JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

An UPLC-MS/MS Method for the Simultaneous Identification and Quantitation of Cell Wall Phenolics in *Brassica napus* Seeds

Andrej Frolov,*[,†,§] Anja Henning,[†] Christoph Böttcher,[‡] Alain Tissier,[†] and Dieter Strack[†]

[†]Department of Cell and Metabolic Biology and [‡]Department of Stress and Developmental Biology, Leibniz Institute of Plant Biochemistry, Weinberg 3, 06120 Halle (Saale), Germany

Supporting Information

ABSTRACT: The seed residues left after pressing of rapeseed oil are rich in proteins and could be used for human nutrition and animal feeding. These press cakes contain, however, antinutritives, with fiber being the most abundant one. The analysis of fiber phenolic component (localized to seed coat cell walls) is, therefore, important in breeding and food quality control. However, correct structure and content assignments of cell wall-bound phenolics are challenging due to their low stability during sample preparation. Here, a novel LC-MS/MS-based method for the simultaneous identification and quantitation of 66 cell wall-bound phenolics and their derivatives is described. The method was internally standardized, corrected for degradation effects during sample preparation, and cross-validated with a well-established UV-based procedure. This approach was successfully applied to the analysis of cell wall phenolic patterns in different *B. napus* cultivars and proved to be suitable for marker compound search as well as assay development.

KEYWORDS: *cell wall phenolics, Brassica napus, quantitation, LC-MS/MS, tandem mass spectrometry, information-dependent acquisition (IDA)*

INTRODUCTION

Oilseed rape (*Brassica napus*) is one of the most important oil-yielding crops in agriculture. The press cakes, left after oil extraction, are rich in proteins and could be used for feeding of domestic animals. However, these residues contain antinutritive components, with fiber among the most important ones.[1] In mature seeds, the fiber is localized mostly to the secondary cell walls of the seed coat and correlates with reduced digestibility in mammals.[2] Despite the high economical importance of antinutritive fiber content reduction, little information on the structure and composition of cell wall-associated phenolics in *B. napus* seeds is available.

The plant cell wall is a strong fibrillar network that gives each cell its stable shape.[3] Generally, the fraction of plant cell wall phenolics mostly consists of lignins and low molecular weight phenolic acids and their derivatives.[4] The latter esterify cell wall matrix biopolymers and serve as lignin precursors.[4,5] The esters can be cleaved by alkaline hydrolysis (saponification).[4,6,7] This procedure is usually performed after removal of intracellular phenylpropanoids. Resulting isolates are referred to as "purified cell walls"[7] or "crude cell wall preparation".[6] In the literature, the phenolic fraction, obtained by saponification of crude cell wall preparation is usually referred to as "cell wall-bound phenolics".[8] It should be distinguished from "cell wall phenolics", the total phenylpropanoid fraction of the cell wall.[9]

Online coupling of reversed phase high-performance liquid chromatography (RP-HPLC) to electrospray ionization mass spectrometry (ESI-MS) is the way to achieve both high selectivity and sensitivity of analysis[10] that can be additionally improved by the ultraperformance liquid chromatography (UPLC) technique.[11] For metabolite identification and quantitation, liquid chromatography−tandem mass spectrometry (LC-MS/MS), that is, targeted metabolite profiling, is the strategy of choice.[10,12,13] The MS/MS instruments based on ion traps (ITs)[12] provide comprehensive structure analysis, whereas triple quadrupole (QqQ) mass analyzers provide highly sensitive, fast, and precise quantitation by multiple reaction monitoring (MRM).[13] The hybrid triple quadrupole-linear ion trap (QqLIT) mass analyzer combines the advantages of both QqQ- and LIT instruments,[14] which makes highly sensitive analyte quantitation and identification in a single chromatographic analysis possible.

Here we present a new RP-UPLC-ESI-MS/MS method for the simultaneous identification and quantitation of phenolic monomers as well as their degradation products in saponified cell wall preparations. We show also the applicability of the method to the analysis of bound phenylpropanoid composition in *B. napus* seeds. The method can be used for the estimation of cell wall phenolic composition in seeds and press cakes and in the search for chemical markers of antinutritive fiber in rapeseed.

MATERIALS AND METHODS

Chemicals, Reagents, and Plant Material. Methanol, *n*-hexane (both Rotisolv, HPLC gardient grade), SDS (≥99%), sodium chloride (p.a.), and fuming hydrochloric acid (37%, p.a.) were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Acetone, sodium hydroxide, ethyl acetate (all p.a.), and universal indicator paper (pH 1−14) were from Merck KgaA (Darmstadt, Germany). Polypropylene glycol solutions were from AB Sciex (Darmstadt, Germany). 5-Hydroxyferulic (the structure was confirmed by MS^n analysis, purity ≥ 95%, as determined by RP-HPLC/MS) acid was kindly provided by

Received: October 5, 2012
Revised: December 22, 2012
Accepted: December 24, 2012
Published: December 24, 2012

Table 1. Annotation of Phenolic Acids, Aldehydes, and Alcohols by t_R and m/z Values and Their Characteristic Fragmentation Patterns[a]

no.	substance[b]	t_R (min)	$[M-H]^-$ (m/z)	fragments (m/z) observed in enhanced product ion scan (relative intensity %)[c]	optimal MRM transition[d]/CE
1	protocatechuic acid	2.0	153.0	91 (5), 108 (31), 109 (100), 153 (44)	153.0 → 109.0/−20
2	4-hydroxybenzoic acid	3.3	137.0	93 (100), 137 (18)	137.0 → 93.0/−20
3	salicylic acid	3.3	137.0	65 (8), 93 (100), 137 (54)	137.0 → 93.0/−20
4	2-(4-hydroxyphenyl) ethanol	4.2	137.1	93 (8), 106 (4), 107 (7), 119 (24), 137 (100)	137.1 → 106.0/−20
IS	orcinol (internal standard)	4.2	123.0	79 (5), 81 (26), 123 (100)	123.0 → 81.0/−30
5	vanillic acid	4.3	167.0	65 (15), 91 (28), 108 (100), 123 (34), 152 (45), 167 (59)	167.0 → 108.0/−15
6	caffeic acid	4.5	179.0	89 (6), 107 (4), 134 (22), 135 (100), 179 (43)	179.0 → 135.0/−22
7	4-hydroxybenzaldehyde	4.6	121.0	91 (5), 92 (56), 93 (12), 121 (100)	121.0 → 92.0/−26
8	syringic acid	5.0	197.1	63 (3), 78 (17), 89 (50), 91 (50), 95 (38), 106 (38), 121 (53), 123 (47), 138 (54), 153 (50), 167 (17), 182 (54), 197 (100)	197.1 → 121.0/−24
9	5-hydroxyferulic acid	5.2	209.0	121 (3), 149 (20), 150 (100), 165 (11), 194 (43), 209 (62)	209.0 → 150.0/−26
10	vanillin	5.6	151.0	92 (31), 108 (28), 136 (100), 151 (39)	151.0 → 136.0/−18
11	*p*-coumaric acid	5.7	163.0	91 (6), 93 (24), 117 (10), 119 (100), 163 (37)	163.0 → 119.1/−20
12	benzoic acid	5.9	121.0	77 (19), 121 (100)	121.0 → 77.0/−16
13	coniferyl alcohol	6.2	179.1	89 (3), 91 (5), 117 (5), 119 (3), 135 (13), 145 (23), 146 (100), 147 (12), 161 (15), 163 (14), 164 (61), 179 (42)	179.1 → 146.0/−18
14	syringaldehyde	6.3	181.1	67 (11), 95 (19), 123 (50), 137 (4), 151 (100), 165 (8), 166 (79), 181 (42)	181.1 → 166.0/−20
15	ferulic acid	6.4	193.1	89 (20), 117 (6), 133 (18), 134 (100), 149 (10), 178 (18), 193 (49)	193.1 → 134.0/−20
16	sinapyl alcohol	6.6	209.1	77 (5), 93 (11), 95 (3), 105 (6), 121 (8), 133 (18), 151 (46), 161 (59), 176 (18), 179 (100), 191 (7), 194 (47), 209 (40)	209.1 → 179.0/−28
17	sinapic acid	6.7	223.1	65 (3), 89 (4), 93 (30), 104 (3), 117 (3), 121 (59), 132 (3), 135 (11), 148 (3), 149 (100), 163 (12), 164 (46), 165 (6), 179 (7), 193 (37), 208 (24), 223 (53)	223.1 → 164.0/−28
18	coniferyl aldehyde	7.2	177.1	133 (3), 134 (5), 161 (10), 162 (100), 177 (45)	177.1 → 162.0/−20
19	sinapaldehyde	7.5	207.1	77 (5), 93 (15), 105 (9), 121 (17), 123 (3), 149 (29), 163 (3), 177 (100), 191 (5), 192 (75), 207 (50)	207.1 → 177.0/−28

[a]Analysis was performed by an ESI-QqLIT mass spectrometer operating in negative ion mode and coupled on-line to an RP-UPLC system. [b]The analytes are listed in the order of their elution in presence of aqueous NH_4CH_3COO, pH 4.0/CH_3OH. [c]The EPI spectra were acquired at CE of 35 eV with CE spread of 15 eV. Only signals with relative intensities of >2% are shown. Precursor ions are underlined. [d]MRM transition produced the highest Q3 fragment intensity among three optimized ones.

Dr. Thomas Vogt (IPB Halle/Saale, Germany). All other chemicals were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Water was purified by a Milli-Q PLUS Ultra-Pure Water System (Millipore GmbH, Schwalbach, Germany) in house (resistance > 18 MΩ). The *B. napus* seeds of cultivars Express, Lisora, and Drakkar were obtained from Norddeutsche Pflanzenzucht Hans-Georg Lembke KG (Holtsee, Germany).

Isolation of Cell Wall-Bound Phenolics. Typically, 100 mg of seeds was ground in liquid nitrogen using a Mixer Mill MM 400 ball mill with ⌀ 3 mm stainless steel balls (Retsch, Haan, Germany) at a vibration frequency of 30 Hz for 2 × 1 min. The ground samples were transferred into 15 mL polypropylene tubes and extracted (twice) with 5 mL of 80% aqueous methanol for 10 min in a Sonorex Super RK 510 ultrasonic bath. After each extraction, the suspensions were centrifuged for 15 min at 3172*g*, and the supernatants were discarded. The residues were subsequently extracted with methanol, water, 0.5% aqueous SDS solution, 1 M NaCl, water, methanol, acetone, and *n*-hexane (5 mL for 10 min in ultrasonic bath, twice as described by Tan and co-workers.[7] After each extraction step, the samples were centrifuged at 3172*g*, and the supernatants were discarded. After the last extraction, the residues were dried under air at room temperature. The resulting material was defined as "cell wall preparation" and was supposed to be depleted of intracellular components.[15]

For cleavage of ester bonds, 10 mg of each cell wall preparation was placed in glass tubes, 2 mL of 1 M NaOH was added, the tubes were tightly sealed, and nitrogen was purged for 10 min before incubation in darkness for 15 h at 20 °C under continuous shaking (500 rpm). Afterwards, the samples were centrifuged at 3172*g* for 15 min, and the supernatants were transferred into new tubes. The residues were washed with methanol twice, dried in a stream of nitrogen, and saponified for 24 h at 80 °C. After centrifugation, both supernatants were acidified (pH 3.0) and extracted with 3 × 6 mL of ethyl acetate containing 5-methylresorcinol (internal standard) at a concentration of 2.2 μmol/L. Organic phases were pooled and dried under vacuum in a Büchi Rotavapor type RE-111 rotary evaporator. The residues were reconstituted in 200 μL of 80% aqueous methanol, transferred to 0.5 mL polypropylene tubes, and stored at −80 °C until analysis.

Standard Solutions. For external calibration and instrument detection (LOD_i) and quantitation (LOQ_i) limit determination, the mixture containing 1 mmol/L of each individual standard was serially diluted with 80% aqueous methanol by 2.0−2.5-fold increment to obtain 23 concentrations steps (0.01 nmol/L−500 μmol/L). For method LOD (LOD_m) determination and internal calibration, 200 μL of each calibration solution was diluted with 1 mol/L NaOH (1:10, v/v, n = 3) and treated further as was described for *B. napus* cell wall preparations in the previous section. Internal calibration was performed with linear sections of analyte/internal standard peak area ratio−concentration curves built individually for each analyte.

RP-UPLC Separations. The separations were performed on a Waters Acquity UPLC HSS T3 column (100 mm × 1 mm i.d., 1.8 μm, 100 Å, Waters GmbH, Eschborn, Germany) at 50 °C using a Waters Acquity UPLC System, equipped with an Acquity Binary Solvent Manager and Acquity Sample Manager with a 10 μL sample loop (1 μL injection volume) operating in partial loop injection mode (Waters GmbH). Eluents A and B were water and 90% aqueous methanol, respectively, both containing 0.01% ammonium acetate adjusted to pH 4.0 with ammonium hydroxide. Elution was performed isocratically for 1 min at 4% eluent B, and then consecutive linear gradients to 40, 80, and 100% eluent B in 6, 6.5, and 0.5 min, respectively, were run. The flow rate was set to 150 μL/min. Phenylpropanoids were detected by ESI-QqLIT- or ESI-QqTOF-MS.

ESI-QqLIT-MS and MS/MS. Linear ion trap (LIT) and triple quadrupole (QqQ) scans were acquired on a 3200 Q Trap LC-MS/MS System hybrid QqQ-LIT mass spectrometer equipped with an ESI-TurboIon-spray interface, operating in negative ion mode and controlled by Analyst 1.5 software (AB Sciex, Darmstadt, Germany). The samples were introduced using LC or by syringe pump infusion. Instrument tuning and mass calibration were performed with 100 μmol/L polypropylene glycol solutions in infusion mode.

For LIT experiments, enhanced MS (EMS) and enhanced product ion (EPI) scans were acquired using the parameters listed in the Supporting Information. QqQ scans were acquired as MRM experiments. Compound-dependent parameters for standards were optimized individually in additional infusion experiments. For detection of unknowns the compound-dependent parameters were set as follows: declustering potential (DP), −35 V; collision cell entrance potential (CEP), −7 V; collision cell exit potential (CXP), −2 V. Selection of Q3 masses and collision potentials (CEs) for individual MRM transitions was done on the basis of targeted EPI experiments with subsequent MRM-based CE optimization using "unit" Q1 and Q3 resolution. Dwell times were defined by the "scheduled MRM" function set as follows: targeted scan time, 3 s; and MRM detection window, 60 s.

For information-dependent acquisition (IDA) runs MRM scans were chosen as survey experiments and EPI scans as dependent ones. EPI scans were acquired with CE of −35 eV with CE spread of −15 eV. Q1 masses resulting in MRM signals with intensity >500 counts per second (cps) were selected for fragmentation in EPI scan.

ESI-QqTOF-MS. For acquisition of high-resolution mass spectra a MicrOTOF-Q II hybrid quadrupole time-of-flight (QqTOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an Apollo II electrospray ion source and operating in negative ion mode was used. Internal mass calibration of each analysis was performed by infusion of 20 μL 10 mmol/L lithium formate in isopropanol/water, 1:1 (v/v), using a diverter valve.

RESULTS AND DISCUSSION

Optimization of ESI Buffer Conditions. Due to the presence of easily ionizable carboxylic and phenolic hydroxyl groups, hydroxycinnamates are typically detected in negative ion mode by their $[M-H]^-$ ions[16] in the presence of basic additives enhancing their ionization.[17] However, low buffer pH is favorable when RP-LC experiments are performed.[18] To find a compromise between analyte separation and ionization, Q1 scan experiments with 0.5−3 mmol/L individual phenolics were performed. The use of 0.1% NH_4OAc (pH 6.8) in 60% aqueous acetonitrile resulted in at least 3-fold higher sensitivity for phenolic acids in comparison to 0.1% formic acid (pH 3.0) in the same solvent with only minimal effect of organic modifier. Reduction of the NH_4OAc concentration to 0.01% (v/v) yielded an additional 3-fold gain in sensitivity. This concentration resulted in overall at least a 9-fold ionization enhancement and was used in further experiments.

Establishment of LC-MS/MS Experiments. The structure information obtained in infusion MS/MS experiments (Table 1) was further applied to quantification of characterized phenylpropanoids by MRM experiments.[19] This approach is usually coupled to LC and referred to as "LC-MS/MS quantitation".[20] The optimized MRM transitions are listed in Table 1. On the basis of this information an initial UPLC-MS/MS (MRM) method comprising up to three transitions for each analyte was designed. Using an UPLC system ensured relatively short analysis time (20 min) and improved the efficiency of separation.[11]

Optimization of the Chromatography System. The standard mixture of 19 phenolics was separated by the preliminary method described in the previous section, using water as eluent A and 90% aqueous CH_3CN as eluent B, both containing 0.01% NH_4OAc with pH of 4.0, 4.5, or 5.0. Acetonitrile was chosen as an organic modifier due to its relatively high elution power, which results in higher chromatography peak efficiencies and, hence, better sensitivity of analysis.[21] The eluent pH values of 4.5−5 did not ensure complete retention of the most polar hydroxybenzoates (**1**, **2**, **3**, **5**, **6**, and **8**), which can be attributed to a dissociated state of acidic compounds under these conditions.[18] Decrease of buffer pH to 4.0 resulted in significant improvement of peak shape. For further optimization of analysis, three strategies could be applied: (i) further reduction of eluent pH by switching to acidic additives; (ii) introduction of ion pair reagents (typically substituted amines) in eluent system;[10] and (iii) replacement of organic modifier with a weaker one.[22] As demonstrated above, the first strategy resulted in an at least 9-fold MS sensitivity loss. Use of alkyl amines seemed to be also undesirable, as these ion pair reagents readily suppress ionization and contaminate ESI source.[23] With these considerations taken into account, the third option was chosen.

Replacement of acetonitrile with methanol resulted in significant improvement in the chromatographic behavior of all analytes (Figure 1). Despite an approximately 2-fold

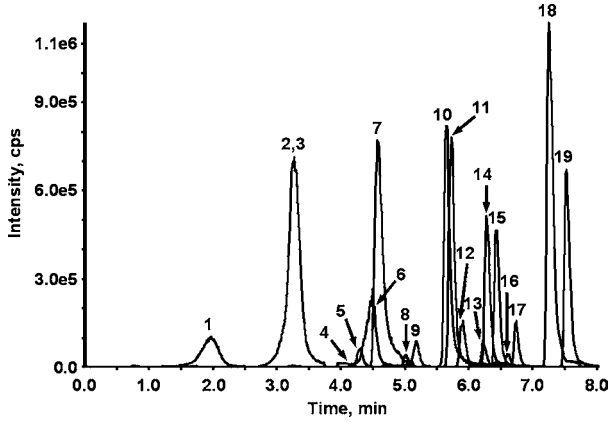

Figure 1. RP-UPLC-MS/MS profile obtained with a standard mixture of 19 phenolic acids and their derivatives (100 pmol each). Separation was performed on a Waters HSS T3 C18 column (1 × 100 mm, 1.8 μm particle size) using water as eluent A and 90% aqueous CH_3OH as eluent B, both containing 0.01% NH_4CH_3COO (pH 4.0). The phenolics are marked according to Table 1.

reduction in efficiencies of acidic analytes, all peaks demonstrated good symmetry, higher retention times (t_R), and better resolution in comparison to the CH_3CN-based system. It decreases the susceptibility of the ionization process to matrix effects[24] and increases, thereby, the reproducibility of the analysis. On the basis of these observations, this system was used in further experiments.

Simultaneous Identification and Quantitation of Phenylpropanoids. The optimized UPLC-MS/MS method could be further applied to quantitation of phenolics by external or internal standardization. Collecting qualitative information, however, requires acquisition of additional product ion scans. An information-dependent acquisition (IDA) experiment comprising MS experiments of two different types, that is, MRM (QqQ scan) and EPI (QqLIT scan), is an elegant way to avoid this.[25] However, the extremely low (up to 1−2 s) widths of UPLC peaks and unfavorable duty cycle of the quadrupole

dx.doi.org/10.1021/jf3042648 | J. Agric. Food Chem. 2013, 61, 1219−1227

instrument in the product ion scan mode bring two limitations: the duration of the total MRM scan is relatively long for such narrow peaks and the sensitivity of the dependent scan is insufficient for identification of components recognized in the survey one. To overcome the first limitation, we applied the "scheduled MRM" function,[20] drastically reducing the duration of the MRM scan in each point of the chromatogram. The use of a QqLIT instrument minimized the second problem due to the high duty cycle of a linear ion trap.[14] The compromise between QqQ and LIT duty cycles in the whole IDA experiment was achieved at the MRM peak intensity of 500−1000 cps taken as a switch criterion from survey to dependent scan. Under these conditions all compounds of the standard mixture could be identified by MS/MS as far as their intensity fulfilled switch criteria.

Optimization of the Alkaline Hydrolysis Procedure. From a chemical point of view, alkaline hydrolysis of esters is a nucleophilic acyl substitution reaction leading to acyl carbon−oxygen cleavage and is expected to be quantitative at room temperature.[4] However, when saponification is performed at elevated temperatures, some phenolic ether bonds (mostly *β*-*O*-4 ones in lignin) can be also cleaved.[26]

Indeed, after incubation at 80 °C, all hydroxybenzoic and hydroxycinnamic acids demonstrated much higher intensities in hydrolysates in comparison to saponification at 20 °C (Figure 2), and this relative abundance increase was in all cases

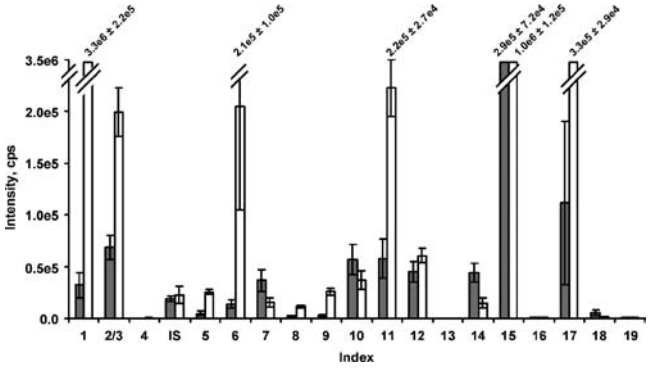

Figure 2. Intensities (expressed as peak areas, cps) of individual phenolics obtained after saponification of 10 mg isolated *B. napus* cell wall material in 1 mol/L NaOH for 24 h at 20 °C (gray bars) and 80 °C (white bars). The analytes are numbered as in Table 1.

significant. At the same time, abundances of aldehydes (**7**, **10**, **14**, and **18**) and, especially, alcohols (**4** and **16**) demonstrated significant reduction after incubation at 80 °C or were even below the detection limit (e.g., coniferyl alcohol, **13**). The most obvious explanation would be degradation of these hydroxycinnamates in alkaline solutions in the presence of oxygen,[27] which brings uncertainties in analysis. Hence, the stability of analytes under hydrolysis conditions must be characterized, and their possible degradation must be taken into account in the standardization procedure.

As all phenolics demonstrated acceptable extraction recoveries (in most cases 90−100%), any further analyte losses could be attributed exclusively to phenylpropanoid degradation during the saponification procedure. Thus, a 24 h incubation at 80 °C resulted in complete loss or drastic (>100-fold) decrease of hydroxycinnamyl alcohols and aldehydes, protocatechuic acid, and caffeic acid. Sinapic acid demonstrated up to a 6-fold decrease. In contrast, abundances of several hydroxybenzoates significantly increased. The degree of these alterations was much lower at 20 °C. It corresponded well to the results presented in Figure 2. Most of the analytes demonstrated acceptable recoveries when simultaneous decreases of incubation temperature and time were applied. However, caffeic acid and cinnamyl alcohols still could not be quantified with desired sensitivity as ≥99% of their initial amounts were degraded even at the lowest incubation time and temperature. Quantification problems could also be expected for protocatechuic acid. However, hydrolyzing predried samples could reduce the losses of this phenolics (approximately 2-fold). Thus, this procedure was used in further analyses.

The behavior of the phenolic standards observed under saponification conditions can be explained by their chemical structures and relative reactivities in aqueous solutions. The oxidation of hydroxycinnamates under such conditions may occur by a radical mechanism through the formation of resonance-stabilized radicals. Due to the increasing stability of such species, amenability of hydroxycinnamates to radical reactions increases in the following direction: acids < aldehydes < alcohols. Because of the stabilizing effect of the additional methoxy group, all 3,5-dimethoxy-4-hydroxycinnamates should be less stable than their 3-methoxy-4-hydroxy counterparts. This mainly explains the observed phenylpropanoid abundance patterns. The intensities of the C_6C_1 cleavage products, syringaldehyde and vanillin, distinctly increased with temperature and time, and for syringaldehyde this alteration was essentially stronger. High amenability of protocatechuic and caffeic acids to degradation can be explained by the formation of the resonance-stabilized catechol radical.

Theoretically, analyte losses could be avoided by using antioxidants during hydrolysis. However, in practice it is not done because of the involvement of antioxidants in side reactions (e.g., oxidation of ascorbic acid).[28] Generally, losses of analytes at the sample preparation step can be corrected by a single internal standard added to the extraction solvent.[29] Internal standardization helps also to discriminate low metabolic differences between lines, plants, organs, etc. Using standardization with saponified authentic standards and using orcinol as an internal one allowed us to correct these losses and increased 2-fold the number of analyzed samples per day. A hydrolysis step was incorporated as overnight (15 h) incubation.

Targeted Analysis of Unknowns. Evaluation of UV chromatograms obtained from saponified cell wall material reveals essentially more signals than those corresponding to the 19 available standards. Many of these substances could be annotated and some tentatively identified. However, complete and unambiguous identification of annotated signals would be possible only after MS^n experiments using corresponding commercially or synthetically available standards or/and nuclear magnetic resonance (NMR) experiments, which was beyond the scope of this study. With this taken into account, all annotated signals, independent of the level of their annotation, were treated here as "unknowns" and are referred to correspondingly.

Annotation and as thorough as possible tentative identification of unknowns was performed using the targeted metabolomics approach.[10] On the basis of available literature data obtained with other plant species and experiment types, we proposed the following compound groups to be relatively abundant in such preparations: (i) intermediate metabolites of lignin biosynthesis pathway;[30] (ii) hydroxycinnamic acid

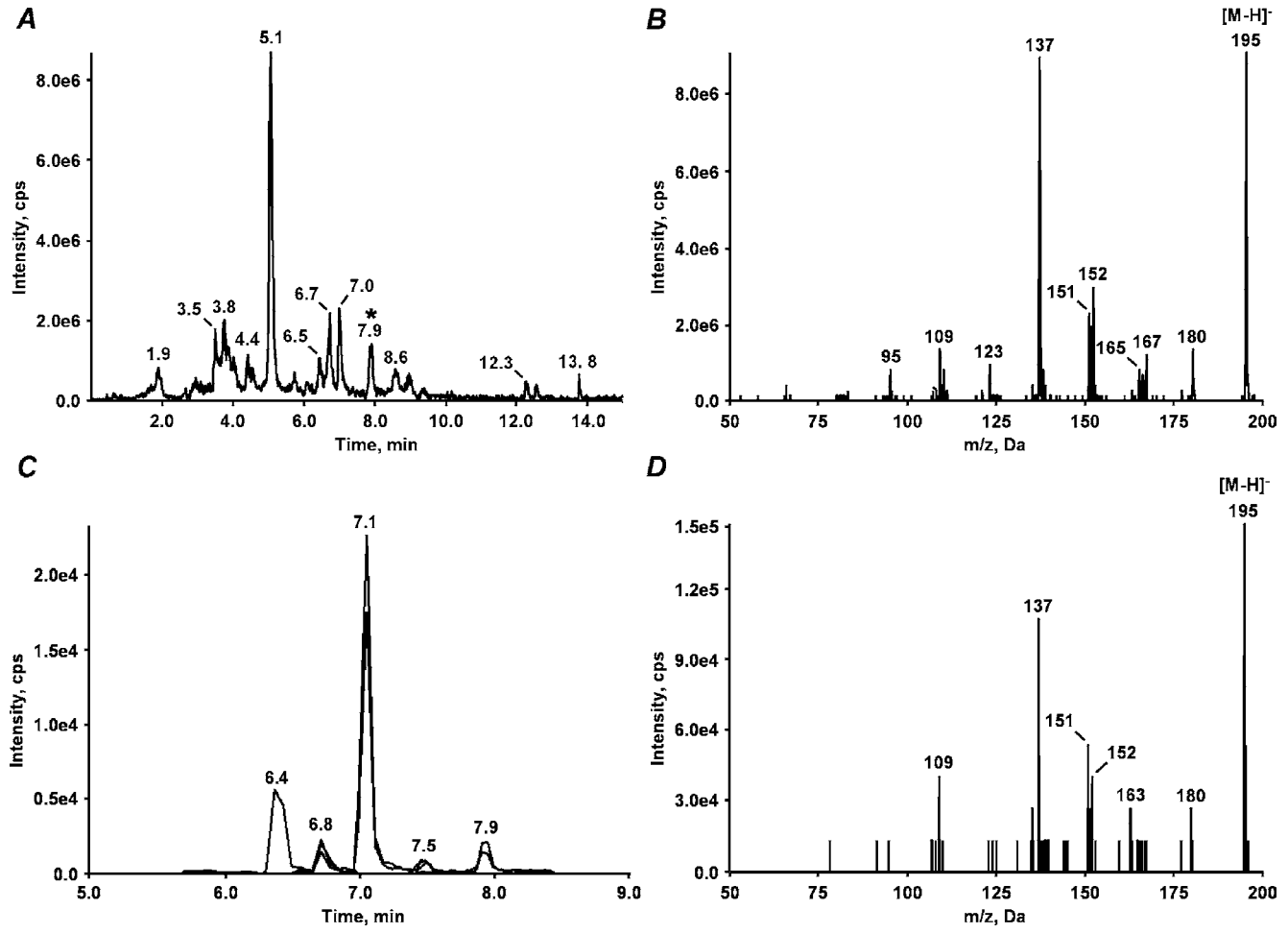

Figure 3. Annotation and identification of unknowns in *B. napus* cell wall material saponified with 1 mol/L NaOH for 24 h at 80 °C: (A) EPI TIC chromatogram of *m/z* 195.1; (B) MS/MS spectrum of *m/z* 195.1 acquired at 7.9 min of EPI scan (A); (C) overlay of XIC MRM chromatograms for MRM transitions 195.1/137.0 and 195.1/152.0 obtained in an IDA experiment; (D) MS/MS spectrum of *m/z* 195.1 acquired at 7.9 min of IDA experiment.

conjugates (mostly dimers);[31] and (iii) thermal degradation products of phenolics including lignin.[32−35]

Our general annotation/identification workflow for the QqLIT instrument platform is presented in Figure 3 and the Supporting Information for putative acetosyringone as an example. This compound is a well-known product of lignin thermal degradation[33] and can be expected to be present in saponified *B. napus* cell wall material. First, an EPI chromatogram at *m/z* 195.1 (putative acetylsyringone $[M-H]^-$ ion) for a concentrated cell wall preparation was acquired (Figure 3A). The MS/MS data of the resulting peaks were manually explored for the theoretically predicted fragmentation pattern of acetylsyringone. The fragment spectrum at t_R 7.9 min (Figure 3B) matched the predicted fragmentation pattern. The obtained results were subsequently confirmed in an IDA experiment (Figure 3C,D). Similarly, all predicted structures were processed. All positive hits were appended to the LC-MS/MS (IDA) method described previously.

To increase the reliability of our data (in terms of false-positive qualitative and quantitative results), we tried to select two MRM transitions for each unknown in all cases when it was possible. However, 11 unknowns could be quantified only by one MRM transition. The final LC-MS/MS method, obtained after this correction, contained altogether 47 unknowns producing no cross-talk with standard hydroxycinnamates. As was already expected from the results obtained with phenolic acids and their derivatives (Figure 2), saponification of cell wall material at 20 °C drastically reduced (up to 153-fold) the yields of unknowns. With this taken into account, saponification for analysis of unknowns was performed at 80 °C.

Additionally, the same saponified preparations were analyzed by an untargeted metabolite profiling approach using an RP-UPLC-ESI-QqTOF-MS analytical platform.[36] However, not all of 47 unknowns were detected using this technique. This can be explained by the lower sensitivity of QqTOF-MS and the difference in ionization efficiencies demonstrated by the two ion sources. Despite these problems, however, for 18 unknowns detected in TOF scans, an elemental formula could be assigned with relatively high mass accuracy (Table 2). This assignment essentially increased the reliability of their tentative identification and made these unknowns the preferable targets for comprehensive identification in future work.

Validation of the Method. By means of the designed IDA-based LC-MS/MS method, altogether 66 species could be identified by their product spectra and quantitated by one or two MRM transitions. For the 19 standards listed in Table 1, the LOD_i and LOD_m values, as well as LOQ_i and LOQ_m, were determined (up to 2.5×10^{-17} and 2.5×10^{-14}, respectively). The LODs and LOQs of unknowns were expressed relative to their intensity in preparation (up to 1:4098 and 1:1024,

Table 2. Identification of Unknowns by Retention Time, Exact m/z Values, and Fragmentation Patterns[a]

		[M-H]$^-$ mass (m/z)					
no (Un)[b]	t_R[c]	nominal[d]	theoretical	observed[e]	Δm (ppm)	fragment ions;[f] m/z (%)	tentative assignment[g]
3	1.8	195	195.0299	195.0304	2.6	106 (5), 107 (51), 109 (51), 123 (5), 135 (4), 151 (100), 195 (54)	carboxyhydroxyphenylacetic acid
4	1.9	181	181.0506	181.0505	0.6	108 (16), 119 (6), 135 (15), 136 (18), 137 (100), 163 (18), 181 (100)	dihydrocaffeic acid
12	3.8	151	151.0401	151.0399	1.3	95 (11), 106 (6), 107 (100), 108 (5), 123 (6), 136 (5), 151 (73)	4-hydroxyphenylacetic acid
13	4.0	181	181.0506	181.0514	4.4	108 (18), 109 (26), 134 (8), 135 (55), 137 (23), 163 (21), 181 (100)	3-hydroxydihydrocaffeyl aldehyde
15	4.5	163	163.0401	163.0403	1.2	107 (8), 119 (4), 134 (12), 135 (6), 145 (4), 161 (31), 163 (100)	caffeoyl aldehyde
20	5.3	151	151.0401	151.0401	17.9	92 (6), 105 (6) 107 (28), 108 (41), 109 (16), 136 (13), 151 (100)	3,4-dihydroxyacetophenone
25	6.1	385	385.0929	385.0920	2.3	158 (8), 173 (10), 267 (68), 282 (48), 326 (22), 341 (100), 385 (11)	ferulic acid dimer 1
27	6.1	193	193.0506	193.0504	1.0	89 (12), 133 (11), 134 (100),135 (4), 149 (12) 178 (11), 193 (55)	*cis*-ferulic acid
29	6.5	149	149.0608	149.0616	5.4	134 (100), 135 (3), 149 (33)	*p*-vinylguaiacol
30	6.7	151	151.0401	151.0392	6.0	92 (3), 107 (95), 108 (6), 109 (4), 136 (10) 151 (100)	anisic acid
31	6.7	195	195.0663	195.0628	17.9	135 (6), 136 (35), 137 (6), 151 (90), 165 (6), 177 (13), 195 (100)	3,5-dimethoxyphenylacetic acid
34	6.9	385	385.0929	385.0953	6.2	108 (25), 158 (64), 159 (100), 173 (29), 266 (27), 281 (25), 385 (47)	ferulic acid dimer 2
35	7.0	385	385.0929	385.0932	0.8	159 (35), 267 (100), 282 (75), 297 (52), 326 (48), 341 (69) 385 (71)	ferulic acid dimer 3
40	7.8	385	385.0929	385.0947	4.7	158 (65), 159 (100), 173 (29), 193 (61), 266 (29),281 (70), 385 (36)	ferulic acid dimer 4
42	7.9	195	195.0663	195.0687	12.3	109 (12), 137 (96), 151 (28), 152 (27), 165 (10), 180 (13), 195 (100)	acetosyringone
44	8.5	385	385.0929	385.0950	5.9	239 (22), 267 (59), 281 (19), 282 (31), 326 (39), 341 (74), 85 (100)	ferulic acid dimer 5
46	8.7	165	165.0557	165.0578	12.7	106 (11), 121 (79), 122 (4), 165 (100)	3-methoxyphenylacetic acid
47	9.8	445	445.1140	445.1193	11.9	203 (12), 297 (16), 242 (20), 355 (12), 357 (80), 401 (20), 445 (100)	sinapic acid dimer

[a]The data were collected by sequentially running ESI-QqTOF-MS and ESI-QqLIT-MS/MS experiments with both mass spectrometers operating in negative ion mode and alternatively coupled on-line to the same chromatography system. [b]The unknowns are labeled as in Table S7 of the Supporting information. [c]t_R values were the same with both QqTOF-MS and QqLIT-MS/MS detection. [d]Nominal masses were determined by LC-ESI-QqLIT-EMS. [e]Exact masses were determined by LC-ESI-QqTOF-MS. [f]Fragment nominal masses were determined by LC-ESI-QqLIT-MS/MS (EPI). [g]Only the compounds assigned by exact m/z values are shown here. For the complete list of unknowns, see Table S7 in the Supporting Information.

respectively). LODs of all unknowns, besides Un21, were within the studied dilution range. In the latter case, quantitation in 1:10 dilution was performed.

All analytes demonstrated good linearity of response, as was calculated on the basis of three independent dilution series (typically $R^2 \geq 0.995$). The linear dynamic ranges were from 1.00×10^3 to 1.00×10^4 ($R^2 > 0.990$). Only 4-hydroxybenzaldehyde showed a narrower linear dynamic range. As was mentioned before, two structural isomers, namely, 4-hydroxybenzoic and salicylic acids (**2** and **3**, respectively), could not be always chromatographically resolved (Figure 1). Unfortunately, under CID conditions, corresponding [M-H]$^-$ ions produced the same fragments, although in different relative intensities (Table 1). For this reason, the peak corresponding to the mixture of these two compounds can be quantitated as equivalents of one of these. We decided to quantitate both acids as 4-hydroxybenzoic acid equivalents, as the peak was represented mostly by this compound, proved by signal intensity ratios for fragments listed in Table 1.

The important feature of the described method was the good intraday and interday precision. The relative standard deviations (RSD) of analyte peak areas were in most cases below 10%, which can be expected for the instruments and MS experiments of this type.[25] Retention times, in contrast, were characterized with much higher reproducibility even at low analyte intensities. It can be attributed to the features of UPLC technology,[11] and it matches well the t_R accuracy values obtained in other RP-UPLC applications.[37] This high t_R precision made it possible to increase dwell times in a "scheduled MRM" experiment to provide higher sensitivity.

Standardization Strategy for Absolute Quantitation of Standard Cell Wall-Bound Phenolics. The designed method was successfully applied to the quantitation of alkali-extractable phenolics in seed cell walls of *B. napus* cv. Express. For absolute quantitation of the phenolics listed in Table 1, two standardization strategies were applied: external calibration with the mixture of authentic standards dissolved in 80% aqueous methanol and internal calibration using 2 μmol/L orcinol (5-methylresorcinol, added in the extraction step) as an internal standard with the same authentic standard mixture incubated with 1 mol/L NaOH solution for 15 h. Although the first approach, relying on untreated authentic standards, is the common quantitation strategy in phenylpropanoid research, the second one (i.e., based on saponified standards), to the best of our knowledge, was applied here for the first time.

The comparison of quantification with and without saponification of calibration standards in parallel to analyzed samples revealed essential differences in analyte behavior. For the majority of low-abundant compounds (≤0.06 nmol/mg cell wall material) the use of the second standardization approach did not result in significant differences or only slight alterations in calculated cell wall analyte contents (Figure 4A). In contrast,

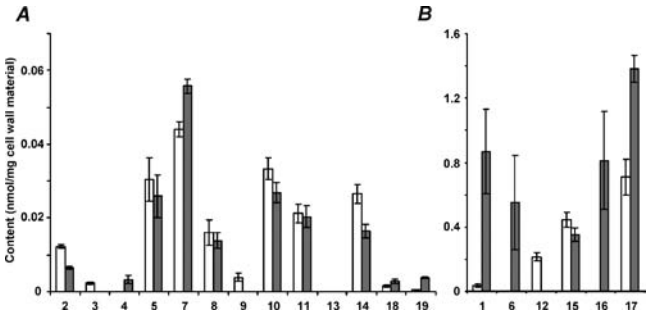

Figure 4. Absolute quantification of low-abundant (≤0.06 nmol/mg, A) and highly-abundant (>0.06 nmol/mg, B) ester-bound phenolics in *B. napus* (cv. Express) cell wall material saponified with 1 mol/L NaOH for 15 h at 20 °C. Quantification was performed in triplicates by external standardization with untreated authentic standards (white bars) and internal standardization with orcinol as an internal standard and saponified authentic standards (gray bars).

only one analyte with an abundance of >0.06 nmol/mg cell wall material (namely, ferulic acid) showed such a behavior (Figure 4B). For other highly abundant analytes (sinapyl alcohol and protocatechuic, caffeic, and sinapic acids) as well as for some low-abundant ones (2-(4-hydroxyphenyl)ethanol, coniferyl aldehyde, and sinapaldehyde) this quantitation approach delivered much lower contents. It clearly indicated overestimation of these analytes under applied hydrolysis conditions when unhydrolyzed standards were used. The above-discussed oxidative degradation of phenolics in aqueous solutions was the most likely reason for this.

We expected that using a "saponified" standard mixture, the components of which undergo degradation to a similar degree as the sample components, for calibration might compensate for these effects and correct the content values. Complementation of this approach with internal standardization ensured correction of the dispersion related to ethyl acetate extraction procedure. In contrast, higher cell wall contents were observed for C_6C_1 compounds when the authentic standards were not saponified (Figure 4). This result is in accordance with the reduced temperature used and the oxidation stability of C_6C_3 compounds. Indeed, we demonstrated that increased degradation of hydroxycinnamates is accompanied with accumulation of corresponding acidic and, especially, aldehydic products of their degradation. Hence, standardization of the method with untreated (not saponified) standards resulted in overestimation of these compounds.

Unfortunately, quantitation of benzoic acid using standards incubated with 1 mol/L NaOH turned out to be impossible because of its high LOQ_m (Figure 4B). Most likely, benzoic acid is the degradation product of other phenylpropanoids and accumulated in relatively large amounts during thermal treatment. Due to the presence of a catechol moiety in its structure, 5-hydroxyferulic acid is highly amenable to oxidative degradation and, because of its relatively low content, it cannot be quantitated using NaOH-treated standard dilution series (Figure 4A). Taking these observations into account we suggest quantitating these substances using untreated standards as benzoic acid and 5-hydroxyferulate equivalents, respectively. One should realize, however, that the origin of these equivalents remains unknown.

Theoretically, oxidative degradation of phenylpropanoids can be, to some extent, reduced and their stability under alkaline hydrolysis conditions increased. Several factors causing oxidative degradation may be involved: incomplete evacuation of air from the reaction mixture, air diffusion through silicone septa during relatively long incubation times, and trace amounts of transition metals in cell wall material capable of participating in Fenton reactions with H_2O_2 produced during sonication of samples.[38] However, it is necessary to take into account the following considerations: (i) better evacuation of air and prevention of its diffusion afterward would be time-consuming and expensive; (ii) the degree of air evacuation cannot be reliably measured; and (iii) these measures will dramatically reduce sample throughput. Keeping this in mind, we have chosen the protocol described here rather than further reduction of dissolved oxygen content. Although relative abundances of phenylpropanoids in cell wall preparations differ from those in standard mixtures, our approach allows adequate estimation of highly temperature-labile and oxidation-sensitive phenolics. Moreover, hydroxybenzoates, typically overestimated in saponifed preparations, could be quantitated correctly. The method has a limitation: the analyzed oxidation-sensitive compounds must be relatively highly abundant (as caffeic and protocatechuic acids in this study); otherwise, oxidative degradation or intensive chemical background produced by the whole procedure will prevent their quantitation (e.g., 5-hydroxyferulic or benzoic acid).

Absolute and Relative Quantitation of Seed Cell Wall-Bound Phenolics in Rapeseed Cultivars. The applicability of the developed method was confirmed in the study of seed cell wall composition performed with different *B. napus* cultivars: Express, Lisora, and Drakkar. Because Express is a winter rapeseed cultivar and the other two are spring ones, metabolic variations due to different acclimatization abilities could be expected.[39] Indeed, differences in phenylpropanoid patterns were observed. The abundances of vanillin, *p*-coumaric acid, coniferyl aldehyde, and sinapaldehyde differed significantly (up to 3-fold, $p < 0.01$). These compounds showed lower abundances in the cell walls of Express seeds and higher ones in Lisora. Thus, similar analyte intensity differences were observed between spring and summer, as well as between two summer cultivars. Such variations can reflect differences in esterification patterns of cellulose, hemicelluloses, and pectins. Remarkably, for the majority of analytes relatively low intensity dispersion (typically, RSD% of ≤30%) among biological replicates was observed. A good method precision can be considered to be the main reason for this. This feature allows distinguishing even minor differences in cell wall composition observed between two plant groups. However, different genetic homogeneity of cultivars should be also taken into account, as it may influence RSD% values in experimental groups.

Surprisingly, quantitative distribution of unknowns in these three cultivars differed from the described pattern. In this case, no significant differences in intensities of individual phenolics between Express and Lisora seeds were observed, whereas both of these cultivars demonstrated higher abundances of prospective lignin degradation products than the third one, Drakkar (Figure 5A), and these differences were significant ($p <$

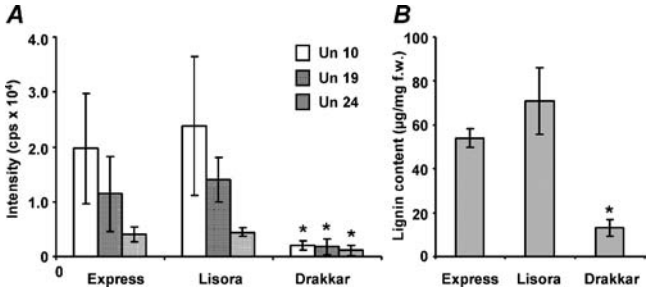

Figure 5. Abundances of putative 2-(3,4-dihydroxyphenyl)ethanol, 3,4-dihydroxy-5-methoxyethylbenzene, and acetovanillon (unknowns **10**, **19**, and **24**, respectively, A) and lignothioglycolic acid (B) in isolated cell wall material obtained from the seeds of different *B. napus* varieties. The asterisk denotes significant ($p < 0.05$) differences between Drakkar and other cultivars.

0.05 or $p < 0.01$). These results might indicate reduced lignin amounts in Drakkar seeds. It is necessary to note that only the levels of so-called "core lignin", corresponding to phenolic ether cell wall fraction, showed this tendency, whereas the levels of "non-core lignin" demonstrated quite a different phenolic pattern. That may, in turn, influence the fiber amounts, as lignin is known to be a component of antinutritive fiber in rapeseed.[1]

To test this supposition, lignin analysis according to the UV-based procedure of Bruce and West[40] was performed. Indeed, lignin contents in Drakkar seeds were 4- and 5.5-fold lower than those in Express and Lisora ones, respectively (Figure 5). The matching of the results obtained with two different methods (Figure 5) means that tentatively identified lignin degradation products may be used as marker compounds of lignin content in *B. napus*. However, additional studies are necessary to reveal quantitative relationships between antinutritive fiber contents, lignin, and corresponding low molecular weight markers. As the whole seeds were analyzed here, seed coat and embryo cell wall phenolics cannot be distinguished. The described method allows, however, complete characterization of prospective marker compounds in separated tissues, to optimize seed processing technology, that is, minimize contents of antinutritives in final products. Such studies aiming at the search for lignin-related tissue-specific biomarkers of antinutritive fiber are in progress.

To summarize, we describe here a novel LC-MS/MS-based method for the simultaneous identification and quantification of cell wall-bound phenolics. The absolute quantitation was performed using orcinol as internal standard with saponified authentic standards. This new analytical strategy allowed us to correct quantitation errors related to oxidative degradation of phenylpropanoids during alkaline hydrolysis and following extraction procedure. Additionally, 47 possible lignin monomers and degradation products or hydroxycinnamic acid dimers were tentatively identified and quantitated on a relative basis. The applicability of the method was verified in the comparative study of cell wall-bound phenolic contents in three different rapeseed cultivars. Phenylpropanoids analyzed in this work can be considered as potential marker compounds of antitinutritive fiber and lignin contents. On the basis of the identified prospective marker compounds fast screening analytical methods to detect and quantitate phenolic antinutritive polymers in *B. napus* can be established. Such assays may be applied in both breeding practice and food quality control.

■ ASSOCIATED CONTENT

Supporting Information

Additional tables and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +49 (0) 341 9731332. Fax: +49 (0) 341 9731339. E-mail: andrej.frolov@bbz.uni-leipzig.de.

Present Address

[§]Leipzig University, Faculty of Chemistry and Mineralogy, Institute of Bioanalytical Chemistry, Centre for Biotechnology and Biomedicine, Deutscher Platz 5, 04103 Leipzig, Germany.

Funding

Financial support from Bundesministerium für Bildung und Forschung [Project GABI-Kanada (CGAT): YelLowSin Rapeseed: Functional genomics approaches for the development of yellow-seeded, low sinapine ('YelLowSin') oilseed rape/canola (*Brassica napus*), Förderkennzeichen: 0313703B] is gratefully acknowledged.

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Wittkop, B.; Snowdon, R. J.; Friedt, W. Status and perspectives of breeding for enhanced yield and quality of oilseed crops for Europe. *Euphytica* **2009**, *170*, 131−140.

(2) Biergegaard, C.; Eggum, B. O.; Jensen, S. K.; Sørensen, H. Dietary fibres in oilseed rape: physioloical and antinutrtional effects in rats of isolated IDF and SDF added to a standard diet. *J. Anim. Physiol. Anim. Nutr.* **1991**, *66*, 69−79.

(3) Cosgrove, D. J. Wall structure and wall loosening. A look backwards and forwards. *Plant Physiol.* **2001**, *125*, 131−134.

(4) Kang, Y. H.; Parker, C. C.; Smith, A. C.; Waldron, K. W. Characterization and distribution of phenolics in carrot cell walls. *J. Agric. Food Chem.* **2008**, *56*, 8558−8564.

(5) Merali, Z.; Mayer, M. J.; Parker, M. L.; Michael, A. J.; Smith, A. C.; Waldron, K. W. Metabolic diversion of the phenylpropanoid pathway causes cell wall and morphological changes in transgenic tobacco stems. *Planta* **2007**, *225*, 1165−1178.

(6) Maier, W.; Peipp, H.; Schmidt, J.; Wray, V.; Strack, D. Levels of terpenoid glycoside (blumenin) and cell wall-bound phenolics in some cereal mycorrhizas. *Plant Physiol.* **1995**, *109*, 465−470.

(7) Tan, J.; Bednarek, P.; Liu, J.; Schneider, B.; Svatoš, A.; Hahlbrock, K. Universally occurring phenylpropanoid and species-specific indolic metabolites in infected and uninfected *Arabidopsis thaliana* roots and leaves. *Phytochemistry* **2004**, *65*, 691−699.

(8) Mandal, S.; Mitra, A. Accumulation of cell wall-bound phenolic metabolites and their upliftment in hairy root cultures of tomato (*Lycopersicon esculentum* Mill.). *Biotechnol. Lett.* **2008**, *30*, 1253−1258.

(9) Gorshkova, T. A.; Salnikov, V. V.; Pogodina, N. M.; Chemikosova, S. B.; Yablokova, E. V.; Ulanov, A. V.; Ageeva, M. V.; Van Dam, J. E. G.; Lozovaya, V. V. Composition and distribution of cell wall phenolic compounds in flax (*Linum usitatissimum*) stem tissues. *Ann. Bot.* **2000**, *85*, 477−486.

(10) Lu, W.; Bennet, B. D.; Rabinowitz, J. D. Analytical strategies for LC-MS-based targeted metabolomics. *J. Chromatogr., B* **2008**, *871*, 236−242.

(11) MacNair, J. E.; Lewis, K. C.; Jorgenson, J. W. Ultrahigh-pressure reversed-phase liquid chromatography in packed capillary columns. *Anal. Chem.* **1997**, *69*, 983−989.

(12) Rochfort, S. J.; Trenerry, V. C.; Imsic, M.; Panozzo, J.; Jones, R. Class targeted metabolomics: ESI ion trap screening methods for glucosinolates based on MS^n fragmentation. *Phytochemistry* **2008**, *69*, 1671−1679.

(13) Zeng, G.; Xiao, H.; Liu, J.; Liang, X. Identification of phenolic constituents in Radix *Salvia miltiorrhizae* by liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 499−506.
(14) Hager, J. W.; Le Blanc, J. C. High-performance liquid chromatography-tandem mass spectrometry with a new quadrupole/linear ion trap instrument. *J. Chromatogr., A* **2003**, *1020*, 3−9.
(15) Chen, M.; Sommer, A. J.; McClure, J. W. Fourier transform-IR determination of protein contamination in thyoglycolic acid lignin from radish seedlings, and improved methods for extaction-free cell wall preparation. *Phytochem. Anal.* **2000**, *11*, 153−159.
(16) Milbury, P. E.; Chen, C.-Y.; Dolnikowski, G. G.; Blumberg, J. B. Determination of flavonoids and phenolics and their distribution in almonds. *J. Agric. Food Chem.* **2006**, *54*, 5027−5033.
(17) Chen, G.; Zhang, L.-K.; Pramanik, B. N. LC/MS: theory, instrumentation, and applications to small molecules. In *HPLC for Pharmaceutical Scientists*; Kazakevich, Y., Lobrutto, R., Eds.; Wiley: Hoboken, NJ, 2007; pp 281−347.
(18) Klimek-Turek, A.; Dzido, T. H. Separation selectivity of some phenolic acids in RP HPLC systems with binary mobile phase comprised various modifiers. *Adsorption* **2010**, *16*, 287−294.
(19) Kitteringham, N. R.; Jenkins, R. E.; Lane, C. S.; Elliott, V. L.; Park, B. K. Multiple reaction monitoring for quantitative biomarker analysis in proteomics and metabolomics. *J. Chromatogr., B* **2009**, *877*, 1229−1239.
(20) Handrick, V.; Vogt, T.; Frolov, A. Profiling of hydroxycinnamic acid amides in *Arabidopsis thaliana* pollen by tandem mass spectrometry. *Anal. Bioanal. Chem.* **2010**, *398*, 2789−2801.
(21) Frolov, A.; Hoffmann, R. Separation of Amadori peptides from their unmodified analogs by ion-pairing RP-HPLC with heptafluorobutyric acid as ion-pair reagent. *Anal. Bioanal. Chem.* **2008**, *392*, 1209−1214.
(22) Frolov, A.; Singer, D.; Hoffmann, R. Site-specific synthesis of Amadori-modified peptides on solid phase. *J. Pept. Sci.* **2006**, *12*, 389−395.
(23) Seifar, R. M.; Ras, C.; van Dam, J. C.; van Gulik, W. M.; Heijnen, J. J.; van Winden, W. A. Simultaneous quantification of free nucleotides in complex biological samples using ion pair reversed phase liquid chromatography isotope dilution tandem mass spectrometry. *Anal. Biochem.* **2009**, *388*, 213−219.
(24) Taylor, P. J. Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography-electrospray-tandem mass spectrometry. *Clin. Biochem.* **2005**, *38*, 328−334.
(25) Leung, G. N. W; Leung, D. K. K.; Wan, T. S. M.; Wong, C. H. F. High throughput screening of sub-ppb levels of basic drugs in equine plasma by liquid chromatography-tandem mass spectrometry. *J. Chromatogr., A* **2007**, *1156*, 271−279.
(26) Imai, A.; Yokoyama, T.; Matsumoto, Y.; Meshitsuka, G. Significant lability of guaiacylglycerol β-phenacyl ether under alkaline conditions. *J. Agric. Food Chem.* **2007**, *55*, 9043−9046.
(27) Janson, J.; Fullerton, T. Influence of carbohydrates and related compounds on the alkaline cleavage of the β-aryl ether linkage in a phenolic lignin model compounds. *Holzforschung* **1987**, *41*, 359−362.
(28) Khan, M. M.; Martell, A. E. Metal ion and metal chelate catalyzed oxidation of ascorbic acid by molecular oxygen. *J. Am. Chem. Soc.* **1967**, *89*, 4176−4185.
(29) Bianco, A.; Buiarelli, F.; Cartoni, G.; Coccioli, F.; Jasionowska, R.; Margherita, P. Analysis by liquid chromatography-tandem mass spectrometry of biophenolic compounds in virgin olive oil. *J. Sep. Sci.* **2003**, *26*, 417−424.
(30) Morreel, K.; Ralph, J.; Lu, F.; Goeminne, G.; Busson, R.; Herdewijn, P.; Goeman, J. L.; Van der Eycken, J.; Boerjan, W.; Messens, E. Phenolic profiling of caffeic acid *O*-methyltransferase-deficient poplar reveals novel benzodioxane oligolignols. *Plant Physiol.* **2004**, *136*, 4023−4036.
(31) Bunzel, M.; Ralph, J.; Steinhart, H. Phenolic compounds as cross-links of plant derived polysaccharides. *Czech J. Food Sci.* **2004**, *22*, 64−67.
(32) Bland, D. E.; Logan, A. F. The properties of syringyl, guaiacyl and *p*-hydroxyphenyl artificial lignins. *Biochem. J.* **1965**, *95*, 515−520.
(33) Concin, R.; Burtscher, P.; Burtscher, E.; Bobleter, O. Sparation and identification of monomeric ligin degradation products by GLC-mass spectrometry. *Int. J. Mass Spectrom. Ion Phys.* **1983**, *48*, 63−66.
(34) Galletti, G. C.; Chiavari, G.; Kahie, Y. D. Pyrolysis gas chromatography/ion-trap mass spectrometry of the "Tooth brush" tree (*Salvadora persica* L.). *Rapid Commun. Mass Spectrom.* **1993**, *7*, 651−655.
(35) Takada, D.; Ehara, K.; Saka, S. Gas chromatographic and mass spectrometric (GC-MS) analysis of lignin derived products from *Cryptomeria japonica* treated in supercritical water. *J. Wood Sci.* **2004**, *50*, 253−259.
(36) Böttcher, C.; Westphal, L.; Schmotz, C.; Prade, E.; Scheel, D.; Glawischnig, E. The multifunctional enzyme CYP71B15 (PHYTOALEXIN DEFICIENT3) converts cysteine-indole-3-acetonitrile to camalexin in the indole-3-acetonitrile metabolic network of *Arabidopsis thaliana*. *Plant Cell* **2009**, *21*, 1830−1845.
(37) Grata, E.; Boccard, J.; Guillarme, D.; Glauser, G.; Carrupt, P.-A.; Farmer, E. E.; Wolfender, J. L.; Rudaz, S. UPLC-TOF-MS for plant metabolomics: a sequential approach for wound marker analysis in *Arabidopsis thaliana*. *J. Chromatogr., B* **2008**, *871*, 261−270.
(38) Harada, H.; Kumagai, H. Effect of dissolved oxygen in water on H_2O_2 production rate by sonication. *Jpn. J. Appl. Phys.* **2003**, *42*, 2958−2962.
(39) Janska, A.; Marsik, P.; Zelenkova, S.; Ovesna, J. Cold stress and acclimation - what is important for metabolic adjustment? *Plant Biol. (Stuttgart)* **2010**, *12*, 395−405.
(40) Bruce, R. J.; West, C. A. Elicitation of lignin biosynthesis and isoperoxidase activity by pectic fragments in suspension cultures of castor bean. *Plant Physiol.* **1989**, *91*, 889−897.