Summary of the extraction yields (% $mg_{pHCA\ release}/g_{initial\ pHCA}$) depending on the treatment options: process 1 the PMW followed by enzymatic hydrolysis; process 2 the PMW alone and process 3 the enzymatic hydrolysis alone on raw biomass.

	Process 1		**Process 2**		**Process 3**	
	FA (%)	*p*CA (%)	FA (%)	*p*CA (%)	FA (%)	*p*CA (%)
Maize 98902	18.2	5.2	5.5	4.2	7.6	0.6
Maize 7025	6.6	1.0	0.3	0.7	4.5	0.6
Miscanthus GIB	1.3	5.6	0.7	4.9	0	0
Miscanthus FLO	0.8	3.8	0.5	3.5	0.5	0
Miscanthus ROT	2.2	7.2	1.6	7.1	0.2	0

Table 3 presents the yield of each process for both FA and *p*CA vary as a function of the biomass. For maize 98902, process 1 appears to be the most efficient process, with a release of 18.2 % FA versus 5.5% for process 2 and 7.6% for process 3. For maize 7025, the microwave pretreatment followed by enzymatic hydrolysis allowed the highest yield of FA release (6.6%), but process 3 allowed a higher FA release compared to process 2; the opposite of what was observed for Maize 98902. In contrast, microwave pretreatment alone (process 2) led to a higher *p*CA yield than enzymatic hydrolysis (process 1) for both maize 98902 and maize 7025), but process 3 (microwave + enzymatic hydrolysis) allowed the highest pCA release yield, as for FA. Similarly, the *p*CA release observed in *Miscanthus* leaves was essentially due to microwave pretreatment (process 2), whereas enzymatic hydrolysis (process 3) had no significant effect.

The pCA release observed in Miscanthus leaves was essentially due to microwave pretreatment (process 2), whereas enzymatic hydrolysis (process 3) had no effect.

Yields of FA release in *Miscanthus* leaves were low, regardless of the process used, while enzymatic hydrolysis was not affective on this raw material: indeed, release yields remained close to 0 %. The Rovabio® enzymatic cocktail was designed to extract FA from maize residues, explaining why it was more efficient on this type of biomass but inadequate for *Miscanthus* leaves, which contain almost twice more lignin. *p*CA was more sensitive to the high temperatures that were reached during microwave treatment (100 °C) rather than to the duration of the enzymatic hydrolysis itself. This explains why the release yields were higher in process 2 than in process 3: indeed, the covalent esterified links between *p*CA and lignin and *p*CA and hemicellulose, are known to be particularly sensitive to heat [49].

In conclusion, the different processes tested in this study, under specific operational conditions, highlighted maize 98902 biomass and *Miscanthus* GIB biomass as the most promising types of biomass for extracting *p*-hydroxycinnamic acids (*p*HCA) through successive microwave - enzymatic processes. Results are summarized in Figure 7.

For details concerning economic aspects of PMW pretreatment, in a previous study [54] Bichot et al. compared pressurized microwave pretreatment costs with that of others thermal treatments.

IV. Conclusion

The present study was designed to assess the enzymatic hydrolysis release of *p*-hydroxycinnamic acids from pressurized microwave pretreated biomass (with pretreatment conditions optimized in a previous study). Two biomass with contrasted cell wall content were chosen and pretreated by pressurized microwave and submitted to enzymatic hydrolysis: three maize stalk genotypes and two *Miscanthus* clones. The enzymatic cocktail employed in the study is a commercially available cocktail designed to facilitate the livestock digestion of corn stover.

Results indicate that pressurized microwave pretreatment was effective for extracting *p*HCA from cell walls on 98902 maize stalks, a *p*HCA-rich biomass with the lowest parietal-content. Indeed, among the types of studied biomass and under the tested conditions, 98902 maize stalks represented the feedstock with the highest FA release yield (5.5 %) after pressurized microwave pretreatment. Subsequent enzymatic hydrolysis then led to a global FA yield of 18%. Maize stalks are thus highlighted as being more easily degradable and with a more accessible *p*HCA than the other types of biomass. *Miscanthus* leaves, which are significantly richer in parietal compounds than maize stalks, were only slightly affected by the process with a global yield (microwave pretreatment yield and enzymatic hydrolysis yield) that did not exceed 5.9 % for *p*CA from *Miscanthus* GIB.

To conclude, the bioprocess designed and developed in this study is dependent on the type of substrate, being more effective on maize, since the enzyme cocktail was specifically formulated for this substrate. Pressurized microwave pretreatment followed by an enzymatic hydrolysis of maize 98902 produced an FA yield of almost 20 %. However, for the other types of biomass (maize 7025 and *Miscanthus* GIB, ROT and FLO) microwave pretreatment followed by enzymatic hydrolysis was not as efficient and did not exceed 6 %. The process therefore still needs to be optimized for *Miscanthus* leaves and probably other types of lignocellulosic biomass. More precisely, in order to optimize the yields, the enzymatic cocktail applied should be adapted to each type of biomass.

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List of figures

Figure 1: Initial and prepared biomass used in the study: A and B, initial biomass (------ 2cm); C and D, prepared biomass ground to 1mm and sieved to 200µm (---- 0.6cm). A and C correspond to maize F 98902, B and D correspond to Miscanthus GIB

Figure 2: Minilabotron 2000 with pressurized microwave reactor

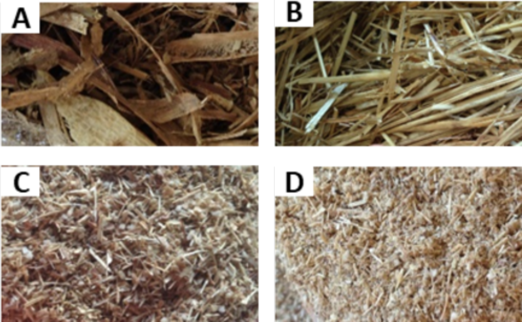
Figure 3: Schema of the treatment conditions tested. NoT: No treatment. Treated and raw biomass samples underwent the same analytical protocol

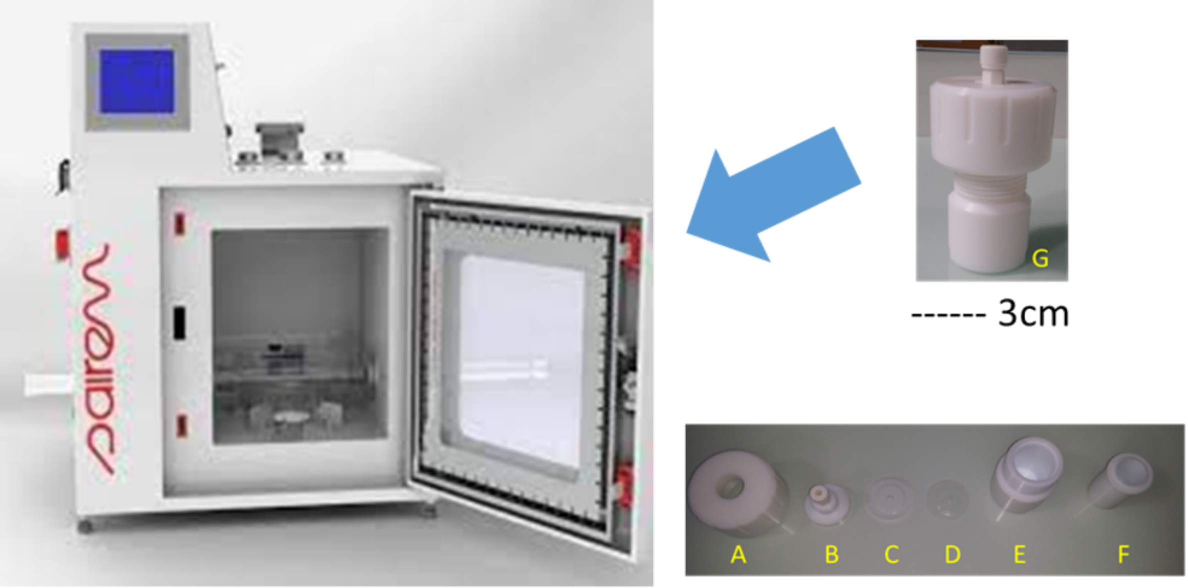
Figure 4: Xylose equivalent reducing ends (mg/g enzymatically pretreated biomass), in hatch raw biomass, in dots pressurized microwave biomass (PMW biomass). A represents maize stalks and B represents miscanthus leaves

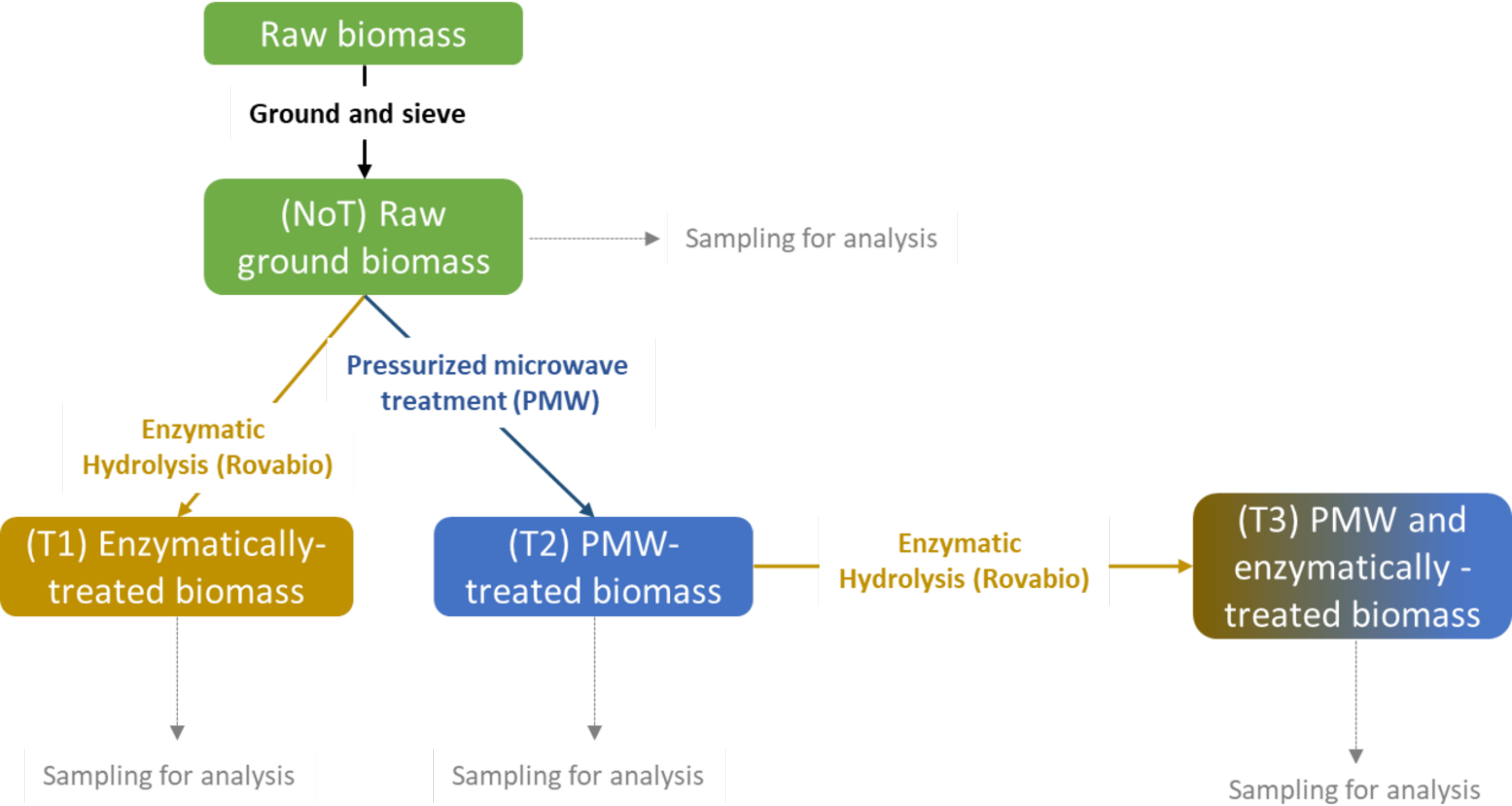
Figure 5: p-hydroxycinnamic acids (pHCA) released after enzymatic hydrolysis (mgpHCA/gDM)

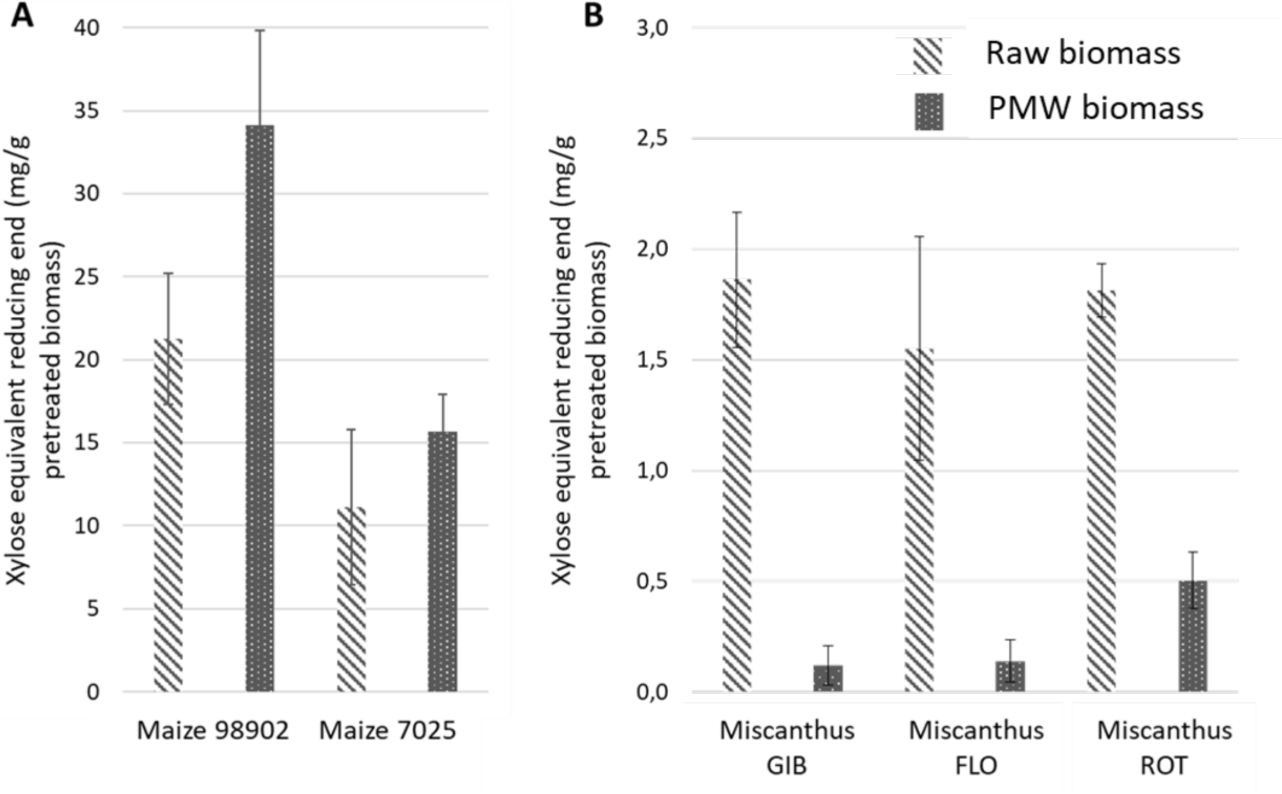
Figure 6: pHCA global yield of after pretreatment and enzymatic hydrolysis

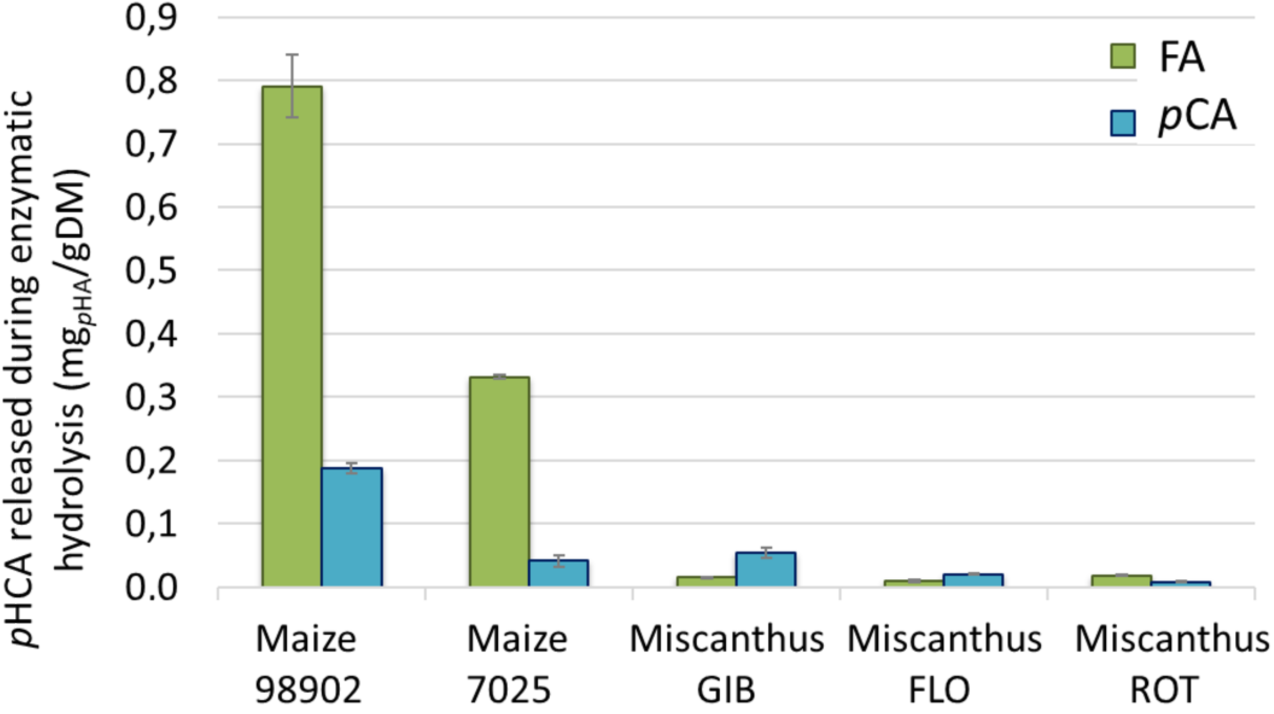
Figure 7: Extraction yield summary for maize 98902 biomass and Miscanthus GIB biomass


A
B
C
D

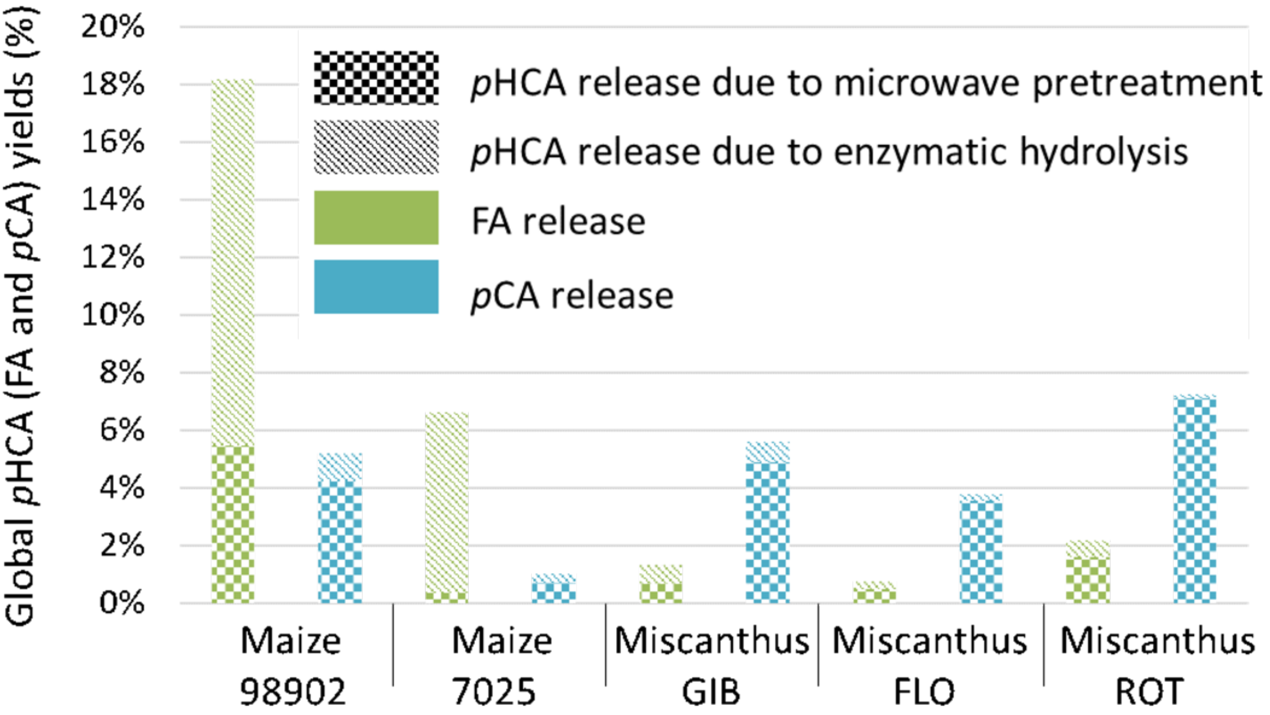

G
------ 3cm
A
B
C
D
E
F

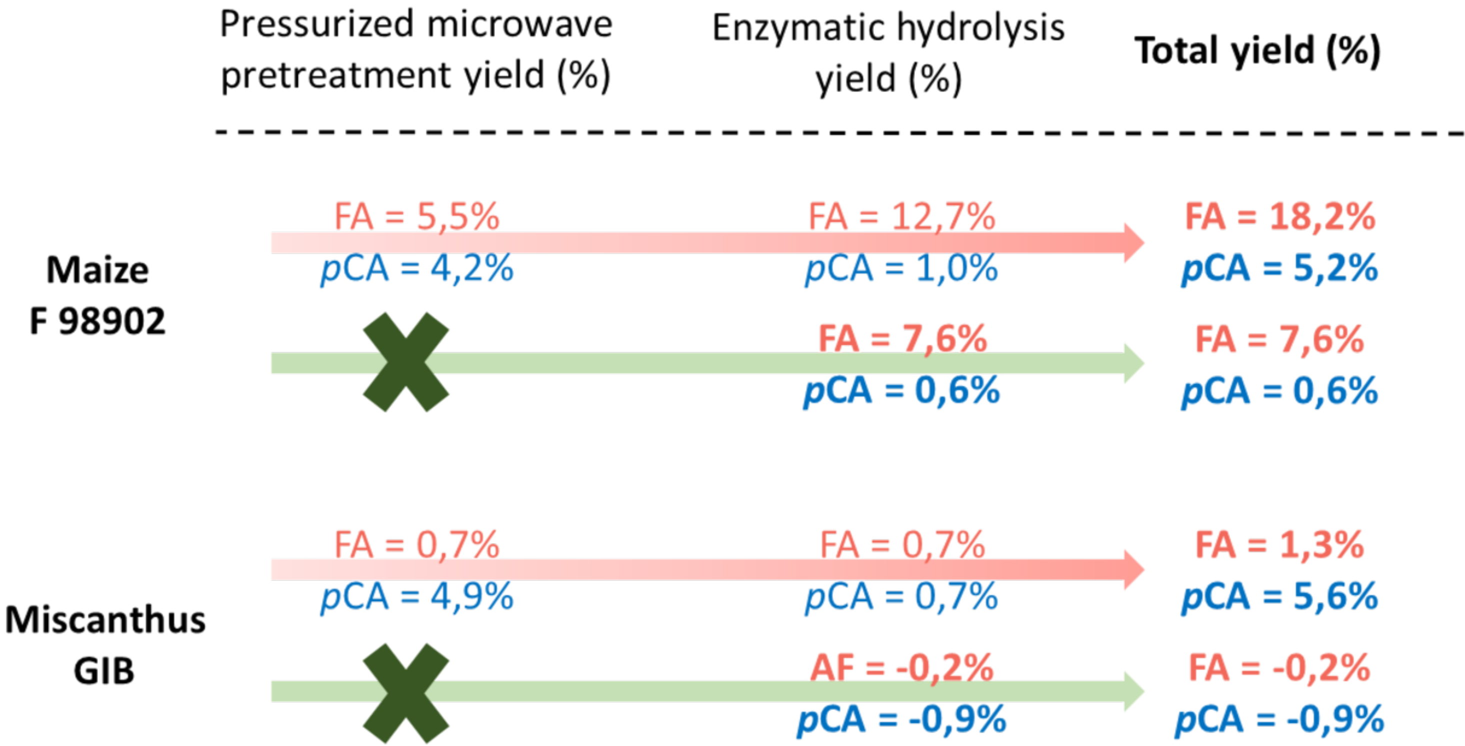

Raw biomass
Ground and sieve
(NoT) Raw ground biomass
Sampling for analysis
Pressurized microwave treatment (PMW)
Enzymatic Hydrolysis (Rovabio)
(T1) Enzymatically-treated biomass
(T2) PMW-treated biomass
Enzymatic Hydrolysis (Rovabio)
(T3) PMW and enzymatically -treated biomass
Sampling for analysis
Sampling for analysis
Sampling for analysis


A
B
Xylose equivalent reducing end (mg/g pretreated biomass)
40
35
30
25
20
15
10
5
0
Maize 98902
Maize 7025
3,0
2,5
2,0
1,5
1,0
0,5
0,0
Raw biomass
PMW biomass
Miscanthus GIB
Miscanthus FLO
Miscanthus ROT



FA	0,791	0,332	0,016	0,010	0,018
*p*CA	0,187	0,042	0,053	0,020	0,008


Global pHCA (FA and pCA) yields (%)
20%
18%
16%
14%
12%
10%
8%
6%
4%
2%
0%
pHCA release due to microwave pretreatment
pHCA release due to enzymatic hydrolysis
FA release
pCA release
Maize 98902
Maize 7025
Miscanthus GIB
Miscanthus FLO
Miscanthus ROT



	Pressurized microwave pretreatment yield (%)	Enzymatic hydrolysis yield (%)	**Total yield (%)**
Maize F 98902	FA = 5,5% *p*CA = 4,2%	FA = 12,7% *p*CA = 1,0%	**FA = 18,2%** ***p*CA = 5,2%**
	✗	**FA = 7,6%** ***p*CA = 0,6%**	**FA = 7,6%** ***p*CA = 0,6%**
Miscanthus GIB	FA = 0,7% *p*CA = 4,9%	FA = 0,7% *p*CA = 0,7%	**FA = 1,3%** ***p*CA = 5,6%**
	✗	**AF = -0,2%** ***p*CA = -0,9%**	**FA = -0,2%** ***p*CA = -0,9%**