



Beyond the wall: High-throughput quantification of plant soluble and cell-wall bound phenolics by liquid chromatography tandem mass spectrometry

Jean-Christophe Cocuron^{a,1}, Maria Isabel Casas^{b,1}, Fan Yang^{c,1}, Erich Grotewold^d, Ana Paula Alonso^{a,*}

^a BioDiscovery Institute and Department of Biological Sciences, University of North Texas, Denton, TX 76203, USA

^b NaPro Research, LLC, Washington, DC, 20018, USA

^c Benson Hill Biosystems, St. Louis, MO, 63132, USA

^d Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824-6473, USA

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ABSTRACT

Plants accumulate several thousand of phenolic compounds, including lignins and flavonoids, which are mainly synthesized through the phenylpropanoid pathway, and play important roles in plant growth and adaptation. A novel high-throughput ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method was established to quantify the levels of 19 flavonoids and 15 other phenolic compounds, including acids, aldehydes, and alcohols. The chromatographic separation was performed in 10 min, allowing for the resolution of isomers such as 3-, 4-, and 5-chlorogenic acids, 4-hydroxybenzoic and salicylic acids, isoorientin and orientin, and luteolin and kaempferol. The linearity range for each compound was found to be in the low fmol to the high pmol. Furthermore, this UHPLC-MS/MS approach was shown to be very sensitive with limits of detection between 1.5 amol to 300 fmol, and limits of quantification between 5 amol to 1000 fmol. Extracts from maize seedlings were used to assess the robustness of the method in terms of recovery efficiency, matrix effect, and accuracy. The biological matrix did not suppress the signal for 32 out of the 34 metabolites under investigation. Additionally, the majority of the analytes were recovered from the biological samples with an efficiency above 75%. All flavonoids and other phenolic compounds had an intra- and inter-day accuracy within a $\pm 20\%$ range, except for coniferyl alcohol and vanillic acid. Finally, the quantification of flavonoids, free and cell wall-bound phenolics in seedlings from two maize lines with contrasting phenolic content was successfully achieved using this methodology.

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1. Introduction

Plants accumulate several thousand of phenolic compounds. These complex metabolites play important roles in plant growth, development and adaptation. For instance, they provide structural support, protection against pathogens and abiotic stresses, and act as pollinator attractants [1,2]. Phenolics are also essential in human

health and industrial applications. They have antioxidant, anti-inflammatory and anti-carcinogenic properties when taken into consumption of fruits, vegetables and their derived products [3]. From the industrial perspective, phenolics play an important role during pulping and biofuel production [1,2].

Phenolics are synthesized *via* the phenylpropanoid pathway. The conversion of phenylalanine to cinnamic acid is the first committed step to the phenylpropanoid pathway (Fig. 1). Cinnamic acid will then branch into the conversion of additional phenolic acids. Alternatively, cinnamic acid can be converted to coumaroyl-CoA, which will lead to additional phenolics and lignin polymerization on the one hand, and on the other hand to flavonoid biosynthesis (Fig. 1). Phenylpropanoids are chemically diverse with phenolics divided into acids, aldehydes and alcohols, which will generate lignin with different cross-linking degrees. Flavonoids can be divided into several sub-classes including the flavanones, flavonols, flavones and anthocyanins. To add to this chemical diversity,

Abbreviations: CE, collision energy; CGA, chlorogenic acid; CXP, collision cell exit potential; DAD, diode array detector; DP, declustering potential; EP, entrance potential; ME, matrix effect; PCA, principal component analysis; RE, recovery efficiency; UHPLC-MS/MS, ultra-high pressure liquid chromatography–tandem mass spectrometry.

* Corresponding author at: BioDiscovery Institute and Department of Biological Sciences, University of North Texas, 1504 W. Mulberry St, Denton, TX 76201, USA.

E-mail address: Anapaula.Alonso@unt.edu (A.P. Alonso).

¹ Equal contribution.

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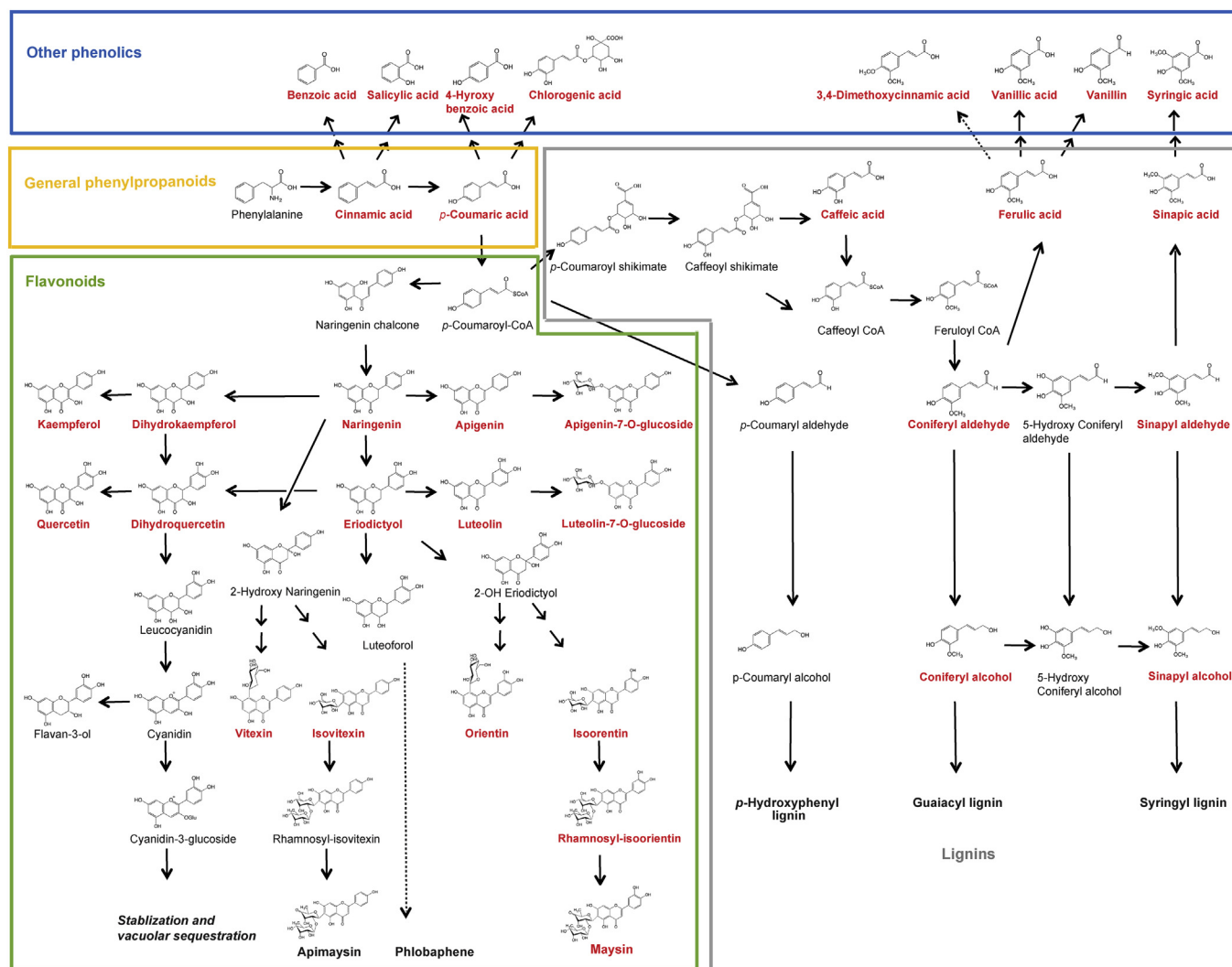


Fig. 1. Schematic of the phenylpropanoid biosynthetic pathway. The different classes of compounds generated from general phenylpropanoids (framed in orange) are presented: flavonoids (green), lignins (grey), and other phenolics (blue). The compounds whose name is red were monitored in this study. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

flavonoids can be further decorated by acylation or glycosylation (Fig. 1), making it difficult to separate, identify and quantify in complex plant biological matrices.

Maize is the most important cereal crop worldwide, with the USA corn grain production in 2016 being 15.1 billion bushels (380 million tons) (<http://www.nass.usda.gov/>), in excess of \$50 billion in value. Agricultural output derived from the development of high-yield varieties of grains combined with technological improvements resulted in food production continuously expanding since the 1960s. To boost pathway discovery, and to guide breeding programs as well as metabolic engineering, it is essential to rely on rapid and sensitive methods to screen for the metabolites synthesized from the phenylpropanoid pathway [4,5].

Several analytical techniques have been applied for the separation and quantification of plant flavonoids and other phenolics, and extensively reviewed [6–9]. Separation of these metabolites is commonly achieved through high performance liquid chromatography (HPLC) using a reverse-phase C18 column. Plant flavonoids and other phenolics are all aromatic compounds and therefore have the ability to absorb in the ultra-violet wavelengths, making them detectable and quantifiable using a diode array detector (DAD). For instance, a dozen of phenolic acids have been quantified in food samples [10], the levels of six flavonoids and four phenolic acids

have been simultaneously determined [11], and cell-wall bound phenolics have been analyzed [12]. Because these compounds are so diverse and often highly decorated, the majority of the studies combine the DAD with a time of flight or an ion trap mass spectrometer, and then use literature to tentatively elucidate their chemical structures [13–16]. In order to specifically quantify targeted compounds, some methodologies coupling HPLC with a triple quadrupole were developed. In general, approaches focus on one class of metabolites, that is to say either on phenolic compounds [17,18] or flavonoids [19,20]. Only a few studies determined the levels of both flavonoids and phenolic acids [21–24]. However, none of them achieved the simultaneous detection and quantification of flavonoids, and other plant phenolics, such as phenolic aldehydes and alcohols that are important intermediaries and components of lignin.

This study describes the development of a novel high-throughput ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC–MS/MS) method to separate and quantify the levels of 19 plant flavonoids and 15 other phenolic compounds, including phenolic acids, aldehydes and alcohols. The chromatographic resolution of these metabolites was achieved in less than 10 min, with the separation of all the isobaric species under investigation with the exception of isovitexin and vitexin.

Table 1

Compound-dependent MS parameters for MRM scan survey.

	Metabolite	Precursor ion	Product ion	Transition (m/z)	DP* (V)	EP* (V)	CE# (V)	CXP§ (V)
OTHER PHENOLICS	3,4-Dimethoxycinnamic ac	C ₁₁ H ₁₁ O ₄ [−]	C ₈ H ₇ [−]	207/103	−90	−10	−18	−47
	4-Hydroxybenzoic acid	C ₇ H ₅ O ₃ [−]	C ₆ H ₅ O [−]	137/93	−80	−10	−26	−39
	Benzoic acid	C ₇ H ₅ O ₂ [−]	C ₆ H ₅ [−]	121/77	−40	−10	−16	−33
	Caffeic acid	C ₉ H ₇ O ₄ [−]	C ₈ H ₇ O ₂ [−]	179/135	−50	−10	−24	−55
	3-CGA, 4-CGA, 5-CGA	C ₁₆ H ₁₇ O ₉ [−]	C ₁₀ H ₇ O ₄ [−]	353/191	−55	−10	−36	−49
	Cinnamic acid	C ₉ H ₇ O ₂ [−]	C ₈ H ₇ [−]	147/103	−50	−10	−18	−45
	Coniferyl aldehyde	C ₁₀ H ₉ O ₃ [−]	C ₉ H ₆ O ₃ [−]	177/162	−90	−10	−22	−43
	Coniferyl alcohol [†]	C ₁₀ H ₁₁ O ₃ ⁺	C ₉ H ₇ O ⁺	163/131	50	10	13	16
	<i>p</i> -Coumaric acid	C ₉ H ₇ O ₃ [−]	C ₈ H ₇ O [−]	163/119	−75	−10	−22	−51
	Ferulic acid	C ₁₀ H ₉ O ₄ [−]	C ₈ H ₆ O ₂ [−]	193/134	−80	−10	−24	−55
	Salicylic acid	C ₇ H ₅ O ₃ [−]	C ₆ H ₅ O [−]	137/93	−80	−10	−26	−39
	Sinapaldehyde	C ₁₁ H ₁₁ O ₄ [−]	C ₉ H ₅ O ₄ [−]	207/177	−80	−10	−28	−29
	Sinapic acid	C ₁₁ H ₁₁ O ₅ [−]	C ₆ HO ₃ [−]	223/121	−100	−10	−40	−51
	Sinapyl alcohol	C ₁₁ H ₁₃ O ₄ [−]	C ₉ H ₅ O ₃ [−]	209/161	−90	−10	−28	−17
	Syringic acid	C ₉ H ₉ O ₅ [−]	C ₆ HO ₃ [−]	197/121	−100	−10	−24	−55
	Vanillic acid	C ₈ H ₇ O ₄ [−]	C ₆ H ₄ O ₂ [−]	167/108	−90	−10	−30	−47
	Vanillin	C ₈ H ₇ O ₃ [−]	C ₇ H ₄ O ₃ [−]	151/136	−70	−10	−20	−55
FLAVONOIDS	Apigenin	C ₁₅ H ₉ O ₅ [−]	C ₈ H ₅ O [−]	269/117	−145	−10	−46	−13
	Apigenin-7- <i>O</i> -glucoside	C ₂₁ H ₁₉ O ₁₀ [−]	C ₁₅ H ₉ O ₅ [−]	431/269	−170	−10	−40	−29
	Dihydrokaempferol	C ₁₅ H ₁₁ O ₆ [−]	C ₆ H ₅ O ₃ [−]	287/125	−100	−10	−30	−55
	Dihydroquercetin	C ₁₅ H ₁₁ O ₇ [−]	C ₆ H ₅ O ₃ [−]	303/125	−120	−10	−38	−51
	Eriodictyol	C ₁₅ H ₁₁ O ₆ [−]	C ₇ H ₃ O ₄ [−]	287/151	−100	−10	−22	−13
	Isoorientin	C ₂₁ H ₁₉ O ₁₁ [−]	C ₁₇ H ₁₁ O ₇ [−]	447/327	−165	−10	−38	−29
	Isovitexin	C ₂₁ H ₁₉ O ₁₀ [−]	C ₁₆ H ₁₁ O ₅ [−]	431/283	−250	−10	−46	−31
	Kaempferol	C ₁₅ H ₉ O ₆ [−]	C ₆ H ₅ O [−]	285/93	−100	−10	−52	−43
	Luteolin	C ₁₅ H ₉ O ₆ [−]	C ₇ H ₃ O ₄ [−]	285/151	−170	−10	−36	−41
	Luteolin-7- <i>O</i> -glucoside	C ₂₁ H ₁₉ O ₁₁ [−]	C ₁₅ H ₉ O ₆ [−]	447/285	−200	−10	−46	−31
	Maysin	C ₂₇ H ₂₇ O ₁₄ [−]	C ₂₁ H ₁₅ O ₉ [−]	575/411	−115	−10	−30	−37
	Naringenin	C ₁₅ H ₁₁ O ₅ [−]	C ₇ H ₃ O ₄ [−]	271/151	−120	−10	−26	−23
	Orientin	C ₂₁ H ₁₉ O ₁₁ [−]	C ₁₇ H ₁₁ O ₇ [−]	447/327	−165	−10	−38	−29
	Quercetin	C ₁₅ H ₁₁ O ₇ [−]	C ₇ H ₃ O ₄ [−]	301/151	−150	−10	−30	−55
	Rhamnosyl-isoorientin	C ₂₇ H ₂₉ O ₁₅ [−]	C ₁₆ H ₁₀ O ₆ [−]	593/298	−150	−10	−60	−1
	Vitexin	C ₂₁ H ₁₉ O ₁₀ [−]	C ₁₇ H ₁₁ O ₆ [−]	431/311	−170	−10	−32	−37

* DP: Declustering Potential.

† EP: Entrance Potential.

CE: Collision Energy.

§ CXP: Collision cell Exit Potential, are depicted for each metabolite.

** Precursor ion [M-H₂O]⁺ is followed for coniferyl alcohol due to a loss of a water molecule at the electrospray ionization source.

Additionally, the method was tested and validated by quantifying free and cell-wall bound compounds present in seedlings from two maize lines with contrasting lignin content.

2. Materials and methods

2.1. Chemicals

Flavonoid and other phenolic standards were purchased from Millipore-Sigma. [1-¹³C₁]-benzoic acid was ordered from Isotec. LC-MS grade acetic acid, formic acid, acetonitrile, and methanol were obtained from Thermo-Fisher. Ultrapure water (>18 mΩ) was generated through a Milli-Q system from Millipore. Phloroglucinol for lignin staining was purchased from Millipore-Sigma.

2.2. Plant materials and growth conditions

Plant selections were performed on the “maize Nested Association Mapping” (NAM) parental panel obtained from the USDA-ARS North Central Plant Introduction Station (Iowa State University, Ames, IA). Two-week-old CML333, Oh7B, and B73 maize seedlings used for soluble and cell wall phenolic analysis were grown in the greenhouse at 27 °C/21 °C day and night temperatures respectively, with a 16 h day/8 h night photoperiod and 60% relative humidity. Three biological replicates (n = 3) were used for the analyses.

2.3. Standard preparation for stock and working solutions

Flavonoid and other phenolic standards as well as [1-¹³C₁]-benzoic acid internal standard were reconstituted in 100% LC-MS grade methanol to a final concentration of 1 mM and stored at −20 °C. Standard curves were generated by serially diluting each metabolite with 100% methanol to give working solutions whose concentrations led to absolute injected quantities in the range of 50–500,000 fmol, and 20,000–5,000,000 fmol, depending on the compound. The limits of detection and quantification were defined as three and 10 times the signal to noise ratio, respectively.

A mixture of flavonoid, other phenolic, and [1-¹³C₁]-benzoic acid standards (1 μM of each metabolite, except 10 μM for sinapyl alcohol) was prepared. This standard mix was run along with the biological samples in order to perform absolute quantification of flavonoids and other phenolics extracted from maize seedlings.

2.4. High-performance reverse phase liquid chromatography

Flavonoids and other phenolics were analyzed utilizing a UHPLC 1290 system from Agilent Technologies. The mixture of metabolites was automatically injected using an auto-sampler kept at 10 °C. The liquid chromatography separation was carried out at 30 °C. In order to obtain an accurate liquid chromatographic method for the quantification of flavonoids and other phenolics, a reverse phase C18 Symmetry column (4.6 × 75 mm; 3.5 μm) with a Symmetry C18 pre-column (3.9 × 20 mm; 5 μm) from Waters was tested for its capacity to resolve the 35 metabolites of interest within a short period of time. For this purpose, a combination of different solvents

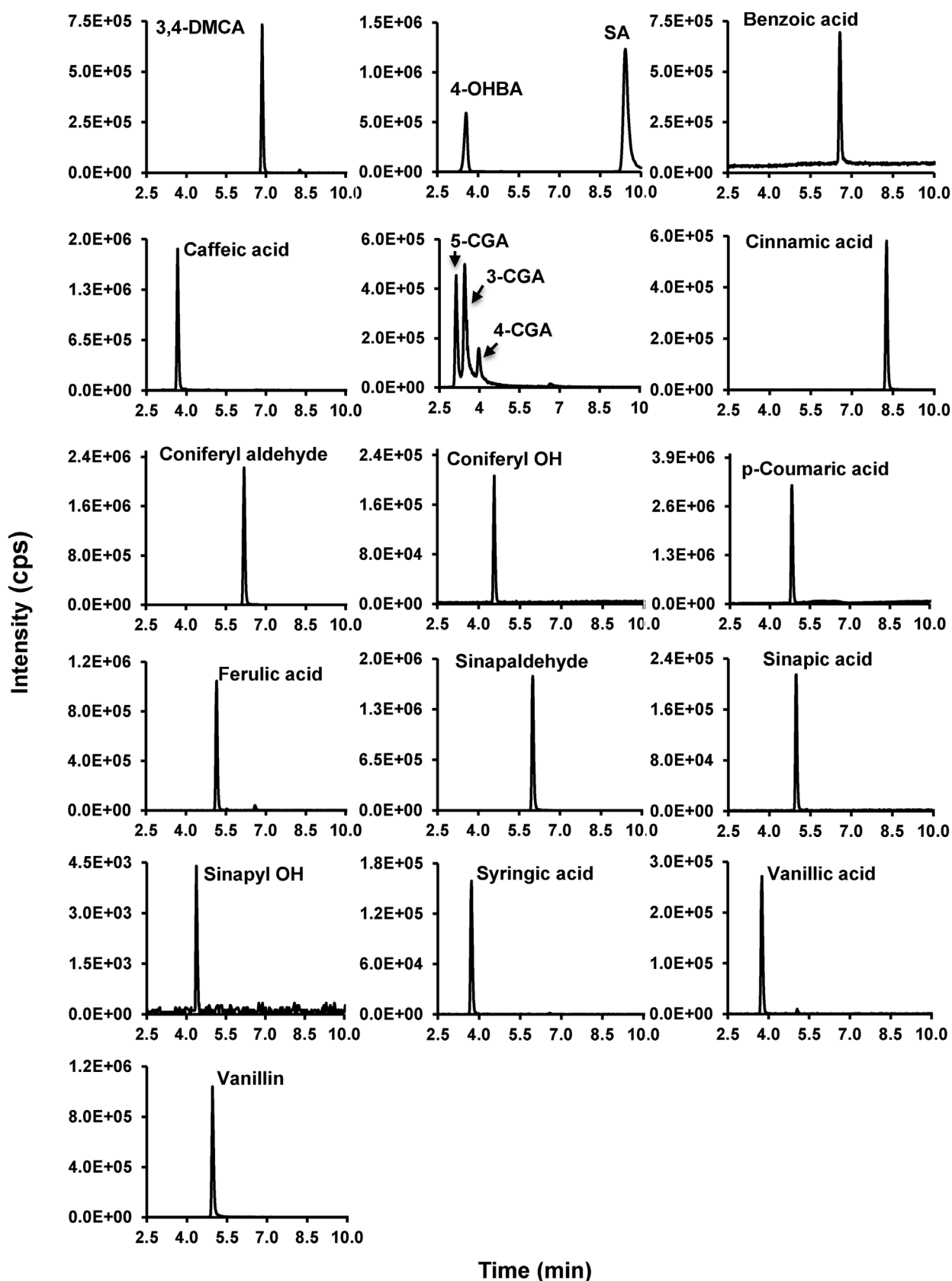


Fig. 2. Analysis of phenolic standards using multiple reaction monitoring. The separation and the assignment of phenolics were conducted as indicated in the Materials and Methods, [Tables 1, and 2](#). Each individual LC-MS/MS chromatogram represents a transition precursor/product ion associated with one or more phenolic(s). A transition with more than one peak depicts the existence of isomers (see 4-OHBA/SA transition). 3,4-DMCA, 3,4-dimethoxycinnamic acid; 4-OHBA, 4-hydroxybenzoic acid; SA, salicylic acid; CGA, chlorogenic acid; coniferyl OH, coniferyl alcohol; sinapyl OH, sinapyl alcohol.

Table 2
LC–MS/MS method sensitivity and linearity for flavonoids and other phenolics.

	Metabolite	Transition (m/z)	RT(min)	Linearity Range (fmol)	R ²	LOD (fmol)	LOQ (fmol)
OTHER PHENOLICS	3,4-Dimethoxycinnamic acid	207/103	6.7	200–50,000	0.9999	5.0	16.7
	4-Hydroxybenzoic acid	137/93	3.6	50–20,000	0.9997	9.4	31.5
	Benzoic acid	121/77	6.4	200–50,000	0.9999	19.1	63.5
	Caffeic acid	179/135	3.8	50–20,000	0.9997	3.1	10.4
	3-O-Caffeoylquinic acid	353/191	3.4	50–20,000	0.9993	1.2	3.9
	4-O-Caffeoylquinic acid	353/191	2.7	200–100,000	0.9987	39.7	132.5
	5-O-Caffeoylquinic acid	353/191	3.6	200–50,000	0.9982	3.9	12.9
	Cinnamic acid	147/103	8.1	200–50,000	0.9998	7.8	25.9
	Coniferyl aldehyde	177/162	6.1	50–20,000	0.9999	0.8	2.6
	Coniferyl alcohol	163/131	4.5	500–500,000	0.9971	54.7	182.5
	p-Coumaric acid	163/119	4.9	50–20,000	0.9998	6.8	22.6
	Ferulic acid	193/134	5.2	200–50,000	0.9972	3.9	13.0
	Salicylic acid	137/93	9.6	50–20,000	0.9993	5.5	18.3
	Sinapaldehyde	207/177	5.9	50–20,000	1.0000	1.0	3.2
	Sinapic acid	223/121	5.0	500–100,000	0.9986	21.0	69.9
	Sinapyl alcohol	209/161	4.3	20,000–5,000,000	0.9989	300.0	1000.0
	Syringic acid	197/121	3.7	500–100,000	0.9974	27.8	92.6
	Vanillic acid	167/108	3.8	500–100,000	0.9981	14.2	47.2
	Vanillin	151/136	4.8	50–20,000	0.9992	4.2	14.1
FLAVONOIDS	Apigenin	269/117	8.7	20–10,000	0.9999	1.5	4.9
	Apigenin-7-O-glucoside**	431/269	5.4	20–10,000	0.9997	0.0**	0.0**
	Dihydrokaempferol	287/125	6.5	50–20,000	0.9997	0.4	1.3
	Dihydroquercetin	303/125	5.4	500–100,000	0.9999	1.2	3.9
	Eriodictyol	287/151	7.4	20–10,000	1.0000	0.1	0.4
	Isoorientin	447/327	3.7	200–100,000	0.9926	2.0	6.7
	Isovitexin*	431/283	4.4	20–10,000	0.9990	1.0	3.2
	Kaempferol	285/93	9.0	500–100,000	0.9994	7.0	23.4
	Luteolin	285/151	7.5	200–100,000	0.9979	3.3	11.1
	Luteolin-7-O-glucoside	447/285	4.6	20–10,000	0.9967	0.1	0.4
	Maysin	575/411	5.5	200–100,000	0.9977	1.4	4.7
	Naringenin	271/151	8.6	20–10,000	0.9992	1.8	6.1
	Orientin	447/327	3.9	20–10,000	0.9962	0.1	0.4
	Quercetin	301/151	7.7	200–50,000	0.9980	0.9	3.1
	Rhamnosyl-isoorientin	593/298	3.5	200–100,000	0.9912	0.1	0.3
	Vitexin*	431/311	4.4	20–10,000	0.9974	0.3	1.0

Limits of detection (LOD) and limit of quantification (LOQ) were obtained based on a signal-to-noise ratio of 3:1 and 10:1, respectively. Retention time (RT), linearity range, and correlation coefficient (R²) were also accessible using the current LC–MS/MS method.

* Isovitexin and vitexin were not resolved chromatographically and spectrometrically, even when different product ions were selected.

** LOD and LOQ for apigenin-7-O-glucoside were 1.5 and 5.0 amol, respectively.

(acetonitrile, methanol, water) with different additives (acetic acid and formic acid) was examined as well as the impact of the flow rate and temperature onto the column. Acetonitrile-water with 0.1% acetic acid outperformed methanol-water and formic acid for the resolution and the sensitivity of the flavonoid and other phenolic isomers studied here (data not shown). The gradient used to separate the flavonoids and other phenolics consisted of 0.1% (v/v) acetic acid in acetonitrile (solvent A), and 0.1% (v/v) acetic acid in water (solvent B). The total UHPLC–MS/MS run was 15 min with a flow rate of 800 µL/min. The gradient applied to resolve the metabolites was as follows: A = 0–1 min 15%, 1–9 min 50%, 9–9.1 min 80%, 9.1–12 min 80%, 12–12.1 min 15%, 12.1–15 min 15%. A mixture of methanol/water (50:50; v:v) was used to rinse the auto-sampler needle after each injection. To generate the standard curves, the injected volumes adopted were 2, 5, and 10 µL.

2.5. Triple quadrupole mass spectrometer

Phenolic compounds and polyphenols were individually and directly infused into a triple quadrupole AB Sciex QTRAP 5500 mass spectrometer in order to optimize their detection parameters. The standards were diluted to 1 µM in 50% (v/v) methanol in ultrapure water. Each metabolite was injected individually, and directly into the mass spectrometer at a flow rate of 7 µL/min. First, the metabolites were tested for both negative and positive ionization modes using full scan detection survey (Q1). Then, a product ion scan survey (MS/MS) was automatically conducted in order to obtain the five most abundant fragments from the molecular ion as well as their associated MS parameters: i) the declustering potential

(DP), ii) the collision energy potential (CE), and iii) the collision cell exit potential (CXP). The parameters for the most abundant precursor/product ions corresponding to a particular compound are reported in Table 1.

Following flavonoid and other phenolic analyte optimization, a flow injection analysis was performed to optimize the parameters of the source/gas such as positive and negative ionization, temperature, and curtain, nebulizer, and heating gases (Materials and Methods). Ultimately, ion polarity switching mode was selected to develop robust liquid chromatographic conditions for the phytochemicals considered in this work.

Mass spectra were acquired using electrospray ionization switching from negative (3000 V) to positive mode (4000 V) with a settling time of 65 msec. Flavonoids and other phenolics were simultaneously detected using multiple reaction monitoring (MRM). The source parameters such as curtain gas (30 psi), temperature (650 °C), nebulizer gas (65 psi), heating gas (60 psi), and collision activated dissociation (Low) were kept constant during MRM. Note that the gas/source parameters cited above were previously optimized by direct flow injection analysis. The dwell time in the mass spectrometer was set to 20 msec. LC–MS/MS data were recorded and processed using Analyst 1.6.1 software (AB Sciex).

2.6. Determination of recovery, matrix effect and accuracy intra- and inter-assay

Four biological maize extracts from B73 seedlings were used to assess the recovery, matrix effect, and intra- and inter-day accuracy. Metabolite recovery was determined as previously described

[25,26], using the following equation: Recovery (%) = 100 x [analyte peak area (sample spiked before extraction) – analyte peak area (sample)]/[analyte peak area (sample spiked after extraction) – analyte peak area (samples)]. Matrix effect (ME) and intra- and inter-day accuracy for the different compounds were assessed following the procedure published [27]. Briefly, the ME was determined using the following equation: ME (%) = 100 x [analyte peak area (sample spiked after extraction) – analyte peak area (sample)]/average analyte peak area (external standard). In these terms, a ME close to 100% depicts no ion suppression. The accuracy was determined as the relative mean error (RME) between the concentration of the analyte in the spiked biological sample and the theoretical concentration (0.25, 0.5, and 1 μ M): RME (%) = [average analyte concentration (sample spiked) – mean analyte concentration (sample) – theoretical concentration]/theoretical concentration.

2.7. Histology

The maize stem was embedded in histology grade wax before sectioning to a thickness of approximately 1–1.5 mm using a hand microtome. Histochemical studies were carried out using phloroglucinol. A 2% (w/v) solution of phloroglucinol dissolved in a 2:1 mixture of ethanol and concentrated HCl was applied to the stem sections for 3 min and rinsed with water to detect lignin [28]. All sections were immediately observed using an SMZ1500 stereomicroscope (Benz). Images were registered using a Digital Sight DS-Fi1 camera (Nikon).

2.8. Extraction of soluble metabolite from biological samples

The biomass from two-week old Oh7B and CML333 plant stems was used to measure soluble phenolics. Stems were freeze-dried and a portion was homogenized using a bead beater with a 5 mm diameter tungsten bead for 5 min at 30 Hz (Restch MM 400). Ten milligrams of stem powder were transferred into a 1.5 mL microcentrifuge tube and 10 nmol [$1\text{-}^{13}\text{C}_1$]-benzoic acid was added as an internal standard at the time of extraction. 1 mL 100% (v/v) methanol at room temperature was added, mixed by vortexing for 30 s, and centrifuged for 5 min at 17,000g at room temperature. The supernatant was recovered and the extraction step was repeated once. A second round of extractions was performed twice using 70% methanol (v/v) in ultrapure water. The four supernatants from each sample were pooled together, and dried to completion using a SpeedVacuum.

2.9. Extraction of cell wall bound phenolics

The biomass from two-week old Oh7B and CML333 stems was used to measure cell wall-bound phenolics. The biomass after soluble metabolites extraction was dried to completion in a SpeedVac. 10 nmol [$1\text{-}^{13}\text{C}_1$]-benzoic acid was then added as an internal standard to the extracted biomass (5 mg), mixed with 500 μ L of 2 M NaOH, and shaken at 1400 rpm for 24 h at 25 °C. The mixture was acidified with 100 μ L of concentrated HCl and subjected to three ethyl acetate partitioning steps. Ethyl acetate fractions were pooled and dried in a SpeedVacuum.

2.10. LC–MS/MS quantification of intracellular metabolites from maize seedlings

For soluble phenolics, extracts were re-suspended in 500 μ L of 50% (v/v) methanol in ultrapure water, then cleared by centrifugation (5 min, 20,000g), and filtered using 0.2 μ m centrifugal filter (Pall Nanosep MF centrifugal device with Bio-Inert membrane;

Table 3

Matrix effect* (ME, %) and recovery efficiency** (RE, %) of methanol soluble flavonoids and other phenolics.

	Metabolite	ME (%) [*] (n = 3)	RE (%) ^{**} (n = 3)
OTHER PHENOLICS	3,4-Dimethoxycinnamic acid	93.8 $\pm$ 1.1	90.0
	4-Hydroxybenzoic acid	82.8 $\pm$ 2.2	116.3
	Benzoic acid	95.4 $\pm$ 1.9	94.0
	Caffeic acid	79.2 $\pm$ 2.9	105.6
	3-O-Caffeoylquinic acid [†]	ND	ND
	4-O-Caffeoylquinic acid	97.6 $\pm$ 5.0	53.6
	5-O-Caffeoylquinic acid	97.2 $\pm$ 6.0	94.7
	Cinnamic acid	96.5 $\pm$ 1.8	90.5
	Coniferyl aldehyde	95.2 $\pm$ 2.8	79.0
	Coniferyl alcohol	93.7 $\pm$ 1.8	79.4
	p-Coumaric acid	99.4 $\pm$ 2.0	98.6
	Ferulic acid	97.4 $\pm$ 1.9	105.7
	Salicylic acid	96.8 $\pm$ 0.8	87.3
	Sinapaldehyde	95.8 $\pm$ 1.6	81.3
	Sinapic acid	93.8 $\pm$ 1.9	97.3
	Sinapyl alcohol	89.8 $\pm$ 3.0	97.2
	Syringic acid	69.6 $\pm$ 2.0	111.0
	Vanillic acid	73.8 $\pm$ 0.9	116.4
	Vanillin	100.8 $\pm$ 0.6	106.4
FLAVONOIDS	Apigenin	99.0 $\pm$ 3.9	47.5
	Apigenin-7-O-glucoside	101.1 $\pm$ 0.8	97.9
	Dihydrokaempferol	102.2 $\pm$ 1.1	81.4
	Dihydroquercetin	100.0 $\pm$ 1.8	85.7
	Eriodictyol	98.7 $\pm$ 3.3	84.6
	Isoorientin	66.6 $\pm$ 2.9	87.4
	Isovitexin [*]	101.1 $\pm$ 0.1	100.5
	Kaempferol	99.3 $\pm$ 2.0	37.2
	Luteolin	100.8 $\pm$ 0.4	42.9
	Luteolin-7-O-glucoside	104.6 $\pm$ 1.1	98.6
	Maysin	100.6 $\pm$ 0.6	95.3
	Naringenin	97.2 $\pm$ 3.6	79.7
	Orientin	98.7 $\pm$ 1.6	98.6
	Quercetin	99.0 $\pm$ 2.0	33.5
	Rhamnosyl-isoorientin	86.7 $\pm$ 2.0	96.6

Values of matrix effect ME < 70% and recovery efficiency RE < 75% are depicted in bold and italic.

[†] 3-O-Caffeoylquinic acid was highly abundant in maize seedling extract which was preventing the determination of the ME and RE. This is depicted by the “ND” abbreviation for “not determined”.

Millipore-Sigma). A 5 μ L aliquot of sample was injected onto the column.

For cell wall bound phenolics, extracts were re-suspended in 500 μ L 50% (v/v) methanol in ultrapure water and filtered at 20,000 g for 5 min using 0.2 μ m centrifugal filter. A 50 μ L aliquot of extract was added to a vial containing 450 μ L of 50% (v/v) methanol in ultrapure water, and 1 μ L of the diluted sample was injected onto the column.

The quantification of intracellular metabolites was accomplished by UHPLC-MS/MS, using: i) [$1\text{-}^{13}\text{C}_1$]-benzoic acid as internal standard to account for any loss of material during sample preparation; and ii) phenolic external standards consisting of known concentrations of phenolics.

2.11. Statistical analysis

For each flavonoid and other phenolic compounds, the mean and standard deviation were calculated from three biological replicates. The principal component analysis (PCA) was performed using MetaboAnalyst v3.0 [29] after the data for each variable were normalized using log2 function, mean-centered, and divided by the standard deviation. Differences between CML333 and Oh7B were tested by two-sided Student *t*-test.

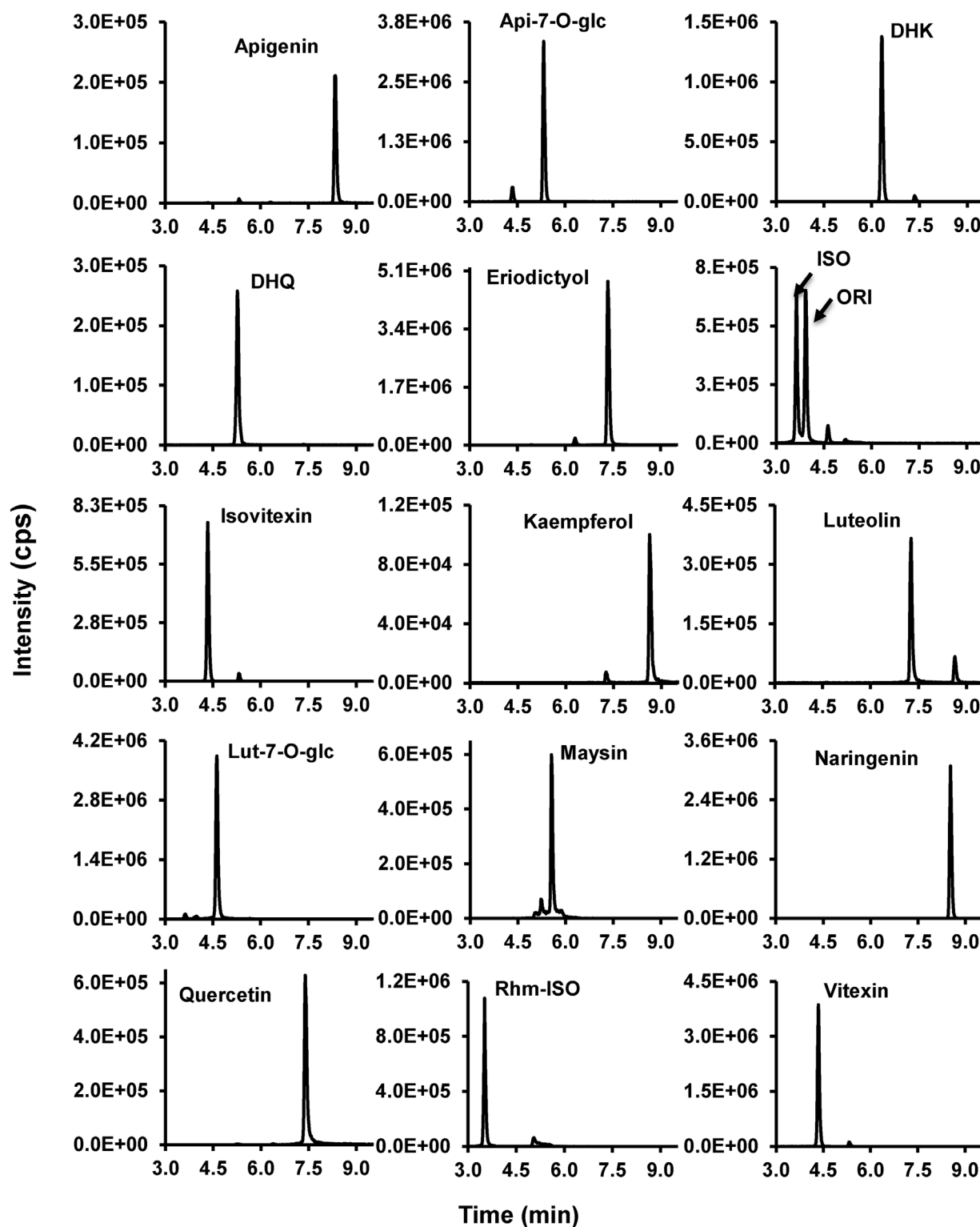


Fig. 3. Analysis of flavonoid standards using multiple reaction monitoring. The separation and the assignment of flavonoids were conducted as indicated in the Materials and Methods, Tables 1, and 2. Each individual LC-MS/MS chromatogram represents a transition precursor/product ion associated with one or more flavonoid(s). A transition with more than one peak depicts the existence of isomers (see ISO/ORI transition). Api-7-O-glc, apigenin-7-O-glucoside; DHK, dihydrokaempferol; DHQ, dihydroquercetin; ISO, isoorientin; ORI, orientin; Lut-7-O-glc, luteolin-7-O-glucoside; Rhm-ISO, rhamnosyl-isoorientin.

3. Results and discussion

3.1. Optimization of mass spectrometry parameters for the quantification of plant phenolic compounds

Phenolic compounds presented in Fig. 1 are commonly found in cereals, fruits and vegetables [6,30]. Among those phytochem-

icals, a set of 35 commercially available metabolites was selected to develop a selective and quantitative LC-MS/MS method using multiple reaction monitoring (MRM). This group of phenolic compounds comprised: i) phenolic acids (3,4-dimethoxycinnamic acid, 4-hydroxybenzoic acid, benzoic acid, 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, caffeic acid, cin-

Table 4

Intra- and inter-day accuracy (%) for methanol soluble flavonoids and other phenolics.

		Accuracy (%)					
		Intra-day assay (n = 4)			Inter-day assay (n = 12)		
	Metabolite	0.25 [†] μM [†]	0.5 [†] μM [†]	1 [†] μM [†]	0.25 [†] μM [†]	0.5 [†] μM [†]	1 [†] μM [†]
OTHER	3,4-Dimethoxycinnamic acid	−3.7	−0.5	1.4	−2.9	−0.4	0.2
PHENOLICS	4-Hydroxybenzoic acid	−8.3	−6.9	−6.7	−10.2	−8.5	−7.3
	Benzoic acid	−10.8	−8.8	−5.7	−15.5	−9.7	−6.3
	Caffeic acid	−6.2	−3.5	1.4	−8.1	−4.5	−1.7
	3-O-Caffeoylquinic acid [*]	ND	ND	ND	ND	ND	ND
	4-O-Caffeoylquinic acid	1.7	5.9	7.5	4.9	5.6	2.8
	5-O-Caffeoylquinic acid	−1.5	0.9	6.1	0.2	0.6	1.0
	Cinnamic acid	−1.8	−1.2	2.4	−3.3	−0.5	1.5
	Coniferyl aldehyde	−0.4	−2.9	−0.4	−0.9	−1.3	−0.9
	Coniferyl alcohol	−23.0	−13.4	−3.6	−29.5	−10.8	−1.9
	p-Coumaric acid	−3.3	−5.4	−0.9	−7.4	−5.0	−0.1
	Ferulic acid	−4.1	−5.8	−1.0	−5.1	−2.4	−0.7
	Salicylic acid	1.5	−0.2	0.4	0.7	−0.1	1.2
	Sinapaldehyde	−2.9	−2.9	−2.4	−2.1	−2.4	−2.5
	Sinapic acid	−0.9	0.8	−2.3	−2.7	−1.2	−0.8
	Sinapyl alcohol	−2.4	−0.5	−2.8	−5.5	−1.6	−2.9
	Syringic acid	−15.8	−11.6	−7.7	−18.3	−15.3	−9.8
	Vanillic acid	−28.6	−23.6	12.5	−27.6	−26.5	−3.4
	Vanillin	−3.5	0.1	0.4	−3.2	−0.5	−0.4
FLAVONOIDS	Apigenin	1.3	−0.5	5.0	1.2	0.6	3.5
	Apigenin-7-O-glucoside	−0.3	0.5	1.6	−1.1	−0.2	1.2
	Dihydrokaempferol	−1.0	0.8	3.5	0.4	1.5	3.1
	Dihydroquercetin	−0.2	1.5	0.5	−1.1	1.6	2.7
	Eriodictyol	−0.3	−1.4	−0.5	−1.1	−1.4	−0.8
	Isoorientin	−13.3	−6.8	−1.2	−13.8	−9.9	−5.4
	Isovitexin	−3.1	−2.8	1.8	−3.3	−1.6	3.0
	Kaempferol	0.1	−1.8	−1.5	−1.1	−0.1	0.9
	Luteolin	2.1	−0.2	0.8	0.0	2.9	2.6
	Luteolin-7-O-glucoside	2.6	2.9	4.2	1.8	4.8	6.8
	Maysin	−2.9	−3.1	2.3	−1.9	−0.6	2.0
	Naringenin	−0.2	−2.4	5.2	−1.2	0.0	2.9
	Orientin	−2.3	−4.8	−3.3	3.0	1.5	1.0
	Quercetin	2.7	−0.3	0.3	−2.3	−2.1	−1.1
	Rhamnosyl-isoorientin	−1.8	−1.3	−1.1	−1.8	−0.8	−2.2

Low accuracies $\pm 20\%$ are depicted in bold and italic.^{*} 3-O-Caffeoylquinic acid was highly abundant in maize seedling extract which was preventing the determination of the accuracies. This is depicted by the “ND” abbreviation for “not determined”.[†] Metabolite concentration (μM) added to maize seedling extract.

amic acid, p-coumaric acid, ferulic acid, salicylic acid, sinapic acid, syringic acid, vanillic acid), ii) aldehyde forms of phenolic acids (vanillin, sinapaldehyde, coniferyl aldehyde), iii) alcohol forms of phenolic acids (coniferyl alcohol, sinapyl alcohol), and iv) flavonoids (apigenin, apigenin-7-O-glucoside, dihydrokaempferol, dihydroquercetin, eriodictyol, isoorientin, isovitexin, kaempferol, luteolin, luteolin-7-O-glucoside, maysin, naringenin, orientin, quercetin, rhamnosyl-isoorientin, vitexin).

Phenolic compounds and polyphenols were individually and directly infused into a triple quadrupole AB Sciex QTRAP 5500 mass spectrometer (Materials and Methods). The best sensitivity for the majority of the phytochemicals was achieved under negative ionization, except for coniferyl alcohol (Table 1). The most abundant product ion (quantifier ion) for each phenolic compound is reported in Table 1. The product ions for the phenolic acids were characterized by a neutral loss corresponding to: i) one CO_2 ($\Delta m/z = 44$ amu) for 4-hydroxybenzoic, benzoic, caffeic, cinnamic, coumaric, salicylic acids, ii) one CH_3 ($\Delta m/z = 15$ amu) and one CO_2 ($\Delta m/z = 44$ amu) for ferulic and vanillic acids, iii) two molecules of formaldehyde ($\Delta m/z = 60$ amu) plus one CO_2 ($\Delta m/z = 44$ amu) for 3,4-dimethoxycinnamic acid, and iv) formic acid ($\Delta m/z = 46$) plus two CH_3 ($\Delta m/z = 30$ amu) for syringic acid [31]. For chlorogenic acids (3-CGA, 4-CGA, 5-CGA), their product ions consisted of deprotonated quinic acid, which was in accordance with a previous work conducted [32]. Neutral losses of one (−15) or two (−30) CH_3 groups were observed for coniferyl aldehyde and vanillin, and for

sinapaldehyde, respectively [33]. A loss of water ($\Delta m/z = 18$ amu) and two CH_3 ($\Delta m/z = 30$ amu) were observed for sinapyl alcohol, and a loss of methanol ($\Delta m/z = 32$ amu) was detected for coniferyl alcohol. The precursor ion $[\text{M}-18]^+$ of coniferyl alcohol was positively ionized in order to achieve the best sensitivity.

Flavonoids have their backbone made of three rings namely A, B, and C, with the cleavage of the C–C bond of the C-ring giving structural information on the chemical groups present on the A- and B-rings. Moreover, flavonoids can be decorated with sugar moiety or moieties, and are called flavonoid glycosides. Nomenclatures previously established [34–40] were utilized to elucidate the structure of the product ion corresponding to each flavonoid under investigation (Table 1). The significance of the letter code and its associated superscript/subscript numbers mentioned herein are defined in Fig. A.1 (Supplemental material) [34]. Most of the flavonoid aglycones had cleavage of the C-ring bonds and generated product ion containing a part of the C-ring plus: i) the A-ring yielding $^{1,3}\text{A}^-$ (eriodictyol, naringenin, luteolin), $^{1,4}\text{A}^-$ (dihydrokaempferol, dihydroquercetin), and $^{1,2}\text{A}^-$ (quercetin) fragments, and ii) the B-ring producing $^{1,3}\text{B}^-$ fragment for apigenin. Kaempferol was the only flavonoid for which the selected product ion (deprotonated phenol) had a cleavage occurring in the C–C bond between the B- and the C-rings. For the flavonoid O-glycosides (apigenin-7-O-glucoside, luteolin-7-O-glucoside), the product ions depicted in Table 1 represented the Z_0^- fragment containing the aglycone moiety. On the other hand, the flavonoid C-monoglycosides were

characterized by mass losses corresponding to the fragmentation $^{0,1}\text{X}_0^-$ (isovitexin) and $^{0,2}\text{X}_0^-$ (isoorientin, orientin, vitexin) in the sugar moieties. Rhamnosylisoorientin and maysin (flavonoid C-diglycosides) had neutral losses consistent with $^{0,1}\text{X}_0^-$ and Z_1^- fragmentations, respectively.

3.2. LC–MS/MS method development for the quantification of plant phenolic compounds

A reverse phase C18 Symmetry column (4.6×75 mm; $3.5 \mu\text{m}$) was tested with different solvents, additives, flow rates and temperatures for its capacity to resolve the 35 metabolites of interest within a short period of time (Material and Methods). The best performing method was able to resolve the 33 out of the 35 metabolites over a total analytical period of 15 min using a gradient of acetonitrile, while acetic acid remained at 0.1%. The initial conditions were 15% acetonitrile for one minute, and then the acetonitrile was linearly increased to 50% for eight minutes, which permitted the elution of all the flavonoids and other phenolics except salicylic acid (Table 2, Figs. 2 and 3). Furthermore, these chromatographic settings were enabling the resolution of isobaric metabolites, specifically 3-, 4-, and 5-CGAs (353/191), 4-hydroxybenzoic acid and salicylic acid (137/93), isoorientin and orientin (447/327), and luteolin (285/151) and kaempferol (285/93) as depicted in Figs. 2 and 3. Unfortunately, the separation of the pair of isomers isovitexin/vitexin (431/283) was not achieved under these conditions, which lead us to only consider isovitexin for the remainder of the study. However, a partial resolution of these flavonoids could be obtained with 0.1% formic acid instead of acetic acid as additive (data not shown).

There was a strong linearity for all the calibration curves of flavonoid and other phenolic compounds from low fmol to high pmol range with correlation coefficients above 0.99 (Table 2). The sensitivity of this LC–MS/MS approach is demonstrated by its limits of detection between 1.5 amol for apigenin-7-O-glucoside to 300 fmol for sinapyl alcohol, and its limits of quantification between 5 amol to 1000 fmol.

In order to further validate the application of this UHPLC–MS/MS method to biological samples, the matrix effect (ME) for each flavonoid and other phenolic compound was investigated, as well as the recovery efficiency (RE) from the soluble and cell wall-bound fractions (Tables 3, and A.1, Supplemental material). Overall, there was no ion suppression from the biological matrix, except for syringic acid and isoorientin whose signal was inhibited by 30.4 and 33.4%, respectively. The efficiency with which each compound was recovered from the biological soluble fraction was found to be above 75% for the majority of them, except 4-O-Caffeoylquinic acid, apigenin, kaempferol, luteolin, and quercetin for which the respective REs were 53.6, 47.5, 37.2, 42.9, and 33.5%. It is noteworthy that ME and RE were not assessed for 3-O-caffeoylquinic acid due to its high abundance in the biological samples. The phenolic compounds bound to the cell wall were recovered with a high efficiency, except for caffeic acid, coniferyl aldehyde, and synapaldehyde (Table A.1, Supplemental material). For cell wall bound vanillin, the recovery was found to be 144%, which may indicate ionization enhancement due to coeluting sample compounds [41]. It is important to note that the extraction treatment consisting of sodium hydroxide (2 M) and concentrated hydrochloric acid is widely applied in the field [42,43]. Our study demonstrates that this treatment results in the degradation of caffeic acid, as well as a partial loss of the phenolic aldehydes.

As part of the validation procedure, the intra- and inter-day accuracy of the analytical method were determined as described by [27] and reported in Table 4. With the exception of coniferyl alcohol

Table 5

Quantitative analysis of methanol-soluble flavonoids and other phenolics in stems from two-week-old Oh7B and CML333 seedlings.

		Average $\pm$ SD (pmol/mg DW)	
		Oh7B	CML333
OTHER PHENOLICS	3,4-Dimethoxycinnamic acid	5 $\pm$ 1	2 $\pm$ 2
	Benzoic acid	139 $\pm$ 19	124 $\pm$ 2
	Caffeic acid	8 $\pm$ 2	12 $\pm$ 7
	Cinnamic acid	NQ	NQ
	Coniferaldehyde	1.6 $\pm$ 0.2	1.4 $\pm$ 0.4
	Coumaric acid	19 $\pm$ 7	16 $\pm$ 7
	3-O-Caffeoylquinic acid	NQ	NQ
	4-O-Caffeoylquinic acid ^c	147 $\pm$ 44	2,233 $\pm$ 162
	5-O-Caffeoylquinic acid ^c	415 $\pm$ 32	6,604 $\pm$ 770
	Sinapyl alcohol	395 $\pm$ 42	394 $\pm$ 8
	Coniferyl alcohol	81 $\pm$ 18	63 $\pm$ 9
	Ferulic acid ^a	139 $\pm$ 50	28 $\pm$ 18
	Salicylic acid	5 $\pm$ 2	6 $\pm$ 2
	4-Hydroxybenzoic acid	NQ	NQ
	Sinapaldehyde	2.1 $\pm$ 0.5	2.0 $\pm$ 0.3
	Sinapic acid	22 $\pm$ 4	11 $\pm$ 7
	Syringic acid	0.9 $\pm$ 0.4	0.7 $\pm$ 0.1
	Vanillin	51 $\pm$ 10	46 $\pm$ 1
	Vanillic acid	9 $\pm$ 2	4 $\pm$ 3
FLAVONOIDS	Naringenin ^b	3.3 $\pm$ 0.5	1.5 $\pm$ 0.4
	Eriodictyol ^c	1.0 $\pm$ 0.1	0.1 $\pm$ 0.0
	Apigenin ^b	14 $\pm$ 3	25 $\pm$ 1
	Apigenin-7-O-glucoside ^c	0.2 $\pm$ 0.0	0.6 $\pm$ 0.0
	Luteolin	1.0 $\pm$ 0.2	0.6 $\pm$ 0.0
	Luteolin-7-O-glucoside	0.6 $\pm$ 0.1	0.3 $\pm$ 0.1
	Dihydrokaempferol ^c	0.2 $\pm$ 0.1	2.5 $\pm$ 0.6
	Dihydroquercetin	NQ	NQ
	Kaempferol ^a	5 $\pm$ 1	39 $\pm$ 33
	Quercetin ^b	10 $\pm$ 1	5 $\pm$ 1
	Orientin ^b	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0
	Isoorientin ^c	38 $\pm$ 5	4 $\pm$ 1
	Isovitexin/Vitexin ^c	22 $\pm$ 3	4 $\pm$ 1
	Rhamnosyl-isoorientin ^c	23 $\pm$ 5	1 $\pm$ 1
	Maysin ^c	2,396 $\pm$ 465	1 $\pm$ 1

Values are means of three biological replicates ($n = 3$). NQ indicates not quantified, either due to absence of metabolite or values below the LOQ. Letters next to each name indicate significant differences between Oh7B and CML333 using two-sided Student's *t*-test.

^a *p*-value below 0.05.

^b *p*-value below 0.01.

^c *p*-value below 0.001.

and vanillic acid, all the flavonoids and other phenolic compounds had intra- and inter-day accuracies in a $\pm 20\%$ range.

3.3. Application of the novel LC–MS/MS method to the quantification of phenolics in seedlings from two maize lines with contrasting lignin content

A panel of twenty-three maize lines was grown to obtain two-week-old seedlings for selection by histochemical analysis with phloroglucinol staining. From these lines, CML333 and Oh7B presented the most contrasting lignin content after staining (Fig. 4). We used our new methodology to test if this difference in lignin was correlated with a variation in the levels of intermediate compounds from the phenylpropanoid pathway. Over the 34 flavonoids and other phenolics monitored, 30 and 16 were within the quantification range in the soluble and cell-wall bound fractions, respectively (Tables 5 and 6). Principal component analysis (PCA) of the complete dataset of the intermediaries of the phenylpropanoid pathway showed that differences in the levels of these metabolites separated Oh7B from CML333 samples (Fig. 5). Principal component 1 (PC1) explained 60.3% of the variance, and the loadings for each compound are reported in Table A.2. The variables that contributed the most (positively or negatively) to the separation were the ones that were found significantly different between the

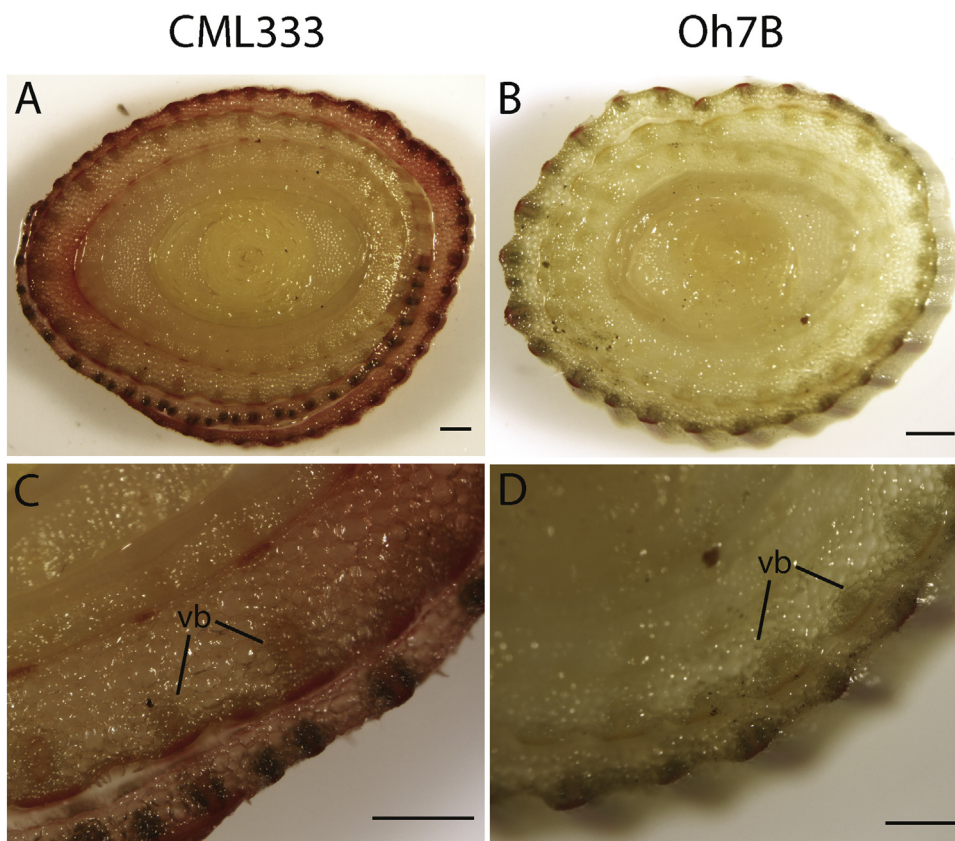


Fig. 4. Histochemical staining of CML333 and Oh7B stems. Stem cross-sections of two-week old seedlings analyzed by phloroglucinol staining under light microscope. Panels A and C correspond to the maize line CML333, and panels B and D to Oh7B. Vb, vascular bundles. Scale bars represent 500 μm.

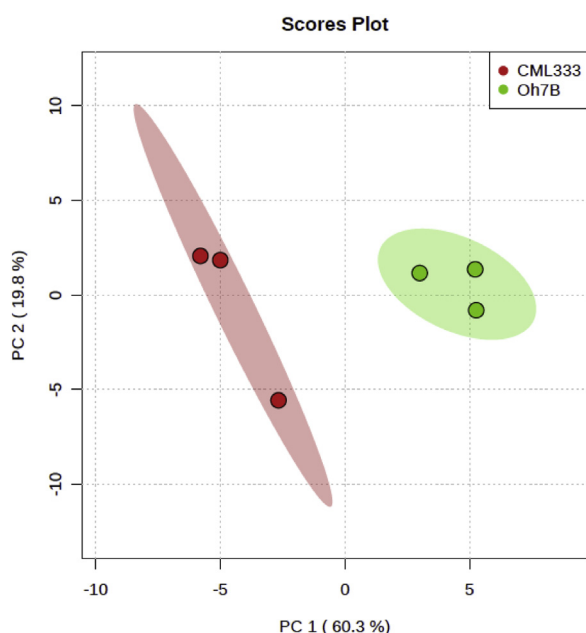


Fig. 5. Principal component analysis of the flavonoid and other phenolic compounds in CML333 and Oh7B seedlings. The shaded red and green ellipses in the PCA plot represent 95 % confidence intervals for the two maize lines CML333 and Oh7B, respectively. Three biological replicates ($n=3$) were used for the analyses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

two maize lines (Tables 5 and 6; ^a p -value<0.05; ^b p -value<0.01; ^c p -value<0.001).

In the soluble fraction, the levels of three phenolic acids were found to be significantly different: 4-CGA and 5-CGA were higher in CML333 whereas ferulic acid was reduced in comparison to Oh7B (Table 5). However, no significant reduction was observed for the content of several other metabolites derived from the lignin pathway, including ferulic and sinapic acids (Table 5). Although most of the free phenolics in the cytosol are similar, the difference in the transport of monolignols to the apoplast for polymerization of lignin and/or the glycosylation for transfer to the vacuole may cause the difference in lignin accumulation of the two lines. All the flavonoids quantified with the exception of luteolin, luteolin-7-*O*-glucoside and dihydroquercetin were statistically significantly different between CML333 and Oh7B (Table 5). It can be inferred from Table 5 that the C-glycosyl flavone pathway leading to maysin accumulation is highly active, given the high values of this metabolite in stems, it remains to be determined if this is the case for apimaysin (Table 5).

With regards to the cell wall bound compounds, we did observe high levels of caffeic, coumaric, vanillic and ferulic acids released after basic hydrolysis. In addition, 4-hydroxybenzoic acid and caffeic acid were statistically significantly different between Oh7B and CML333 (Table 6). However, it is important to note that the recovery of cell wall bound caffeic acid was very low (Table A.1, Supplemental material), and therefore the difference between the two lines may not be relevant. All flavonoids with the exception of rhamnosylisoorientin were 'not quantified' either because they were absent or the levels detected were below the limit of quantification (Table 6).

Table 6

Quantitative analysis of cell-wall bound metabolites in stems from two-week-old Oh7B and CML333 seedlings.

	Metabolite	Average $\pm$ SD (pmol/mg DW)	
		Oh7B	CML333
OTHER	3,4-Dimethoxycinnamic acid	NQ	NQ
	Benzoic acid	1,056 $\pm$ 131	846 $\pm$ 252
PHENOLICS	Caffeic acid ^b	19,773 $\pm$ 6,812	2,229 $\pm$ 1,538
	Cinnamic acid	NQ	NQ
	Coniferaldehyde	84 $\pm$ 28	101 $\pm$ 76
	Coumaric acid	138,308 $\pm$ 8,298	77,542 $\pm$ 29,723
	3-O-Caffeoylquinic acid	NQ	NQ
	4-O-Caffeoylquinic acid	NQ	NQ
	5-O-Caffeoylquinic acid	NQ	NQ
	Sinapyl alcohol	5,590 $\pm$ 1,136	4,269 $\pm$ 2,082
	Coniferyl alcohol	335 $\pm$ 91	221 $\pm$ 118
	Ferulic acid	206,245 $\pm$ 10,781	109,038 $\pm$ 62,517
	Salicylic acid	NQ	NQ
	4-Hydroxybenzoic acid ^c	235 $\pm$ 29	2,077 $\pm$ 161
	Sinapaldehyde	12 $\pm$ 3	25 $\pm$ 23
	Sinapic acid	218 $\pm$ 49	152 $\pm$ 68
	Syringic acid	46 $\pm$ 8	27 $\pm$ 14
	Vanillin	8,629 $\pm$ 977	6,632 $\pm$ 1,164
	Vanillic acid	1,146 $\pm$ 236	786 $\pm$ 223
FLAVONOIDS	Naringenin	NQ	NQ
	Eriodictyol	NQ	NQ
	Apigenin	NQ	NQ
	Apigenin-7-O-glucoside	NQ	NQ
	Luteolin	NQ	NQ
	Luteolin-7-O-glucoside	NQ	NQ
	Dihydrokaempferol	NQ	NQ
	Dihydroquercetin	NQ	NQ
	Kaempferol	NQ	NQ
	Quercetin	NQ	NQ
	Orientin	NQ	NQ
	Isoorientin	NQ	NQ
	Isovitexin/Vitexin	NQ	NQ
	Rhamnosyl-isoorientin	1.8 $\pm$ 0.4	1.0 $\pm$ 0.2
	Maysin	NQ	NQ

Values are means of three biological replicates (n = 3). NQ indicates not quantified, either due to absence of metabolite or values below LOQ. Letters next to each name indicate significant differences between Oh7B and CML333 using two-sided Student's *t*-test

^a *p*-value below 0.05.

^b *p*-value below 0.01.

^c *p*-value below 0.001.

4. Conclusions

This work reports the development and validation of a high-throughput UHPLC-MS/MS method using MRM scan survey for the simultaneous separation and quantification of flavonoids, and phenolic acids, aldehydes, and alcohols. A total of 33 compounds were resolved, including the isobaric species chlorogenic acids, and hydroxybenzoic acids, with high accuracy and sensitivity. This method was successfully applied to determine the *in vivo* levels of flavonoids and other phenolics (free and cell wall-bound) in seedlings from two maize lines with contrasting lignin content. We foresee that this high throughput approach will allow for a more accurate and faster selection in breeding projects and its applicability towards other crops of economical relevance.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.chroma.2018.12.059>.

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