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Qualitative Characterization of Benzoxazinoid Derivatives in Whole Grain Rye and Wheat by LC-MS Metabolite Profiling

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Benzoxazinoids are metabolites occurring in a restricted group of plant species including crops such as rye, wheat, and maize. Focus on the analysis of benzoxazinoid metabolites has typically been due to their importance to plant biochemistry and physiology as highly bioactive molecules that plants use as alleochemicals to defend themselves against predators and infections. However, the potential dietary contribution of these compounds has not been addressed. This study conducted a detailed qualitative characterization of benzoxazinoid metabolites present in the whole grain rye and processed fractions of rye bran, and their presence was also detected in whole grain wheat samples. Several novel benzoxazinoid metabolites of the hydroxamic acids (2,4-dihydroxy-1,4-benzoxazin-3-one, DIBOA; 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, DIMBOA), lactams (2-hydroxy-1,4-benzoxazin-3-one, HBOA), and benzoxazolinones (1,3-benzoxazol-2-one, BOA) were identified, including double-hexose derivatives of DIBOA, DIMBOA, and HBOA. This paper presents an important addition to the information on the phytochemical composition of rye and wheat grains, which deserves attention in the discussion of the potential health-promoting effects of these grains.

KEYWORDS: Rye; *Secale cereale*; whole grain; benzoxazinoids; phytochemicals; metabolite profiling; metabolomics; LC-MS

INTRODUCTION

Benzoxazinoids are natural products that were first characterized in the 1950s in plants of the family Graminae including rye (*Secale cereale*), maize (*Zea mays*), and wheat (*Triticum aestivum*) (*1*, *2*). A variety of derivatives with different molecular structures of benzoxazinoid chemicals as well as their sugar derivatives have been reported to be present in cereals. Benzoxazinoids are found in all parts of plant and are most studied in monocots, but are also reported in some dicots (*3*, *4*). The compositions and concentrations of different benzoxazinoids vary on the bases of plant part, development stage, cultivar, and growing conditions (*5*−*7*). So far, only one paper exists on the presence of benzoxazinoids in seed tissue, namely, in wheat grain (*7*).

Benzoxazinoids are known to exhibit a wide range of biological properties including antimicrobial, antifeedant, and insecticidal effects. By far the most intensively addressed topic in research on benzoxazinoids is their importance as alleochemicals, that is, natural products having a role in the communication (or interaction) of plants with other plants, insects, or microbes (*8*, *9*). Owing to specific bioactivity against both microbial pests and weeds, benzoxazinoids are studied for their potential agronomic utility as natural herbicides in weed management, for example, by incorporation of the green plant parts to soil (*10*). The molecular structures of benzoxazinoids also serve as models for industrial development of candidates for pesticides (*9*, *11*). Benzoxazinoids are known for their unique chemical structure with potential pharmaceutical bioactivity, and a subgroup of benzoxazinoids, benzoxazolinones, has been acknowledged as "privileged scaffold" in terms of the design of drug chemicals (*12*). Reports on benzoxazinone metabolites include analyses on the water-soluble fraction of cernilton, which has been shown to selectively inhibit the growth of a prostate cancer cell line, and the effect was postulated to be due to the hydroxamic acids present in the extract (*13*). This finding was not supported by a later analysis showing that the most abundant compound present in the extract, 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), does not selectively inhibit the growth of the prostate cell line; instead, the inhibitory effect was suggested to be due to the ability of the compound to cause cell death (*14*). Furthermore, benzoxazinoids have been reported to possess aneugenic activity in human-derived cell lines (*15*, *16*). Although the biochemical properties, synthesis, and biodegradation of benzoxazinoids are well studied, the molecular mechanisms effecting their bioactivity have not been resolved in detail (*8*, *9*, *17*, *18*).

In this study we have identified several benzoxazinoid derivatives in whole grain rye by UPLC-qTOF-MS metabolite analysis and verified the presence of the same metabolites also in whole grain wheat, showing that they are part of the phytochemical pool of the two important cereal crops. Furthermore, our study

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indicates that these compounds are present also after enzymatic treatment, fractionation, and column purification in the water-soluble fraction of bran metabolites. The most abundant signal in the LC-MS analysis was observed for double-hexose derivatives, which are to our knowledge reported here for the first time. Our finding that they are present in whole grain rye and wheat suggests that they may contribute to our diet by affecting the potential health impact of whole grain products.

MATERIALS AND METHODS

Plant Material and Extraction. Whole grain rye samples of Finnish origin were used for the study. The metabolite extraction was performed as described previously (*19*). In brief, the whole grain samples (500 mg) were ground to a fine powder in liquid nitrogen, and 1.5 mL of 75% methanol with 0.1% formic acid was added. After sonication at room temperature for 15 min, the samples were centrifuged (10000*g*) and filtered. The samples were stored at −20 °C prior to analysis.

The metabolite extraction was performed also from the isolated rye bran. The rye bran samples were extruded and hydrolyzed by xylanase treatment to release components from the bran matrix. A 10% water suspension of extruded rye barn was subjected to xylanase enzyme (Econase, AB Enzymes GmBH, Darmstadt, Germany), 5 U/g bran, 40 °C, for 21.5 h and cooled to 12−16 °C, followed by centrifugation and separation to extractable (water phase) and nonextractable (residue) fractions (*20*). The metabolites in the extractable fraction were further purified by column chromatography (Amberlite XAD 8 HP) and eluted with ethanol. Both fractions were freeze-dried and stored at −80 °C.

The metabolite extraction from the dried bran fractions was performed as follows: The extractable fraction was directly dissolved in 75% methanol with 0.1% formic acid in the ratio of 6 μL/mg of dried sample. The residue was hydrolyzed by 1 N sodium hydroxide and incubated at 70 °C for 1 h, after which the pH was adjusted to 1−2 by adding 6 N hydrochloric acid. The sample was extracted three times with equal volumes of ethyl acetate and dried under vacuum. The sample was redissolved to 50% methanol and filtered prior to LC-MS analysis.

LC-MS Metabolite Analysis. Metabolite analysis was carried out using a UPLC-PDA-qTOF-MS system: a UPLC Waters Acquity instrument connected in-line to an Acquity PDA (photodiode array) detector and a Synapt HDMS detector (tandem quadrupole/time-of-flight mass spectrometer). The Synapt HDMS system was operated in the standard qTOF mode, without using the ion mobility capabilities. Separation of metabolites was performed using a 100 × 2.1 mm i.d., 1.7 μm UPLC BEH C18 column (Waters Acquity). The mobile phase consisted of 0.1% formic acid in acetonitrile/water (5:95, v/v) (phase A) and 0.1% formic acid in acetonitrile (phase B). The linear gradient program was as follows: 100−72% A over 22 min, 72−60% A over 0.5 min, 60−0% A over 0.5 min, held at 100% B for a further 1.5 min, then returned to the initial conditions (100% A) in 0.5 min, and conditioning at 100% A. The flow rate was 0.3 mL/min; the column temperature was kept at 35 °C. The UV spectra (Waters Acquity PDA detector) were recorded between 210 and 500 nm, or the UV trace was measured at 240 nm (Waters Acquity UV detector). Eluting compounds were detected by the qTOF equipped with an electrospray ionization (ESI) source. Acquisition was performed in the ESI-positive and ESI-negative modes. The following settings were applied during the LC-MS runs: capillary voltage at 3.0 kV; cone voltage at 30 eV; collision energy at 3 eV and 20 eV; argon used as collision gas. For the LC-MS/MS analysis 20 and 35 eV collision energies were used. The *m*/*z* range was 50−1500 Da. The MS was calibrated using sodium formate, and leucine enkephalin was used as the lock mass. A standard mixture that contained 40 μg/mL of each of the following compounds was used to monitor the quality of the chromatogram and reproducibility of the retention time throughout the runs and to aid in metabolite identification: L-tryptophan, L-phenylalanine, *p*-coumaric acid, caffeic acid, sinapic acid, benzoic acid, quercetin dehydrate, kaempferol, rutin, and *trans*-resveratrol (all purchased from Sigma); naringenin, chlorogenic acid hemihydrate, *trans*-cinnamic acid, and isorhamnetin (Fluka); ferulic acid (Aldrich); and tomatine (Apin chemicals). MassLynx software version 4.1 (Waters) was used to control all instruments and calculate the accurate masses.

LC-MS Data Analysis. The chromatograms obtained from UPLC-PDA-qTOF-MS analysis were processed by the MarkerLynx 4.1 software (Waters) for mass signal extraction and alignment. The identification of metabolites was performed as described previously (*19*). In short, the accurate mass and molecular formula predictions were screened for putative molecules from the Dictionary of Natural Products (Chapman and Hall/CRC) and the SciFinder Scholar databases (SciFinder Scholar 2007). The MS/MS fragmentation and UV-absorbability of the metabolites were compared with those of candidate molecules found in databases and verified with earlier literature on similar compounds.

RESULTS AND DISCUSSION

Identification. Analysis of the benzoxazinoid metabolites in whole grain rye and processed bran fractions was carried out as part of a larger study aiming at resolving the phytochemical composition of several whole grain varieties in detail. The identification of the individual benzoxazinoid metabolites was based on analytical data and comparison to published LC-MS analyses on similar compounds. The most common chemical structures of benzoxazinoids include hydroxamic acids (2,4-dihydroxy-1,4-benzoxazin-3-one, DIBOA; 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, DIMBOA), lactams (2-hydroxy-1,4-benzoxazin-3-one, HBOA; 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one, HMBOA), and benzoxazolinones (1,3-benzoxazol-2-one, BOA; 6-methoxy-1,3-benzoxazol-2-one, MBOA) (**Figure 1**). In plants these are stored as glucosides in vacuolar storage, and the bioactive aglycons are released from the sugars by enzymatic activity upon environmental challenge (*17*, *21*). In water solutions the benzoxazinoid aglycons are unstable and are transformed spontaneously to benzoxazolinones (*17*). For the analysis of benzoxazinoid derivatives in plant material several extraction protocols have been developed, as well as analytical detection methods optimized, and can be carried out by routine LC-MS analysis (*22*−*25*). The structural characterization of different benzoxazinoid chemicals has been conducted by 2D-NMR analysis (*3*).

The benzoxazinoid metabolites in whole grain rye were identified among the most intense signals in the nontargeted LC-MS metabolite profiling, being noticeable in the total ion chromatograms (**Figure 2**). The most notable metabolite signal in the methanol extract of whole grain rye kernel comes from an ion with *m*/*z* 504.136 (**Figure 2**). The ion gives the elemental composition of $C_{20}H_{27}NO_{14}$, which does not correspond to any compounds listed in the Dictionary of Natural Products (DNP) database. The molecular ion shows in ES(−) the neutral loss of 324.106 amu, which corresponds to the loss of two hexose sugar units (162.053 each) (*19*). In the MS/MS analysis the aglycon fragment of 180.030 is visible as a trace, and the more abundant fragments are *m*/*z* 162.020 (−18 amu, loss of water) and 134.024 (−28 amu), which corresponds to loss of a CO moiety (**Table 1**). In the positive ionization mode, the corresponding aglycon fragments (182.045, 164.036, 136.041, 80.050) are visible in the in-source fragmentation (**Figure 3**). In the positive mode, the fragment corresponding to the loss of one hexose moiety (*m*/*z* 344.098) is also visible, although this was not observed in the ES(−) mode. The absorbance in the PDA detector gives a double peak shaped spectrum exhibiting UV−visible λ_{max} at 255 and 280 nm (**Figure 4**).

The molecular ion *m*/*z* 342.082 eluting at 5.4 min indicated an elemental composition of $C_{14}H_{17}NO_9$, which corresponds to five molecules in the DNP, of which four are hexose sugar derivatives of benzoxazinone metabolites. The MS/MS analysis in ES(−) gives fragmentation showing the neutral loss of 162.05 (hexose) and fragmentation of the aglycon molecule that is identical to the one observed for the ion of *m*/*z* 504.136. Such fragmentation follows the pattern that has been reported to occur typically in the LC-MS/MS of benzoxazinoids (*23*, *24*). The smaller aglycon fragments observable in the analysis include 118.030 and 108.046 (collision energy = 25 eV) (**Figure 5**), and further fragmentation at 35 eV

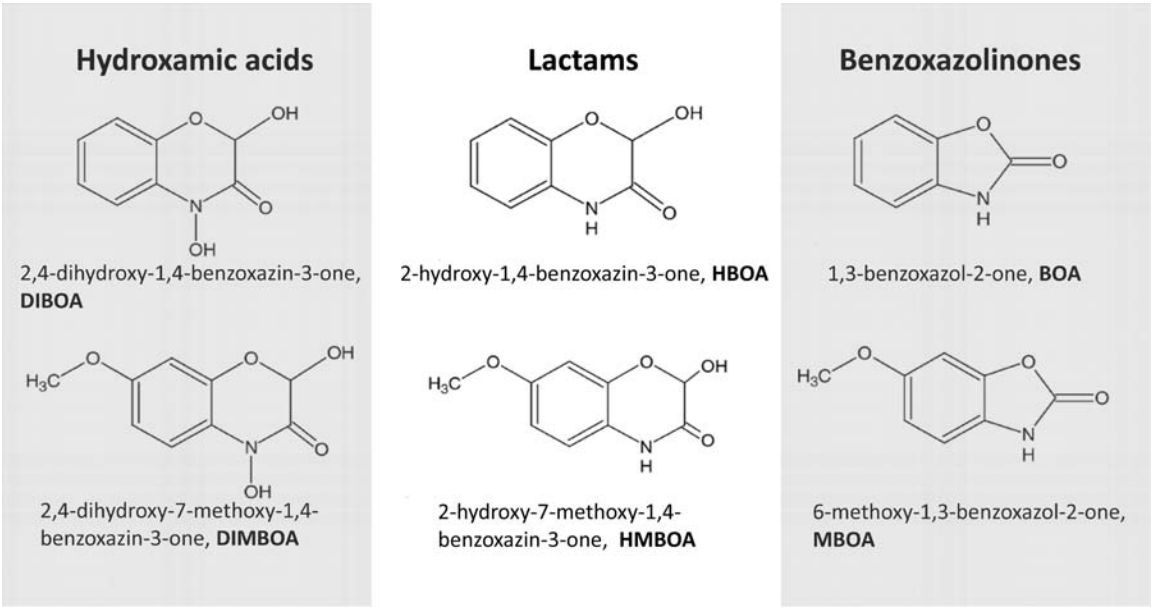

Figure 1. Chemical structures of the most commonly occurring benzoxazinoid compounds.

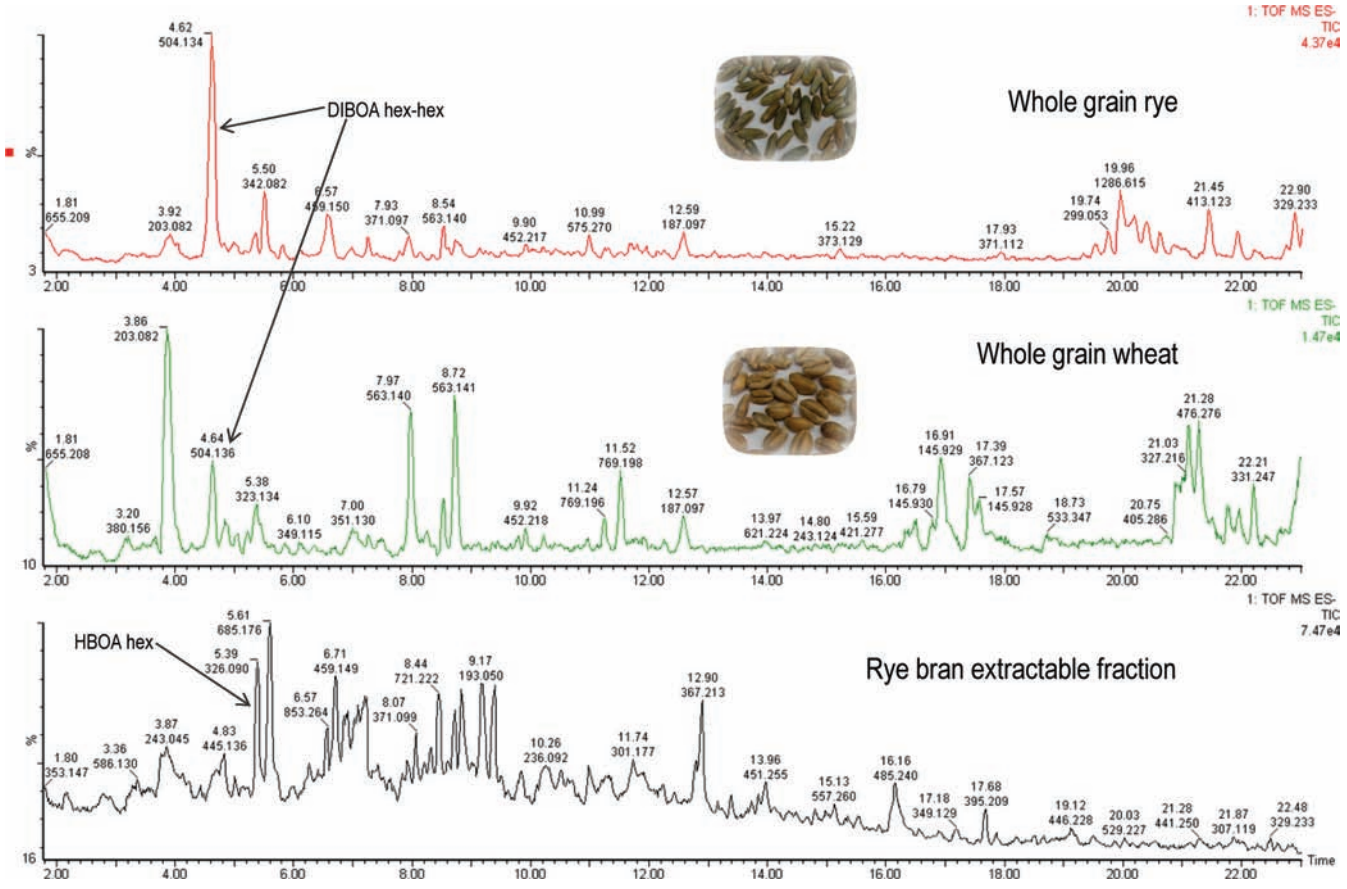

Figure 2. Total ion chromatograms of crude methanol extract from whole grain rye (upper panel), whole grain wheat (middle panel), and the extractable fraction of rye bran (lower panel). The most abundant signals tentatively identified as benzoxazinoid metabolites in each extract are depicted in the chromatograms.

results in *m*/*z* 101.024, 85.031, 78.036, and 59.012 (**Table 1**). Of those, at least 108 (*22*) and 78 (*22*, *24*) have been reported for the fragmentation of the DIBOA aglycon molecule in ES(−) MS/MS analysis. The UV spectrum of the metabolite resembles the one observed for the *m*/*z* 504.136 peak. Such a UV spectrum has been typically reported for DIBOA molecules (*26*). On the basis of these observations, the two metabolites are tentatively identified to be the hexose (*m*/*z* 342.082) and dihexose (*m*/*z* 504.136) derivatives of the hydroxamic acid DIBOA.

A similar fragmentation pattern is observable for two peaks eluting slightly before the DIBOA metabolites, namely, *m*/*z* 488.140 and 326.089. These two ions show the loss of two and one hexose sugars, respectively, resulting in the aglycon molecule of *m*/*z* 164.035. MS/MS analysis in ES(−) includes fragments of *m*/*z* 136.040

Table 1. Identified Benzoxazinone Derivatives in the Crude Methanol Extract of the Whole Grain Rye and Rye Bran Extractable Fraction

t_R (min)	$[M-H]^-$ (m/z)	putative ID	molecular formula	λ_{max}[a] (nm)	ES(−)[b] (m/z)	ES(+)[c] (m/z)
		hydroxamic acids				
4.6	504.136	DIBOA-hex-hex	$C_{20}H_{27}NO_{14}$	255, 280	180.030, 162.020, 134.024, 118.030, 108.046, 101.024, 85.031, 78.036, 59.012	506.152, 344.098, 325.113, 182.045, 164.036, 136.041, 80.050
5.5	342.085	DIBOA-hex	$C_{14}H_{17}NO_9$	255, 280	180.029, 162.019, 134.024, 118.029, 108.044	344.102, 182.055, 164.040, 136.045
5.8	534.147	DIMBOA-hex-hex	$C_{21}H_{29}NO_{15}$	262, 283sh	221.060, 192.036, 164.035, 149.013, 133.016, 121.016, 101.025, 98.029	536.151, 374.116, 212.065, 194.054, 166.057
		lactams				
4.5	488.141	HBOA-hex-hex	$C_{20}H_{27}NO_{13}$	n.a.[d]	221.072, 164.037, 136.042, 118.029, 108.045	166.050, 148.041
5.2	326.087	HBOA-hex	$C_{14}H_{17}NO_8$	250, 276sh, 284sh	164.035, 136.040, 118.029, 108.045	328.103, 166.051, 148.041, 120.046
		benzoxazolinones				
8.6	134.025	2-benzoxazolol	$C_7H_5NO_2$	n.a.	112.131, 91.022, 78.034	136.041

[a] λ_{max} is the absorbance maximum in the UV−visible range. [b] ES(−) fragments are obtained from MS/MS analysis of the molecular ion. [c] ES(+) fragments are observed as in source fragmentation for each of the compounds. [d] n.a., not available.

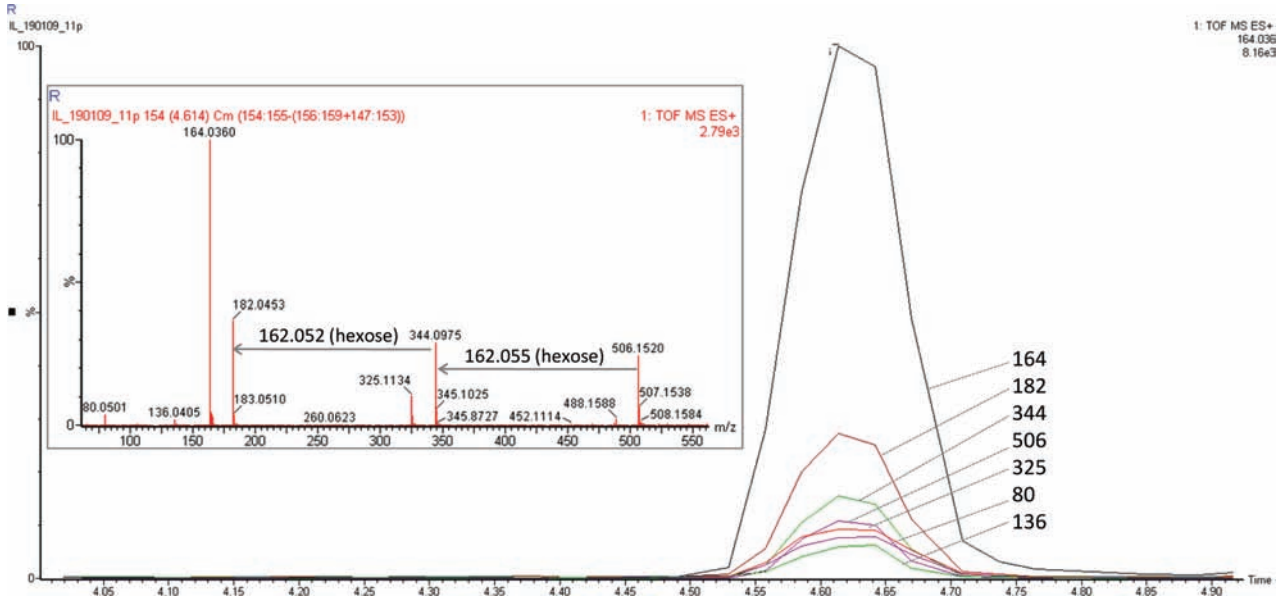

Figure 3. In-source fragmentation of the metabolite tentatively identified as DIBOA-dihexose in the ES(+) ionization mode.

(−28 amu, CO moiety) and m/z 108.045, indicating the loss of another CO moiety (**Figure 5**). Such fragments have been reported in the LC-MS/MS analysis for HBOA lactam benzoxazinoids (*22*, *24*). The UV spectrum for the m/z 326.089 peak is similar to the one observed for the DIBOA metabolites (**Figure 4**). For the ion of m/z 488.140 a clear UV spectrum was not achievable due to the partly coeluting metabolite m/z 504.136. The elemental composition suggested for the m/z 488.140 ion is $C_{20}H_{27}NO_{13}$ with no matching compounds in the DNP, whereas the suggested molecular form for the m/z 326.089, $C_{14}H_{17}NO_8$, corresponds to only two metabolites in the DNP, both of which are hexose sugar derivatives of HBOA lactam molecules. The peaks with molecular ions of m/z 488.140 and 326.089 were thus suggested to contain dihexose and hexose derivatives of HBOA, respectively.

Several benzoxazinoid metabolites typically occur concomitantly in plants capable of producing them. After identification of the DIBOA and HBOA compounds, also other members of the benzoxaxinoid metabolite family were searched from the raw LC-MS data on the basis of molecular weight and fragmentation information available from earlier LC-MS studies (*22*−*24*, *27*, *28*). In this analysis, the molecular ion of m/z 534.147 showed properties suggesting it to be a benzoxazinoid compound. The most abundant aglycon fragments in the ES(−) MS/MS analysis were m/z 192.036 (loss of 18 amu of aglycon), 164.035 (loss of 28 amu), and 149.013 (loss of 15 amu, corresponding to methoxy radical) (**Figure 5**). Such fragmentation has been reported for the methylated hydroxamic acid derivative DIMBOA (*22*, *24*). In the positive ionization mode corresponding fragments are visible together with an additional signal of 212.065 amu, which is the aglycon metabolite after the loss of two 162.05 hexose sugar units. The UV spectrum resembles the one observed for DIBOA and HBOA, the peak having λ_{max} at 267 nm (**Figure 4**). The suggested molecular formula for the ion was $C_{21}H_{29}NO_{15}$ with no hits in the DNP. On the basis of the fragmentation and UV spectral information the metabolite is suggested to be a methylated hydroxamic acid DIMBOA with dihexose derivatization. In contrast to the DIBOA and HBOA metabolites, no single sugar-bearing derivative was observed for DIMBOA.

Additionally, at a retention time of 8.6 min a metabolite of m/z 134.025 with the elemental composition of $C_7H_5NO_2$ eluted (**Figure 4**). The formula matches the one for the benzoxazolinone metabolite benzoxazol-2-one (BOA).

For all of the benzoxazinoid metabolites tentatively identified here, the elution order in the chromatographic separation is logical, as the highly hydrophilic double-sugar derivatives of DIBOA, HBOA, and DIMBOA elute earlier than the corresponding single-hexose forms of DIBOA and HBOA (**Figure 4**). Additionally, the methyl-substituted derivative, DIMBOA dihexose, elutes slightly later than the corresponding derivatives of DIBOA and HBOA.

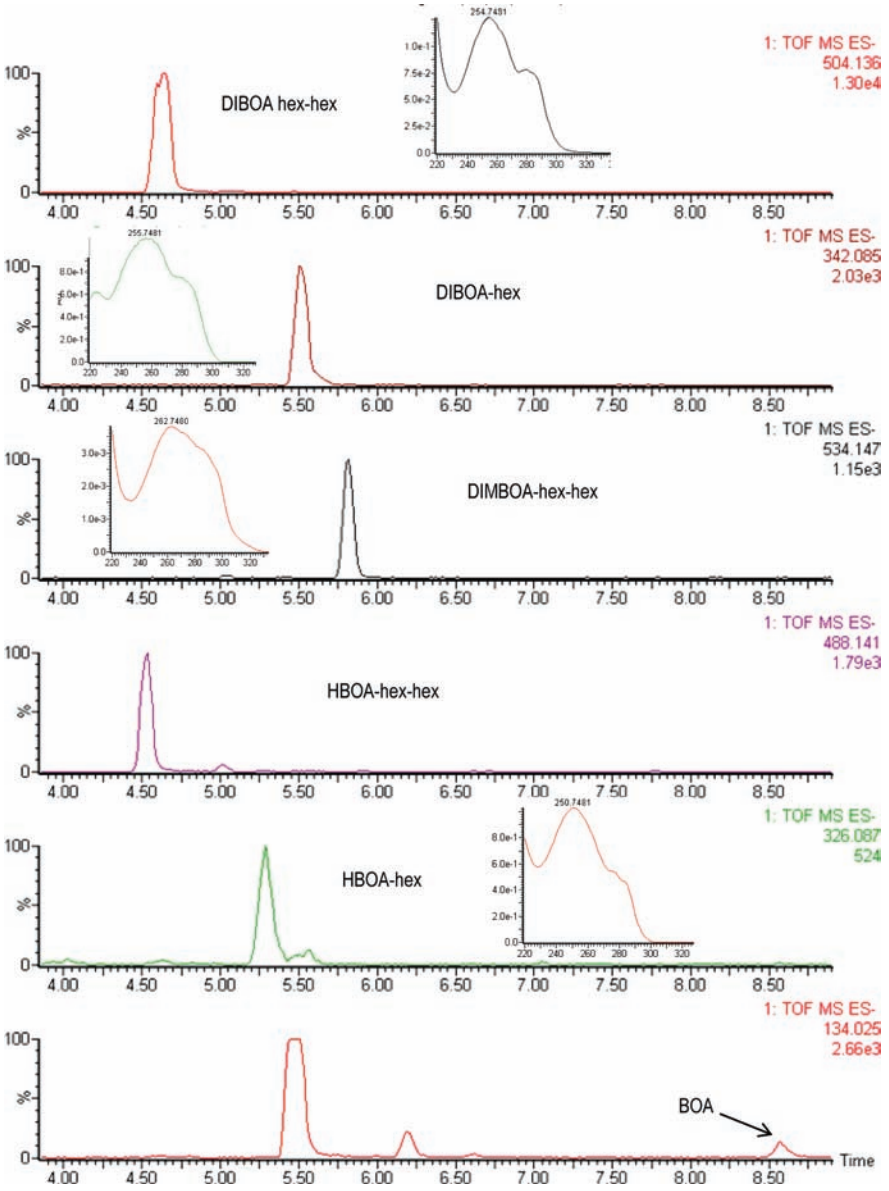

Figure 4. Reconstructed ion chromatograms of detected benzoxazinoid derivatives, with UV spectrum inserted when detectable. All of the chromatograms are from the whole grain rye sample except BOA (lowest panel), which was detectable only in the extractable rye fraction. The signals eluting 5.5 and 6.2 min in the lowest panel (BOA) result from degradation of the other benzoxazinoid metabolites.

The only metabolite without sugar substituents, BOA, elutes clearly later due to the lack of the hydrophilic sugar units (**Figure 4**).

Occurrence in Rye and Other Whole Grain Varieties. The double-hexose derivatized metabolites of DIBOA, HBOA, and DIMBOA do not result in any corresponding compounds when searched against the metabolites listed in the DNP, and they are not reported in any of the publications focusing on the analysis of this metabolite class. Thus far, studies on benzoxaxinoids have reported only the single-hexose-containing derivatives in plant species such as rye, wheat, and maize (*7*, *8*, *11*). In this analysis the presence of two hexose units in the DIBOA, HBOA, and DIMBOA molecules is observed in the LC-MS/MS analysis. The location of the hexose units cannot be determined on the basis of the LC-MS/MS analysis, but the single-glucose unit of benzoxazinoid metabolites is typically in the hydroxyl group of the 2-carbon (*11*) (**Figure 1**). It may be that in the purification procedures typically applied, the relatively harsh conditions employed in previous studies (*27*, *28*) degraded multiple-sugar-containing benzoxazinoid derivatives. By contrast, our simple and straightforward extraction method performed on the whole grain kernels results in a metabolite yield containing more of the different derivatives. Whether the double-hexose derivatized forms of benzoxazinoids occur also in other crop species reported to produce benzoxazinoids should be addressed. Similarly, it would be valuable to determine whether such derivatives are specific to grains or whether they are produced also in the other parts typically synthesizing benzoxazinoids, such as shoots and roots. These issues are important for the quantitative analyses of benzoxazinoids determining their amounts in different crops and plant parts.

In the whole grain rye sample the signal for the metabolite containing two sugar moieties (m/z 504.136) is by far more intense than that for the one containing a single hexose (m/z 342.082) (**Figure 6A**). In the isolated rye bran fraction these two metabolites occur with similar intensities, which might be a result of the fragmentation of the double-sugar derivatized form in the processing of the bran fractions. In the extractable fraction of rye bran the dominating benzoxazinoid derivative is the putative HBOA hexose. The difference of the hydroxamic acids and lactams in these samples may be due to modification occurring during the extraction or results from different distribution of the metabolites in different

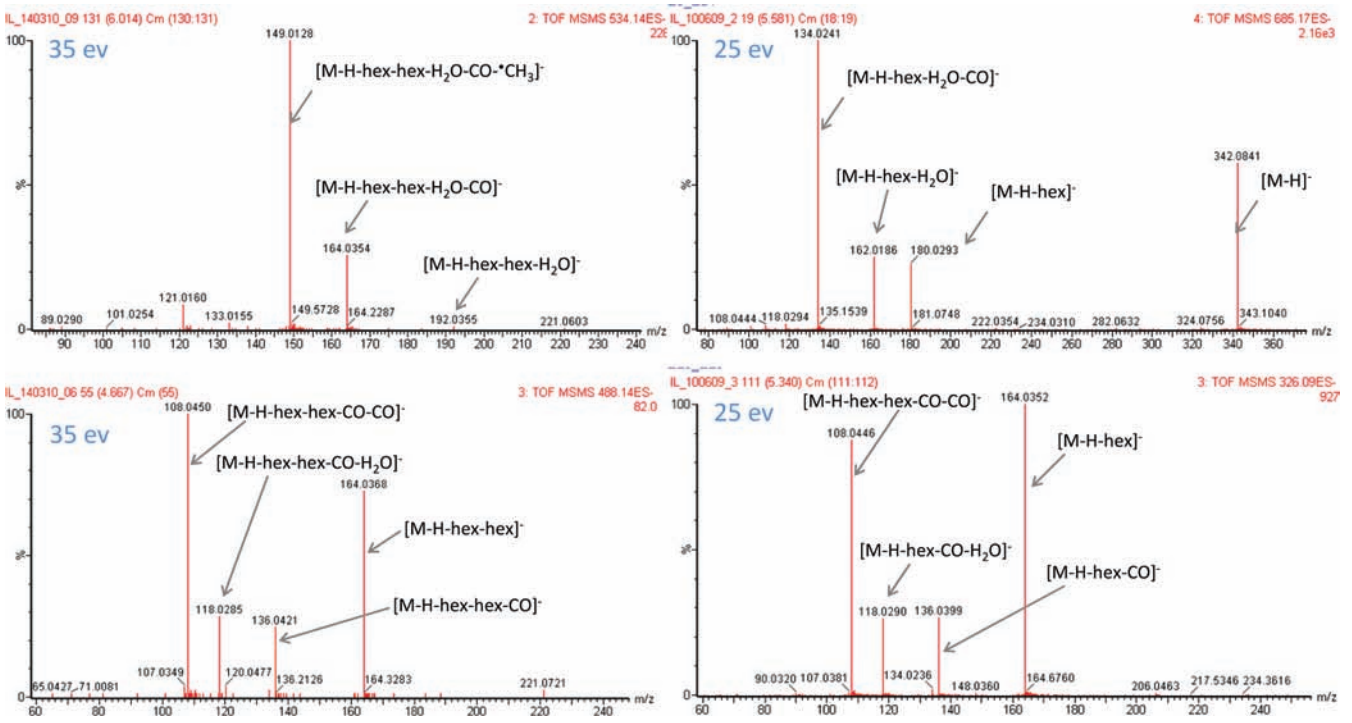

Figure 5. MS/MS spectra of benzoxazinoid derivatives. The collision energies used in the analysis are depicted on the spectra. The identified fragment ions are included.

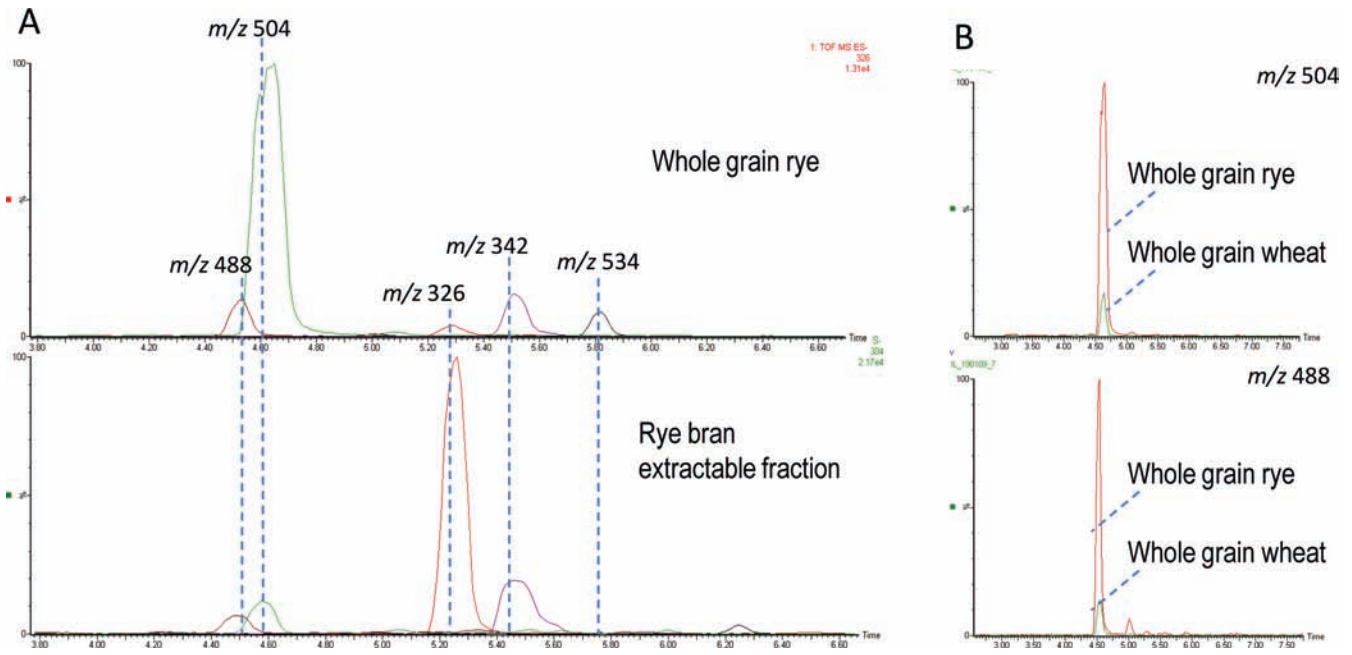

Figure 6. (**A**) Reconstructed ion chromatograms of each of the benzoxazinoid derivatives from the whole grain rye (upper panel) and extractable rye bran fraction (lower panel) showing the relative intensities of each of the metabolites in the two different samples. (**B**) Metabolite signals of benzoxazinoid molecules in the whole grain wheat sample compared to the signals observed in rye samples for the metabolites DIBOA dihexose (*m*/*z* 504; upper panel) and HBOA dihexose (*m*/*z* 488; lower panel).

parts of the kernel. The degradation of the benzoxazinoid metabolites is known to occur relatively easily; that is, the hydroxamic acids are converted to benzoxazolinones spontaneously (*9*, *17*). It is likely that the benzoxazolinone metabolite BOA observed in the extractable fraction of rye bran, but not in the crude methanol extract of whole grain rye, results from those precursors.

Following the detailed qualitative analysis of benzoxazinoid metabolites in the rye samples, also other whole grain species (wheat, barley, and oat) were screened for the presence of similar compounds. The metabolite extraction from whole grain samples was performed in the same way as for rye, and the same analytics was applied. Of the samples studied, only wheat showed signals for benzoxazinoid metabolites when examined from the crude methanol extract (**Figure 2**). All of the metabolites identified in rye were also present in wheat sample, although the metabolite signal intensities were much smaller than the corresponding signals observed in the case of rye, as exemplified by the two most abundant metabolites DIBOA dihexose and HBOA dihexose (**Figure 6B**).

Significance in Human Nutrition. Cereal grains, mainly rye, wheat, and maize, are the only reported agricultural crops to produce benzoxazinoids. The fact that benzoxazinoid compounds are present also in the edible part of rye and wheat, as shown here, deserves further attention. In our analysis the compounds are shown to be present in the water-soluble fraction of the rye bran and may thus be absorbed in the intestine as part of the diet. The bioavailability, stability, absorbability, glucurono-/sulfoconjugation, and possible impact of gut microbial processing of benzoxazinoid metabolites are totally unknown.

Consumption of whole grain has consistently been linked to reduced risk of several chronic diseases in epidemiological studies (*29*). Diets rich in whole grain rye are believed to positively affect insulin and glucose metabolism, thereby potentially lowering the risk of metabolic syndrome and type 2 diabetes, although the underlying molecular mechanisms are not known (*30*, *31*). To date, however, benzoxazinoid metabolites have not been posited to contribute to such health-promoting properties of whole grain cereals (*29*), but from this study, it is apparent that the potential bioactivity of these compounds warrants further study.

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