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Adaptation of the Carrez procedure for the purification of ferulic and *p*-coumaric acids released from lignocellulosic biomass prior to LC/MS analysis

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ABSTRACT

The objective of this study was to adapt and improve an environmentally friendly and fast routine method for the analysis of ferulic and *p*-coumaric acids released from grass cell-walls by alkaline hydrolysis. This methodological development was performed on maize samples selected for their contrasted contents in ferulic and *p*-coumaric acids as a consequence of their different maturity stages (from stage of 7th leaf with visible ligule to stage of silage harvest). We demonstrate that the Carrez method is an efficient substitute to the common solvent-consuming extraction by ethyl acetate for the preparation of samples suitable for HPLC–ESI-MS analysis. We prove that it is possible to replace methanol by ethanol in the Carrez step and at last we propose a scale reduction of this procedure that offer a first step towards high throughput determinations. The new method leads to a solvent consumption reduced by a factor 100 and only requires ethanol as organic solvent.

1. Introduction

The lignocellulosic biomass is an abundant worldwide renewable resource available for the production of biofuels with little impact on plant food uses. Indeed, lignocelluloses are constitutive of agricultural nonfood residues such as corn stover or wheat straw. Their conversion into ethanol, energy, chemical intermediates and materials is at the heart of the development of biorefinery strategies [1]. Lignocelluloses are composed of the polysaccharidic (cellulose and hemicelluloses) and phenolic (lignins and *p*-hydroxycinnamic acids (*p*HAs)) constituents of plant cell-walls. These two kinds of constituents are associated into a complex network covalently cross-linked by the phenolic compounds. Ferulic (FA) and *p*-coumaric (*p*CA) acids are the major *p*HAs found in grass cell-walls and are particular abundant in maize cell wall [2]. They are covalently linked to polysaccharides by ester bonds and to lignin by ester or ether bonds. As cross-linking agents they limit the accessibility of polysaccharides to bioconversion tools such as cellulases. A consequence is the reduction of cell-wall degradability, as shown by *in vitro* investigations [3]. The amount of *p*HAs linked to cell-wall polymers turns out as one structural criteria for the assessment of the quality of biomass regarding its technical ability to bioconversions.

In this context, there is a need for a routine analytical method allowing the determination of *p*HAs in lignocellulosic biomass. Such a determination has to include two steps: (1) the cleavage of the ester and ether bonds involving the acids and (2) the determination of the released acids. The first step is commonly performed by alkaline hydrolysis with different intensities in order to selectively cleave ester bonds (mild conditions) or both ester and ether bonds (severe conditions) [4]. Liquid chromatography combined to mass spectrometry with electrospray ionization (HPLC–ESI-MS) is a powerful more and more widespread technique used for the identification and determination of phenolic structures in the second step [5,6]. It requires however the purification of the phenolic compounds released in the alkaline reaction medium. Indeed, this reaction medium contains polymers such as hemicelluloses, lignins and proteins that are likely to precipitate in the chromatographic system and then hinder the electrospray ionization.

The different strategies reported for the purification of *p*HAs are liquid–liquid extraction [7–9], solid-phase extraction SPE [10,11] and selective precipitation of the contaminating polymers [12–14]. The first strategy has the drawback to use organic solvents such as ethyl acetate [8], chloroform [9], diethyl ether [7], whereas SPE leads to less reliable and reproducible results [12]. Moreover, these strategies are expensive, both in terms of time and solvent consumption, and are not environmentally friendly. The third

Abbreviations: CWR, cell wall residue; FA, ferulic acid; HPLC–MS, high pressure liquid chromatography combined to mass spectrometry; HPLC–ESI-MS, high pressure liquid chromatography combined to mass spectrometry with electrospray ionization; *p*CA, *p*-coumaric acid; *p*HAs, *p*-hydroxycinnamic acids; a.m.u., atomic mass unit.

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strategy consists in the selective precipitation of colloidal material to leave only *p*HAs in the supernatant. This precipitation can be performed by complexation using the technique developed by Carrez for the analysis of urine [15]. This technique is based on two main purification steps, one with methanol for the precipitation of polysaccharides and the other with Carrez solution I (zinc acetate solution) in order to precipitate proteins. Addition of Carrez solution II (potassium ferrocyanide solution) allows the excess of zinc acetate to precipitate. This technique was applied to the analysis of phenolic compounds in different food materials, such as coffee [12,13] and sweet potato [14], but it has never been applied, to our knowledge, to phenolic compounds in lignocelluloses.

The objective of this study was to develop an environmentally friendly and fast routine method for the analysis of FA and *p*CA released from grass cell-walls by alkaline hydrolysis. In this aim, we wanted to test the "Carrez" method as substitute to the common solvent-consuming extraction by ethyl acetate. In order to reduce the environmental impact and to enhance the safety of the procedure we checked that methanol could be replaced by ethanol for the preparation of the reagents. Finally, we proposed a scale reduction of this procedure that offer a first step towards high throughput determinations. This methodological development was performed on maize samples selected for their contrasted contents in FA and *p*CA as a consequence of their different maturity stages.

2. Materials and methods

2.1. Chemicals and preparation of reagents

Methanol and acetonitrile were of HPLC grade and absolute ethanol was of analytical grade. All solvents were purchased from Carlo Erba Reactifs-SDS (Val de Reuil, France). Formic acid, ethyl vanillin, *p*CA and FA were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France).

Zinc acetate and potassium ferrocyanure were obtained from Carlo Erba (Val de Reuil, France). Carrez I solution and Carrez II solution were prepared by dissolving 21.9 g of zinc acetate in 100 mL of distilled water and 10.6 g of potassium ferrocyanure in 100 mL of distilled water, respectively.

2.2. Plant material.

A maize hybrid (F7019*F2) was cropped in summer 2008 at INRA Lusignan (86 – France). Five internodes, below the node from which the ear arises, were collected at 5 stages. Stages 1–5 (stage 1: when 7th leaf with visible ligule, stage 2: tassel emerging in the whorl, stage 3: silking, stage 4: 10 days after silking, and stage 5: silage harvest) cover the whole plant development from seventh ligulated leaf to silage maturity. Collected internodes were carefully mixed, and then chopped. The samples were oven-dried (70 °C) and ground with a hammer mill to pass through a 1 mm screen. Prior to chemical analysis samples were soxhlet extracted sequentially with toluene, ethanol and water in order to recover cell wall residue (CWR).

2.3. Alkaline hydrolyses

The *p*HAs were released after by treating CWR fractions with NaOH using the method described in Mechin et al. [8], with minor modifications, which involves two alkaline treatments differing in their severity. Esterified *p*HAs were released by subjecting CWR samples (50 mg) to a mild alkaline hydrolysis (5 mL of 2 M NaOH, room temperature, 20 h with mechanical stirring). Both esterified and etherified *p*HAs were released using severe alkaline treatment (5 mL of 4 M NaOH, 170 °C for 2 h). Due to the drastic reaction conditions, CWR samples (50 mg) were specifically placed in a Teflon screw-cap vial and then sealed in a stainless steel reaction vessel.

The samples recovered from mild and severe alkaline hydrolyses were subjected to the same subsequent extraction or precipitation procedure.

2.4. Purification of hydrolysates prior to HPLC–MS analysis of pHAs

2.4.1. Purification by extraction of pHAs with ethyl acetate

An internal standard, 1 mL of ethyl vanillin (1 mg/mL in methanol), was added to the samples recovered from alkaline hydrolyses prior to centrifugation (1700 rpm for 10 min). Then, the supernatants were acidified to pH 2 with concentrated HCl and kept at 4 °C for 16 h before another centrifugation step (1700 rpm for 10 min). *p*HAs were extracted from the aqueous supernatant with 3 × 30 mL of ethyl acetate. The ethyl acetate extracts were evaporated under reduced pressure. The extracts were re-dissolved in 2 mL methanol and stored at 4 °C until HPLC–MS analysis. Prior to HPLC–MS analysis, all samples were diluted by 4 and filtered using Acrodisc 13 mm Syringe Filters (Pall Corporation) with 0.45 mm pore size.

2.4.2. Purification by precipitation with Carrez reagents

An internal standard, 1 mL of ethyl vanillin (1 mg/mL in methanol), was added to samples recovered from alkaline hydrolyses prior to acidification to pH 5–6 with concentrated HCl. It is important to respect this pH since a more acid pH can cause the formation of toxic cyanhydric acid vapors with Carrez II solution. According to the proportion used by Nandutu et al. [14], 12 mL of methanol was added to acidified hydrolysates in order to obtain about 70% v/v of aqueous methanol. We also tested the replacement of methanol by ethanol conserving exactly the same conditions. Both solvents were tested and the results were compared. Then, 840 μL of Carrez I reagent was added, mixed for 2 min and left for 1 min. 840 μL of Carrez II reagent was then added and mixed for 20 s. The mixture was centrifuged for 10 min at 10,000 rpm. The supernatant was stored at 4 °C until HPLC–MS analysis. Prior to HPLC–MS analysis, all samples were filtered as previously described but without any dilution. In order to test the linearity of the method, the same treatment was applied to a series of standard aqueous solutions containing pure commercial FA and *p*CA in concentrations ranging between 2 and 25 mg/mL. The linearity was assessed through the R^2 coefficient between theoretical and experimental data.

In order to reduce at minima the volume of used solvent, we decided to test the robustness of a precipitation procedure with reduced volumes. Ethanol (1 mL) was added to 0.75 mL of alkaline hydrolysate and then 70 μL of Carrez I and Carrez II treating the samples as described above.

2.5. HPLC–MS analysis

The following instrumentation was used for HPLC–MS analysis: autosampler/injector (Spectro System AS300), pump (Spectro System P4000), PDA/UV vis detector (Spectro System UV 6000 LP from Thermo Finnigan). The HPLC system was coupled to an LCQ deca (Thermo Quest) mass spectrometer detector equipped with an electrospray ionization (ESI) interface. Xcalibur version 1.4 was used for the control of the equipment and the acquisition and treatment of data. Phenolic compounds were separated using a 150 mm × 4.6 mm Thermo HyPURITY C18 (5 μm) column equipped with guard column (10 mm × 4 mm, 5 μm). Mobile phase consisting of: (A) water–formic acid (99.9:0.1 v/v) and (B) acetonitrile–formic acid (99.9:0.1 v/v) delivered at a flow-rate of 0.8 mL/min. The injection volume was 5 μL. The analysis was carried out in the following

gradient elution mode: eluent A increased to 5 at 9% in 5 min and maintained for 10 min at 9%, then increased to 11% in 7 min and again to 18% in 16 min and maintained for 2 min at 18%. Finally, eluent A increased to 80% in 1 min and maintained for 8 min at 80%. After the run was completed, the column re-equilibration was 10 min. UV detection was performed between 190 and 600 nm and MS detection was performed in negative mode with a m/z between 120 and 1200 a.m.u. (desolvating capillary temperature: 350 °C; needle voltage: 4 kV).

FA and *p*CA determination was based on the internal standard method, using reference solutions to determine response coefficient for FA and *p*CA.

2.6. Statistical analysis

Variance analyses were carried out to test (i) the effect of EtOAc extraction replacement by Carrez MeOH precipitation and (ii) the effect of MeOH replacement by EtOH in the Carrez precipitation step. Variance analysis was performed following standard procedures of the fixed following model:

$$Y_{ij} = \mu + S_i + P_j + e_{ij}$$

where Y_{ij} is the value (esterified, total or etherified FA or *p*CA) of the sample at stage i after purification by the method j (EtOAc versus Carrez MeOH or Carrez MeOH versus Carrez EtOH), μ is the overall mean, S_i is the effect of stage i, P_j is the effect of the purification method j and e_{ij} is the random residual term.

3. Results and discussion

3.1. Variation of p-coumaric and ferulic acid contents along development

We selected 5 developmental stages along maize development from the emerging of the 7th ligulated leaf to the silage harvest stage. These samples were characterized in a first step using the classical organic solvent extraction purification method. Purification of the *p*-hydroxycinnamic acids released either by mild or severe alkaline hydrolysis was thus performed by solvent organic extraction using ethyl acetate. Ethyl acetate is a solvent of medium polarity suitable to solubilize phenolic compounds. It has the advantage of lower toxicity and volatility over chloroform and other chlorinated solvent and also lower flammability over diethyl ether. The organic solvent extraction procedure involves the use of 3 × 30 mL ethyl acetate. Whatever the extraction solvent, an evaporation step is required prior dissolution of the residue in a solvent suitable for reverse-phase liquid chromatography. In the common procedure, the residue is dissolved in 1–2 mL of HPLC-grade methanol.

From stages 1–5, total *p*CA content increased by 94% (Table 1 and Fig. 1a) whereas total FA content increased by 31% (Table 1 and Fig. 1b). These results are in agreement with the fact that FA, in opposition to *p*CA, is mainly encountered in young cell wall [16,17]. FA is thus present at very young stages before the beginning of lignification of the plant cell wall while *p*CA accumulates during all the lignification process. As expected, total *p*CA content thus rises its maximum at stage 4 (Fig. 1a) whereas total FA content rises its maximum very earlier (stage 2, Fig. 1b).

The temporal evolution of esterified *p*CA content along development together with its absolute value is comparable in every way to that of total *p*CA content (Fig. 1a). This result confirms that *p*CA is mainly present in its esterified form (to syringyl lignin units [18]) in plant cell wall. By contrast, esterified FA content did not present any temporal variation and displayed lower absolute values than total FA content (Fig. 1b). This highlighted the well known involvement of FA both in esterified and etherified links in the plant cell wall [19]. Indeed, at young stages of plant cell wall, FA is esterified to hemicelluloses [20] and is thus yet present in the cell wall when lignification occurs. FA can thus act as a linking point for lignin etherifying to the guaiacyl lignin subunit. Ester-linked to hemicelluloses on the one hand and ether-linked to lignin on the other, FA acts as a bridge between the two cell wall constituents. If we look at etherified FA content evolution along development, we observed an increase in etherified FA content up to stage 4 (Table 1) during the lignification period.

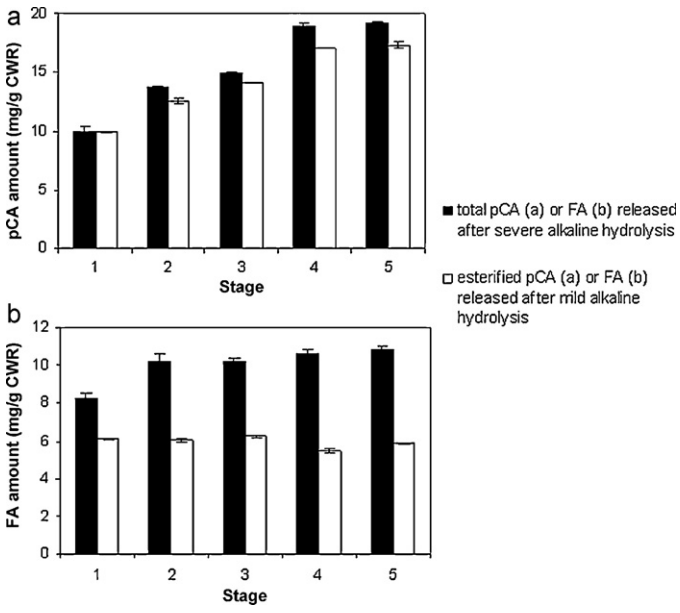

Fig. 1. The amounts of total (■) and esterified (□) *p*CA (a) and FA (b) released after severe and mild alkaline hydrolyses performed on maize samples collected at different developmental stages (1, when 7th leaf with visible ligule; 2, tassel emerging in the whorl; 3, silking; 4, 10 days after silking; and 5, silage harvest). The samples recovered from mild and severe alkaline hydrolyses were subjected to a purification step by extraction with ethyl acetate. The extracts were then re-dissolved in methanol and analyzed by HPLC–MS.

This series of maize samples recovered at different stages allows covering a wide range of contents in FA esters and ethers and *p*CA esters. For this reason, we selected them to test the Carrez purification method associated to HPLC–ESI-MS analysis.

3.2. Validation of the Carrez purification method

The alternative purification procedure proposed in this paper only requires Carrez reagent and methanol. In a first step, methanol (12 mL) is added to the ~9 mL acidified alkaline hydrolysate (to recover an in fine 70% v/v aqueous methanol medium) to make polysaccharides precipitate. In a second step 840 μL of each aqueous Carrez reagent solutions I (zinc acetate) and II (ferrocyanure) are added in order to make proteins precipitate. This method has previously been used and validated with coffee and potatoes matrix. Its transfer to a lignified matrix such as grass cell wall addressed at least two questions: Are there interactions between lignins and phenolic acids during the treatment that could lead to an underestimation of FA and *p*CA? Is the treatment efficient to make cell-wall cellulose and hemicelluloses precipitate and is the supernatant stable after treatment?

In order to answer these questions and validate the method, four mild alkali hydrolyses and four severe alkali hydrolyses were performed each on 50 mg cell wall residue recovered from the five maize samples. For each alkaline hydrolysis type, and each maize sample, two reaction medium were submitted to the classical

Table 1
Content and relative proportion (in parentheses) of (FA est) esterified- and (FA eth) etherified FA determined by alkaline hydrolyses of maize CWR followed by the ethyl acetate extraction purification procedure. Maize samples are collected at different developmental stages (1, when 7th leaf with visible ligule; 2, tassel emerging in the whorl; 3, silking; 4, 10 days after silking; and 5, silage harvest). Relative proportions are expressed as percentage of total FA content.

Stage	1	2	3	4	5
FA est (mg/g CWR)	6.09 (74%)	6.05 (59%)	6.22 (61%)	5.48 (52%)	5.89 (54%)
FA eth (mg/g CWR)	2.15 (26%)	4.21 (41%)	4.04 (39%)	5.12 (48%)	4.93 (46%)

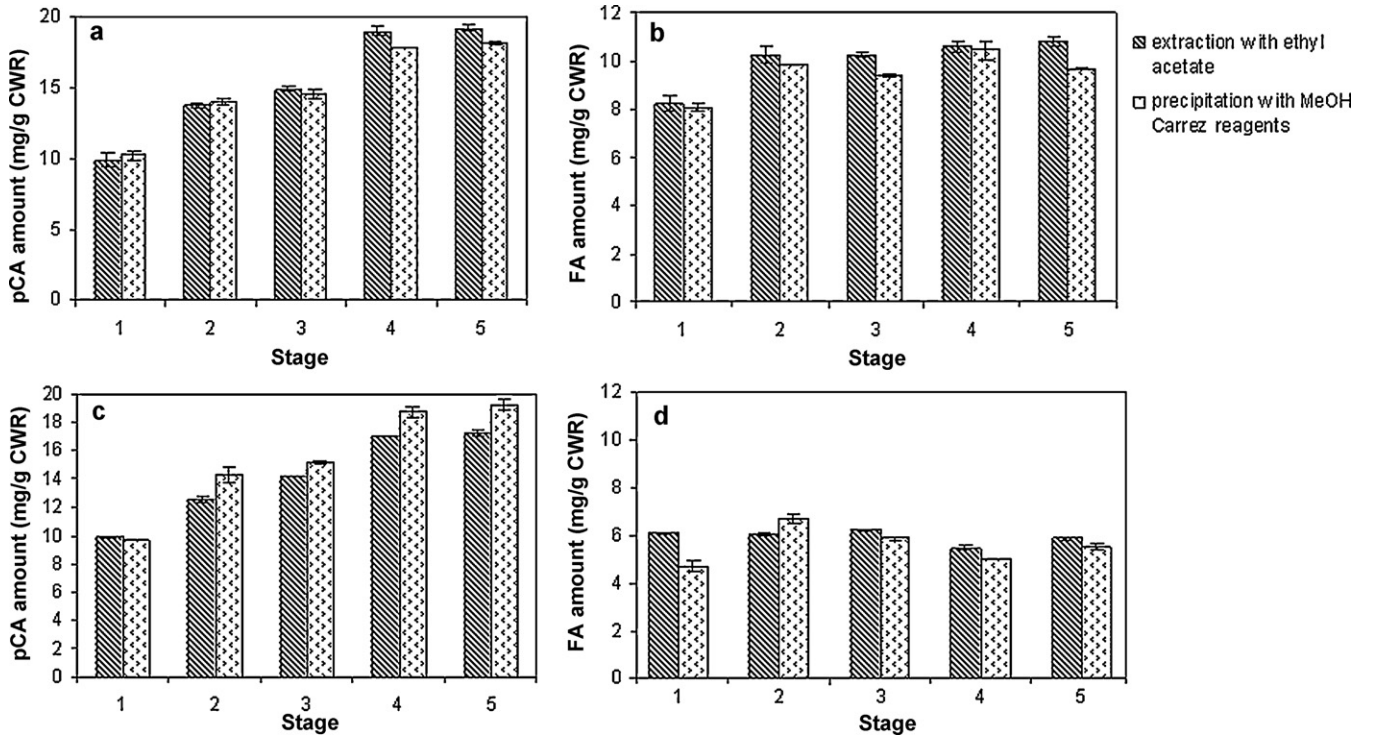

Fig. 2. The amounts of total *p*CA (a), total FA (b), esterified *p*CA (c) and esterified FA (d) released after severe and mild alkaline hydrolyses performed on maize samples collected at different developmental stages (1, when 7th leaf with visible ligule; 2, tassel emerging in the whorl; 3, silking; 4, 10 days after silking; and 5, silage harvest). The samples recovered from mild and severe alkaline hydrolyses were subjected to a purification step by extraction with ethyl acetate (▨) or by precipitation with MeOH Carrez reagents (⊡). The extracts were then re-dissolved in methanol and analyzed by HPLC–MS.

organic solvent extraction and the two other to the Carrez purification method. Each phenolic acid solution recovered after the purification step was analyzed by a single HPLC–ESI-MS injection. The values reported in Fig. 2 are average values each corresponding to a hydrolysis – purification – analysis sequence performed in double on one of the 5 maize samples.

Changing the purification procedure from solvent extraction to Carrez method leads to similar results, whatever the maize sample, the phenolic acid and the alkaline hydrolysis type (Fig. 2a–d). Indeed, the variation coefficient does not exceed 5.5%. A closer look at the comparison between the two purification procedures indicates that in the specific case of *p*CA esters, slightly higher con-

Table 2
Comparison of the classical solvent extraction procedure and the optimized purification procedure with the Carrez reagent.

	Solvent extraction	Optimized procedure
Experimental steps	(1) Centrifugation (2) Acidification (pH 2) (3) Storage at 4 °C (>4 h) (4) Centrifugation (5) 3-Times extraction (6) Evaporation (7) Dissolution (8) Filtration	(1) Acidification (pH 5–6) (2) Addition of the reagents (3) Centrifugation (4) Filtration
Solvent consumption[a]	9 L ethyl acetate + 200 mL methanol	100 mL ethanol
Reagent consumption[a]	None	2.2 mg (low human toxicity)
Solvent and reagent cost[a]	50 euros	<0.5 euros
Time consumption[b]	>5 h	30 min

[a] Calculated for 100 analyses.
[b] Calculated for 4 analyses performed in parallel.

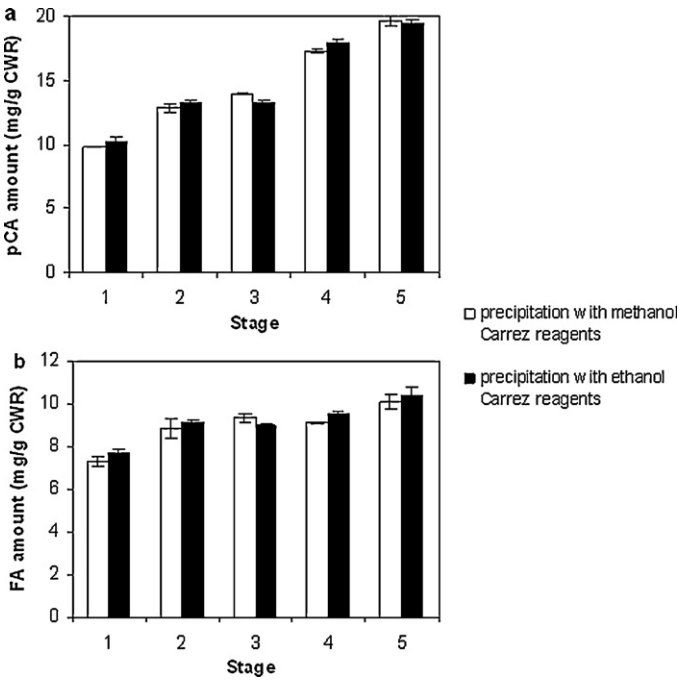

Fig. 3. The amounts of total *p*CA (a) and total FA (b) released after severe alkaline hydrolysis performed on maize samples collected at different developmental stages (1, when 7th leaf with visible ligule; 2, tassel emerging in the whorl; 3, silking; 4, 10 days after silking; and 5, silage harvest). The samples recovered from severe alkaline hydrolysis were subjected to a purification step by precipitation with methanol Carrez reagents (⊡) or by precipitation with ethanol Carrez reagents (■). The extracts were then re-dissolved in methanol and analyzed by HPLC–MS.

tents are observed with Carrez extraction for all the maize samples (Fig. 2c). This case is the only one which presents a significative P_j effect (data not shown). This could suggest that the extraction of *p*CA with ethyl acetate is not exhaustive.

Assessed using standard aqueous solutions of pure FA and *p*CA, the linearity of the method was found excellent (R^2 between the theoretical and experimental concentrations higher than 0.999 for both FA and *p*CA).

3.3. Optimization of the procedure: change of methanol into ethanol and scale reduction

In order to reduce the cost of the procedure and its impact on health, we tried to use ethanol instead of methanol. The impact of this solvent change was assessed by submitting in parallel two aliquots of the same hydrolysates to a methanol and ethanol Carrez procedure. The test was performed for all the maize samples and with the severe alkaline hydrolysis conditions. The results show that using ethanol instead of methanol does not impact the results (variation coefficients between 1.8 and 3.35% and Fig. 3a and b). The P_j effect was never significant in variance analysis (data not shown).

Moreover, repeated injections of the solutions stored at 4 °C over 40 days (maize samples corresponding to stages 2 and 4) shows that the ethanol Carrez purification method does not compromise the stability of the samples. On the contrary, high reproducibility is observed between the HPLC–ESI-MS analyses.

In order to further reduce solvent consumption, the ethanol-Carrez purification method was transpose to an hydrolysate aliquot of 0.75 mL by adding 1 mL ethanol then 70 μL Carrez solutions. Centrifugation was then performed in 2 mL eppendorf tubes using a lab bench centrifuge. This volume modification allowed reducing by a factor 12 the solvent consumption while leading to the same results.

3.4. Gain of the new method

Comparison between the ethyl acetate extraction procedure and the low scale purification procedure with the Carrez reagent (new method) is shown in Table 2. First, the new method offers gain in terms of time (reduction by a factor ≥10) and solvent (reduction by a factor 100) consumption. It requires only 4 steps instead of 8 and avoids the tedious extraction and evaporation steps of the solvent extraction method. Moreover, the strong reduction of solvent consumption induces a financial gain (cost reduction by a factor 100) and ensure a high safety level, since the reagents used with the Carrez method are both food additives with low human toxicity (zinc acetate: E650; potassium ferrocyanide: E536). Finally, the considerable reduction of waste volumes represent an environmental benefit: whereas the solvent extraction produces generates about 10 L/100 analyses of organic solvent wastes to be evacuated, the new method generates only a few milligrams of salts that can be easily stored in the laboratory. Although ethyl acetate can be recycled by distillation, this operation induces high energy costs and cannot be performed at the laboratory for time concerns. According to this comparison, the new method can be claimed as a green routine method for the purification of phenolic acids prior to their LC–MS analysis.

4. Conclusion

The objective of this study was to get rid of high volumes solvent consumption for the analysis of the *p*-hydroxycinnamic acids involved in grass cell-wall cross-linking. For this purpose, we proposed to test a purification method based on a precipitation by the Carrez reagents and the use of ethanol instead of methanol.

Carrez reagents were found efficient to make cellulose, hemicelluloses and lignins precipitate, leading to a stable solution that can be stored for weeks at 4 °C before analysis. Moreover, the statistical analysis of the results shows that neither interactions between colloids and pHA_S during precipitation nor reactions of *p*HAs with Carrez reagents, if any, impact the *p*HAs determination, as compared to the classical purification method.

This study shows that precipitation of colloids using Carrez I and II reagents can advantageously be substituted to the classical ethyl acetate extraction in order to provide a green routine procedure to purify *p*-hydroxycinnamic acids recovered from lignocellulosic substrates. This method will be of great help for a screening approach aiming at assessing the quality of biomass towards industrial hydrolytic conversion processes.

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