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Bioactive Benzoxazinoids in Rye Bread Are Absorbed and Metabolized in Pigs

Khem B. Adhikari,[†] Bente B. Laursen,[†] Helle N. Lærke,[‡] and Inge S. Fomsgaard*[,†]

[†]Department of Agroecology, Aarhus University, Forsøgsvej 1, DK-4200 Slagelse, Denmark

[‡]Department of Animal Science, Aarhus University, Blichers Allé 20, DK-8830 Tjele, Denmark

ABSTRACT: Recently, bioactive benzoxazinoids were discovered in cereal grains and bakery products. In this study, we studied the uptake, distribution, and metabolism of these secondary metabolites using a pig model. Twelve benzoxazinoid compounds and their 4 transformation products were quantified in the pigs' diets and biofluids using high-performance liquid chromatography coupled to electrospray ionization triple quadrupole mass spectrometry. The 2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one (DIBOA-glc) was the most dominant benzoxazinoid (232 nmol/g DM) seconded by the double-hexose derivative of DIBOA (provisionally characterized here as DIBOA-glc-hex) in the rye-based diet. DIBOA-glc (derived from the diet and intestinal deglycosylation of DIBOA-glc-hex) was apparently reduced to 2-β-D-glucopyranosyloxy-1,4-benzoxazin-3-one (HBOA-glc), the most dominant benzoxazinoid in the blood (829 nmol/L). The benzoxazinoid compounds were excreted in the urine, with HBOA-glc (18 μmol/L) as a major metabolite. In this study, we determined for the first time the bioavailability of dietary benzoxazinoids that have high digestibility, distribution, and metabolism in mammals. These findings could be a milestone for the exploitation of healthful and pharmacological properties of benzoxazinoids.

KEYWORDS: *rye, benzoxazinoids, DIBOA-glc-hex, DIBOA-glc, HBOA-glc, BOA, uptake, metabolism, bioavailability*

INTRODUCTION

Benzoxazinoids are a group of naturally occurring plant secondary metabolites with remarkable allelopathic, pharmacological, and health-promoting attributes. The most common chemical structures of benzoxazinoids (Table 1) include benzoxazolinones (2-benzoxazolinone, BOA; and its 6-methoxy derivative, MBOA), lactams (2-hydroxy-1,4-benzoxazin-3-one, HBOA; 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one, HMBOA; and their corresponding glucosides), and hydroxamic acids (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, DIMBOA; its demethoxylated analogue, DIBOA; and their respective glucosides). Major known microbial transformation products of the benzoxazinoids are 2-amino-3*H*-phenoxazin-3-one (APO), 2-amino-7-methoxy-3*H*-phenoxazin-3-one (AMPO), 2-acetylamino-3*H*-phenoxazin-3-one (AAPO), and 2-acetylamino-7-methoxy-3*H*-phenoxazin-3-one (AAMPO) (Table 1).[1] Several studies have detected and quantified benzoxazinoids in the roots and shoots of certain cereal plants,[2−4] reported on their bioactivity, and discussed their usefulness as an alternative means for suppressing weeds, insects, and diseases.[2,3,5−7] Contrary to previous reports,[8,9] the above-mentioned bioactive benzoxazinoids including double-hexose derivatives of DIBOA, DIMBOA, and HBOA were recently detected in mature whole grains of cereals and bakery products,[10−12] suggesting that they might contribute to the potential healthful impact of whole grain products.

Recently, considerable attention has been given to benzoxazinoids to exploit their pharmacological capacities. Some of these bioactive compounds have been reported to possess antiallergic, anti-inflammatory,[13,14] anticancer,[15,16] weight loss, and appetite suppressing[17] effects. The transformation product of BOA, 2-amino-3*H*-phenoxazin-3-one (APO), was reported to be a potent antibiotic compound[18] containing anti-inflammatory, immunoregulatory,[19] and antimicrobial[20] properties. The potential pharmacological effects of dietary benzoxazinoids, to some extent, depend on whether they are converted to BOA, MBOA, APO, and other bioactive compounds and are available to the body. Thus, studies of the bioavailability of benzoxazinoids in whole grain rye bread, which have higher contents of benzoxazinoids than other cereal foods, are important to assess the healthful effects of rye staple foods.

The metabolic fate of benzoxazinoids in the mammalian body could be expected to be similar to that in soil or plants, as demonstrated for other phytochemicals, such as certain flavonoids.[21,22] Benzoxazinoids are very dynamic and change their structures in different plant stages,[2,23] in soil,[1,3,5,24,25] and during food processing.[10,11] DIBOA-glc, one of the most abundant benzoxazinoids in rye bread,[11] and DIMBOA-glc undergo enzymatic degradation into their unstable active agluconic forms upon insect or pathogen attack or cell damage.[2,5,25] Furthermore, these agluconic hydroxamic acids spontaneously or microbially transform into corresponding benzoxazolinones.[2,5,7,23,26−29]

The main microbial degradation pathway of benzoxazinoids in soil/plants/solvents is centered on the 2-aminophenol (AP) from BOA and HBOA, and its subsequent oxidation into APO.[1,3,24,25,27,28] A number of other transformation products originate after the acetylation of APO and the subsequent oxidation, hydroxylation, and methoxylation.[1,25,28] Similarly,

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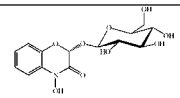
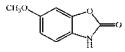
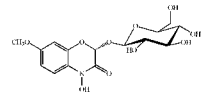
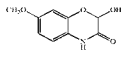
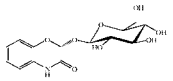
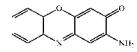
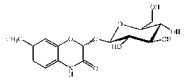
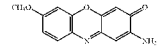
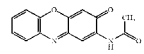
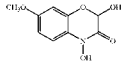
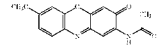
Table 1. Common Benzoxazinoid Compounds and Their Transformation Products

Acronym	Systematic name	Structural formula	Acronym	Systematic name	Structural formula
BOA	2-Benzoxazolinone		DIBOA-glc	2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one	
MBOA	6-Methoxy-benzoxazolin-2-one		DIMBOA-glc	2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one	
HBOA	2-Hydroxy-1,4-benzoxazin-3-one		DIBOA-glc-hex	- [a]	
HMBOA	2-Hydroxy-7-methoxy-1,4-benzoxazin-3-one		HBOA-glc-hex	- [a]	
HBOA-glc	2-β-D-glucopyranosyloxy-1,4-benzoxazin-3-one		APO	2-Amino-3H-phenoxazin-3-one	
HMBOA-glc	2-β-D-glucopyranosyloxy-7-methoxy-1,4-benzoxazin-3-one		AMPO	2-Amino-7-methoxy-3H-phenoxazin-3-one	
DIBOA	2,4-Dihydroxy-1,4-benzoxazin-3-one		AAPO	2-Acetylamino-3H-phenoxazin-3-one	
DIMBOA	2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one		AAMPO	2-Acetylamino-7-methoxy-3H-phenoxazin-3-one	

[a]Not known.

MBOA transforms into 2-amino-7-methoxy-3*H*-phenoxazin-3-one (AMPO)[24,26,29] and its acetylated analogue AAMPO.[28]

Generally, dietary phytochemicals are metabolized by intestinal microorganisms, intestinal epithelial cells, the kidney, or the liver. The mechanisms associated with the gut metabolism and absorption of phytochemicals vary depending upon their structural features.[30−32] For instance, methylated flavones are more efficiently absorbed and transported than demethylated flavones.[30] There are inconsistent reports regarding the mechanism of intestinal absorption of glycosidic phytochemicals.[31] Some glycosidic phytochemicals are actively absorbed in intact forms across the intestinal brush border by the sodium-dependent glucose transporter 1 (SGLT1).[33,34] However, deglycosylation by β-glucosidase in the gut and the consequent passive absorption of aglucones has also been mentioned.[35,36] The liver is another main site of metabolism. Methoxylated flavonoids, for instance, are demethylated to their oxidized analogues by cytochrome P450 enzymes in rat liver.[37]

There is a paucity of information regarding the uptake of dietary benzoxazinoids. MBOA accumulates in grazing animals when they are maintained in benzoxazinoid-rich pastures.[17] DIMBOA-glc, another benzoxazinoid compound, is passively absorbed and excreted into the honeydew of aphids without being metabolized.[38] DIMBOA reduces the growth rate and changes the activities of enzymes that affect the growth and digestive physiology of some insects.[39] MBOA, which is structurally similar to melatonin, is found to stimulate the reproductive system in a number of animal species fed with the pure compound.[40,41] To our best knowledge, no reports exist regarding the uptake of benzoxazinoids in the human body, and the metabolism of dietary benzoxazinoids has not been studied in any organism. To fully understand the implications of dietary benzoxazinoids in human and animal health, it is essential to determine their bioavailability and transformation in vivo.

The purpose of the present study was to elucidate the uptake, distribution, and metabolism of dietary benzoxazinoids in a pig model.

MATERIALS AND METHODS

Animals and Feeding. Twelve pigs ($n = 6$), weighing 70 kg at the start of the experiment, were fed a wheat flour-based washout diet for 3 weeks. The pigs were then assigned to one of two experimental diets based on high-dietary fiber buns made of rye or wheat flour (Table 2) for 7 weeks. After 7 weeks on the diet, the breads were minced, and chromic oxide was added as a digestibility marker for the remaining 2.5 weeks of the study. During week 9, after transfer to the experimental diets, the pigs were moved to metabolic cages for 7 days for daily quantitative collection of feces and urine. Subsequently, the pigs were returned to their pens and slaughtered 2−4 days after the end of the balance period, as described previously.[42]

Sampling. After 8 weeks on the experimental diets, feces and urine were collected quantitatively for 7 days on a daily basis and pooled per pig. Ascorbic acid was added as an antioxidant to the urine collection bottle (4 g per bottle per day). Subsequently, the pigs were euthanized as described by Lærke et al.,[42] and blood from 4 different parts of the circulatory system (portal vein, hepatic vein, lateral auricular artery, and jugular vein) and bile were sampled 3 h postprandially. The samples were stored frozen at −80 °C until chemical analyses.

The animal experiments were conducted according to protocols approved by the Danish Animal Experiments Inspectorate and complied with Danish Ministry of Justice Law number 382 (June 10, 1987) and Acts 739 (December 6, 1988) and 333 (May 19, 1990).

Chemicals. The standards for 10 benzoxazinoids and 4 transformation products, as listed in Table 3, were obtained as gifts or were synthesized as described in Carlsen et al.[4] The compounds were used for the preparation of 4 standard mixtures. Analytical grade methanol and acetonitrile were obtained from Rathburn (Walkerburn, Scotland). Glacial acetic acid was obtained from Baker (Griesheim, Germany).

Table 2. Ingredients of Experimental Diets (g/kg)

ingredients	wash out diet	experimental diets[a]	
		wheat	rye
wheat flour	815	528	0
rye whole meal	0	0	310
rye bran	0	0	400
cellulose	60	157	0
whey protein concentrate	69	25	0
yeast	0	20	20
sugar	0	15	15
egg powder	0	150	150
rape seed oil	28	20	20
lard	0	50	50
cholesterol	0	5	5
vitamin−mineral mix[b]	28	30	30

[a]Before water addition and baking. [b]Provided in mg/kg in the diet: 6642 $Ca(H_2PO_4)_2$, 4122 NaCl, 16 580 $CaCO_3$, 286 $FeSO_4{\cdot}7H_2O$, 114 ZnO, 41 Mn_3O_4, 92 $CuSO_4{\cdot}5H_2O$, 0.3 KI, 0.8 $Na_2SeO_3{\cdot}5H_2O$, 2.1 retinoacetate, 0.03 cholecalciferol, 69 α-tocopherol, 2.52 menadione, 4.58 riboflavin, 12.59 D-pantothenic acid, 0.025 cyanocobalamine (B_{12}), 2.52 thiamin (B_1), 25.2 niacin, 3.78 pyridoxine (B_6), and 0.063 biotin.

Table 3. LC-MS/MS Identification[a] of 10 Known Benzoxazinoids (Standard Mixtures A, B, and C), 2 Provisionally Characterized Benzoxazinoids, and Their 4 Possible Transformation Products (Standard Mixture G) Analyzed in the Samples

standard	acronym	mass	*m/z* values	t_R (min)
A	BOA	135.12	134/78.2	18.4
A	MBOA	165.15	164.3/149	23
A	HBOA	165.15	164/108	10.7
A	HMBOA	195.17	193.9/138.2	13.3
C	HBOA-glc	327.29	326.4/164.3	6
C	HMBOA-glc	357.31	356/194	7.3
C	DIBOA	181.15	180.2/134.3	10.8
C	DIMBOA	211.17	210.1/164.3	14
B	DIBOA-glc	343.29	342/134	6.6
B	DIMBOA-glc	373.31	372/164.3	7.7
-[b]	DIBOA-glc-hex	505.43	504.1/134	4.9
-[b]	HBOA-glc-hex	489.43	488.1/164.3	4.9
G	APO	212.2	213/185	36.9
G	AMPO	242.23	243.6/228.3	38.6
G	AAPO	254.24	255/213.5	39.7
G	AAMPO	284.27	285/243	40.5

[a]Molecular weight, MRM transition ion pair, and retention time (t_R) on chromatographic column. [b]Not available.

Chemically and microbially inert Ottawa sand (particle size 20−30 mesh) was purchased from Fisher Scientific (Leicestershire, UK). The water used in the experiment was purified with a Milli-Q Millipore instrument.

Extraction of Diet and Fecal Samples. All of the extractions were performed in duplicate. Freeze-dried samples of the diets and feces were ground to a particle size of less than 0.5 mm prior to analysis. Then, the samples were extracted using an accelerated solvent extraction 350 system (ASE) from Dionex following the method described in Pedersen et al.[11]

Extraction of Plasma, Bile, and Urine Samples. Because of limitations in sample volumes, the liquid samples were only extracted once, except for the recovery experiment, which was performed using 6 replicates. The impurities in the samples were separated by solid phase extraction using Oasis cartridges (wat094226, 3 cm^3/60 mg) for plasma and C8 cartridges (wat036780, 3 cm^3/500 mg) for bile and urine. A solution containing methanol/acetic acid/water (80:1:19) was the eluent for the benzoxazinoid compounds in standard mixtures A, B, and C (Table 3). For the transformation products in standard mixture G, an Oasis cartridge (wat094226, 3 cm^3/60 mg) was used with acetonitrile as the eluent. The samples were stored at −18 °C and diluted with an equal volume of water before analysis.

LC-MS/MS Detection and Analysis. The samples were analyzed using an AB SCIEX 3200 liquid chromatography triple quadrupole mass spectrometer (LC-MS/MS) and electrospray ionization in the multiple reaction monitoring (MRM) mode. Negative mode ionization was used for benzoxazinoid compounds, and positive mode ionization was used for their 4 possible transformation products. The instrument parameters were as follows: curtain gas, 12 psi (except for the metabolites in standard G, 11 psi); temperature, 475 °C; ion source gas 1, 60 psi; ion source gas 2, 60 psi; interface heater, on; collision gas, medium; and ion spray voltage, −4500 V. Five compound-dependent MS/MS parameters, as stated in Table 4, were applied. Nitrogen was used as a collision and source gas. Before identification, the instrument and compound-dependent MS/MS parameters were optimized using flow injection analysis of individual authentic compounds except for double-hexose derivatives of DIBOA and HBOA.

Table 4. Mass Spectrometry Compound-Dependent Parameters[a]

compd	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
BOA	−36	−7	−17.74	−20	−1.0
MBOA	−24	−4	−17.74	−20	−1.0
HBOA	−28	−3	−17.72	−23	−1.5
HMBOA	−28	−3	−18.84	−19	−2.5
DIBOA	−12	−1.5	−18.32	−10	−2.5
DIMBOA	−12	−1.5	−19.43	−9	−3.5
HBOA-glc	−34	−4	−23.73	−20	−4.9
HMBOA-glc	−30	−4	−24.83	−22	−1.1
DIBOA-glc	−36	−4	−24.32	−24	−4
DIMBOA-glc	−22	−4	−25.42	−18	−5
*HBOA-glc-hex	−36	−4	−29.72	−32	−4
*DIBOA-glc-hex	−36	−4	−30.31	−32	−4
APO	22	7	16.12	26	2
AMPO	22	6	16.96	26	2
AAPO	16	5	17.28	22	4
AAMPO	18	6	18.12	24	2

[a]DP, declustering potential; EP, entrance potential; CEP, cell entrance potential; CE, collision energy; CXP, cell exit potential. *Not optimized with standards.

The columns used for the chromatographic separations were from Phenomenex Synergi Polar RP-80A, 250 × 2 mm id, 4 μm particle size (Allerød, Denmark). Two mobile phases (A, 7% acetonitrile in water; and B, 78% acetonitrile in water, each containing 20 mM of acetic acid) were used in a linear gradient system as follows: 0−5 min, 84:16; 5−22 min, 70:30; 22−30 min, 50:50; 30−32 min, 30:70; 32−35 min, 30:70; 35−39 min, 84:16; and then 39−48 min back to 84:16 of A and B at a flow rate of 200 μL/min with an injection volume of 20 μL. The 10 benzoxazinoids and their 4 transformation products were identified based on comparisons of mass spectra and the selectivity of the LC separation producing matching peaks at the predetermined retention times for the authentic standards listed in Table 3. The double-hexose metabolites (DIBOA-hex-hex and HBOA-hex-hex) were tentatively identified based on the elution order in the chromatographic separation and fragmentation pattern as described by Hanhineva et al.[12] On the basis of the plasma metabolites (see Results and Discussion), these compounds were provisionally characterized here as DIBOA-glc-hex and HBOA-glc-hex.

Table 5. Extraction Efficiency with the Spiked Concentration, as Well as Limits of Detection and Quantification for Analytical Methods of 10 Benzoxazinoids and Their 4 Possible Transformation Products in Plasma, Urine, and Feces[a]

	plasma[b]					urine[b]					feces[c]				
analyte	conc	R	RSD	LD	LQ	conc	R	RSD	LD	LQ	conc	R	RSD	LD	LQ
BOA	37.0	103	5	6.0	20.1	74.0	82	14	26.2	87.3	39.4	108	3	4.0	13.4
MBOA	30.3	101	5	4.9	16.4	60.6	65	5	6.1	20.4	32.2	117	2	1.8	6.0
HBOA	30.3	105	9	8.7	28.8	60.6	74	7	10.1	33.8	32.2	114	3	3.3	10.9
HMBOA	25.6	108	5	4.4	14.5	51.2	79	10	13.0	43.4	27.3	90	6	4.4	14.7
HBOA-glc	15.3	95	7	3.0	10.1	30.6	103	8	7.7	25.6	16.3	99	3	1.3	4.4
HMBOA-glc	14.0	104	5	2.1	7.0	28.0	123	5	4.8	15.9	14.9	89	4	1.6	5.4
DIBOA	55.2	99	5	8.8	29.3	55.2	47	7	5.3	17.6	29.4	101	8	6.9	23.1
DIMBOA	47.4	106	17	25.7	85.6	47.4	69	2	2.2	7.3	25.2	67	12	6.1	20.4
DIBOA-glc	14.6	89	9	3.5	11.6	14.6	117	8	4.1	13.8	15.5	69	5	1.6	5.2
DIMBOA-glc	13.4	99	9	3.7	12.5	26.8	74	17	9.3	31.0	14.2	61	10	2.6	8.7
APO	47.1	71	2	2.0	6.6	47.1	93	1	1.7	5.5	25.1	137	5	4.6	15.5
AMPO	41.3	59	11	8.0	26.6	41.3	97	5	6.0	20.0	22.0	145	7	5.9	19.6
AAPO	39.3	82	1	1.1	3.8	39.3	107	3	3.8	12.7	20.9	76	4	2.0	6.5
AAMPO	35.2	95	6	5.8	19.2	35.2	103	7	7.8	26.1	18.7	62	4	1.5	4.9

[a]R, recovery percent; RSD, relative standard deviation; conc, concentration; L_D, limit of detection; and L_Q, limit of quantification. [b]nmol/L. [c]nmol/g DM.

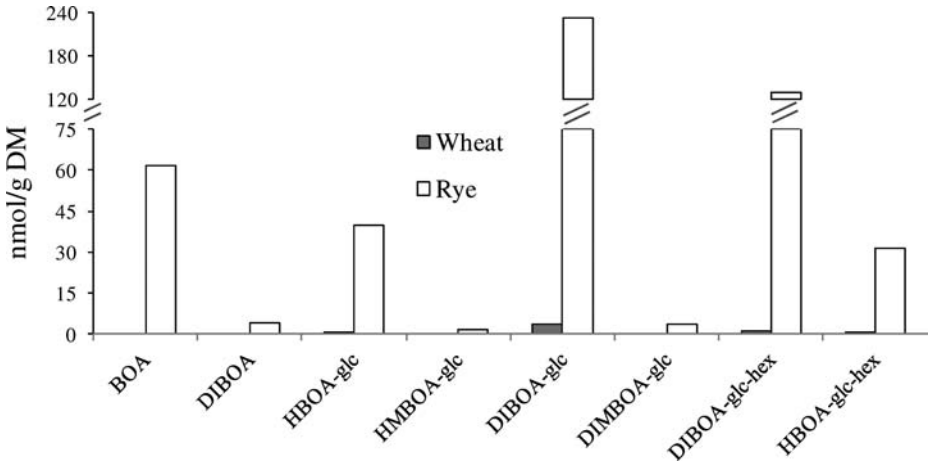


Figure 1. Concentration of benzoxazinoids in the experimental wheat and rye diets.

Standard Curves. The standard calibration curves for all compounds (except for DIBOA-glc-hex and HBOA-glc-hex) were prepared by serial dilutions of pure stock solutions in approximately 25% acetonitrile to quantify metabolites in the range of 0−800 ng/mL. The standard curves were applied to a linear function with a weight of $1/x$ because there were more data points at the lower part of the curve ($R > 0.99$). The peak areas were measured using Analyst, version 1.5.1, software from AB SCIEX. The standard mixtures A, B, C, and G (Table 3) were prepared individually to ensure that any transformation of individual compounds would be detected. Because of the unavailability of pure standards for DIBOA-glc-hex and HBOA-glc-hex, a semiquantitative method was applied to quantify them in the diets and biofluids. The peak areas of these compounds were compared to the standard curves of their respective authentic monoglucosides (DIBOA-glc and HBOA-glc) applied to a quadratic function with a weight of $1/x$ ($R > 0.99$).

Extraction Efficiency of the Analytical Method. The plasma, urine, and feces of the wheat-fed pigs, which had been confirmed previously to not contain most of the metabolites, were used for the recovery studies. Each of the six repeated samples (0.5 mL plasma and urine, 0.1 g feces) was spiked with a low concentration of the standards near the lower limit of the calibration curve and extracted as described above. The samples were analyzed, and the recovery was reported as the mean value (Table 5). Recoveries and limits of detection and quantification were assessed following EURACHEM guidelines.

Statistical Analyses and Calculations. All statistical analyses were carried out using SAS for Windows (version 9.2; SAS Institute Inc., Cary, NC, USA). The level of significance was set at $P < 0.05$ for all analyses. The effects of diet and the site of blood collection on plasma concentrations of benzoxazinoids were examined by PROC MIXED repeated measurements.[43]

$$Y = \mu + \alpha_d + \omega_s + (\alpha\omega)_{ds} + (U)_i + \varepsilon_{dti}$$

where α_d denotes the effect of diet, ω_s is the effect of site, $(\alpha\omega)_{ds}$ is the interaction between diet and site, and i refers to an individual pig. The variance component $(U)_i$ $N(0,\omega^2)$ accounts for the fact that the repeated measurements were made on the same individual, thereby rendering these observations correlated, whereas the term ε_{dti} $N(0,\sigma^2)$ represents the unexplained random error.

The effects of diet on fecal, urine, and bile concentrations, fecal digestibility, and daily excretion in feces were subjected to one-way analyses using the PROC MIXED with the GROUP option to account for variance heterogeneity.[43]

The apparent digestibility of the individual dietary benzoxazinoids was calculated according to Lærke et al.[32] The results are reported as the least-squares means (LSMeans) with standard errors of the means (SEM). In the cases where the compounds were detected only in

Table 6. Concentration (nmol/L) of Benzoxazinoids in Plasma Collected 3 h Postprandially from Different Sites[a] of Pigs Fed Wheat or Rye Diets

site	diet	b	BOA	HBOA	HBOA-glc	HMBOA-glc	DIBOA-glc	DIBOA-glc-hex	HBOA-glc-hex	total
PV	rye	LSMean	83.4	100.2	1042.5	2.1	40.8	15.5	22.2	1320.3
		SEM	14.4	15.8	103.3	0.6	5.9	1.3	1.5	127.6
	wheat	LSMean			17.2		0.8	1.6	4.4	24.1
		SEM			103.3		5.9	1.3	1.5	127.6
HV	rye	LSMean	15.6	6.1	889.6	1.0	32.2	12.4	23.9	971.6
		SEM	3.9	2.9	39.2	0.3	3.8	1.5	2.1	44.2
	wheat	LSMean			15.1		0.8	1.6	12.4	29.9
		SEM			39.2		3.8	1.5	2.1	43.9
LA	rye	LSMean	12.3	1.9	724.7	1.2	17.3	7.5	13.1	778.0
		SEM	6.7	0.7	34.8	0.2	1.9	0.8	1.0	30.5
	wheat	LSMean	2.3		14.6		2.1	2.1	3.3	24.3
		SEM	6.7		34.8		1.9	0.8	1.0	30.5
JV	rye	LSMean	40.3	2.0	829.0	1.3	19.0	6.8	12.2	911.0
		SEM	6.6	0.5	45.7	0.3	2.0	0.6	0.6	48.8
	wheat	LSMean			14.4	0.2	0.7	1.4	2.4	19.2
		SEM			45.7	0.3	2.0	0.6	0.6	48.8
*P*diet			0.0016	0.0006	<0.0001	0.0146	<0.0001	<0.0001	<0.0001	<0.0001
P site			0.0017	0.0003	0.0019	NS	0.0002	0.0002	<0.0001	0.0006
P diet × site			0.0011	0.0003	0.002	NS	0.0007	0.0002	0.0007	0.0006

[a]portal vein, PV; hepatic vein, HV; lateral auricular artery, LA; jugular vein, JV. [b]The values are the least squares means (LSMean) with standard errors of the means; $n = 6$.

certain pigs in a group, the mean value was calculated based on all six pigs.

RESULTS AND DISCUSSION

Validation of Analytical Methods and Limits of Detection. Recovery was assessed following EURACHEM guidelines, and most of the compounds were recovered in the range of 70−120% except for a few compounds (Table 5). The extraction efficiency of benzoxazinoids from bread samples was in the range of 80−120% for all compounds except for DIMBOA.[11] The recovery estimates were not used to correct the data from the regular experiment. The limits of detection (L_D) and quantification (L_Q) were obtained by determining the standard deviation (S) of the spiked compounds in the blank samples of the replicate recovery experiments. Following EURACHEM guidelines, L_D was thus determined to be $3S$, while L_Q was set to $10S$. The detailed results from the recovery experiments, including the detection limits, are shown in Table 5. Not all of the examined benzoxazinoids and their derivatives had concentrations above the L_D, but the results were included nonetheless because quantitation with higher uncertainties than the p-value defined by EURACHEM are possible, and the error in the overall conclusion would be higher if such numbers were omitted than if they are included in the statistical evaluation.[44] The precision of the quantification was high, with relative standard deviations less than 10% in most of the cases.

Benzoxazinoid Composition in the Diets. The experimental diets were formulated to be similar in macronutrient and dietary fiber levels[42] but had varying benzoxazinoid levels and composition (Figure 1). Eight benzoxazinoid compounds were detected in the rye diet, while only 4 compounds were detected in the wheat diet. DIBOA-glc was the most dominant in the rye diet (232 nmol/g DM), accounting for 46% of the total benzoxazinoids, followed by DIBOA-glc-hex > BOA > HBOA-glc > HBOA-glc-hex > DIBOA > DIMBOA-glc > HMBOA-glc. The concentrations of the latter three compounds, however, were less than 1% of the total benzoxazinoid concentrations.

The total benzoxazinoid content in the wheat diet was 78-fold lower than that in the rye diet, the former containing only a modest amount of DIBOA-glc (3.93 nmol/g DM) followed by DIBOA-glc-hex > HBOA-glc > HBOA-glc-hex. Therefore, the results discussed below refer to the rye-fed pigs, unless otherwise specified. The experimental rye bread enriched with rye bran had a benzoxazinoid content (excluding DIBOA-glc-hex and HBOA-glc-hex) that was nearly two times lower than that previously found in ordinary rye bread made from the cultivar Picasso (573 nmol/g DM).[11] Although benzoxazinoids are known to be present in whole grain, bran extracts,[10,12] and embryo, it remains unclear in which portion the majority of the benzoxazinoid content is contained in the grain and warrants a detailed study. The different concentrations of benzoxazinoids in rye bread in the present study and in a previous study can partly be attributed to different cultivars used in bread making and partly to the bread preparation processes.[11]

Levels of Benzoxazinoids and Their Metabolites in Plasma and Bile. Interestingly, LC-MS/MS analyses revealed different levels and compositions of benzoxazinoids in the plasma 3 h postprandially, indicating that they are absorbed from the gut and metabolized. As a result of the higher dietary content, the plasma contents of all benzoxazinoid compounds in the rye-fed pigs were significantly ($P < 0.01$) higher than that in wheat-fed pigs (Table 6). HBOA-glc in the portal vein (1042 nmol/L), as well as in all other sites (hepatic vein, lateral auricular artery, and jugular vein), was the primary circulating benzoxazinoid, accounting for 80−93% of the total benzoxazinoid content in plasma obtained from all four sites of collection. Since no other HBOA-hex or DIBOA-hex was detected in plasma, we believe that part of plasma HBOA-glc was derived from dietary DIBOA-hex-hex, HBOA-hex-hex (which we therefore considered to be provisionally characterized as DIBOA-glc-hex and HBOA-glc-hex, respectively), and DIBOA-glc. Some flavonoid diglucosides are directly

absorbed in the intact glycoside form.[33] Contrarily, DIBOA-glc-hex and HBOA-glc-hex were deglycosylated (losing hexose unit) by intestinal metabolism since only a minor amount of each were detected in the portal blood (Table 6). The DIBOA-glc was apparently reduced to HBOA-glc and subsequently absorbed in the blood. However, it is not clear from this experiment whether the hydrolysis of the hexose unit is caused by cytosolic β-glycosidase or lactase phlorizin hydrolase (LPH) in the lumen or by intestinal microbes. In some cases, deglycosylation by small intestinal β-glucosidase is a critical step for absorption and metabolism[45] but not in others.[33] However, the catalytic efficiency for the hydrolysis of glucosides differs depending upon the position of the glucose attachment in the molecule.[36] DIBOA-glc, in which the glucose is attached to the hydroxyl group in the second position of the DIBOA, was apparently reduced into HBOA-glc without further hydrolysis (Table 6). To our knowledge, this type of conversion has not been previously demonstrated in benzoxazinoid degradation studies in soil or plants, in which hydroxamic glucosides are usually deglycosylated by β-glucosidase into their respective aglucones during the first transformation step.[25,46,47] It remains unclear how and where the reduction of DIBOA-glc to HBOA-glc takes place. There are several mechanisms of intestinal metabolism and absorption of different glucosidic phytochemicals that have been described; however, none of these studies describe a reduction.[48] Certain gastrointestinal microbes can reduce the N−OH group to N−H, at least in rats.[49] HBOA-glc may be actively absorbed in the intact form by SGLT1, as are some flavonoid glucosides.[33,34]

A minor fraction of DIBOA-glc-hex and DIBOA-glc did not undergo reduction; thus, it was still present in the portal blood (Table 6). It is possible that some glucosides might be involved both in interaction with SGLT1 and luminal hydrolysis[35] or partially escape the metabolic process in the intestine. Plant lignans can also escape microbial conversion in the intestine and can be absorbed directly.[32] HBOA, which was not detected in the diets, was another important circulating benzoxazinoid in the portal blood. Dietary DIBOA (1.12 nmol/g DM) (Figure 1) was also not detected in the plasma (except in the portal veins of two rye-fed pigs, 44.2 and 22.6 nmol/L), which suggests that it has undergone reduction to HBOA. This finding is in accordance with the speculated mechanism of reduction of DIBOA into HBOA, as suggested by Krogh et al.[3] Alternatively, HBOA could have been formed by deglucosylation of its dietary glucoside. Certain flavonoid and isoflavone glucosides have been shown to be hydrolyzed by intestinal microbes[50] or β-glucosidase at the intestinal brush border and are passively absorbed.[36] A recent study using a rat model demonstrated that the deglycosylation of some flavonoids starts in the stomach, with major absorption occurring in the duodenum, independently of metabolism by colonic microflora.[51]

MBOA (not present in the diet) was detected in the plasma from the portal veins of 2 rye-fed pigs (7.8 and 6 nmol/L) out of 6. MBOA could have been formed via the methylation of BOA by intestinal microbes or an enterocyte-based methyltransferase or via enzymatic cleavage of the glucosidic bond in dietary DIMBOA-glc, followed by rapid hydrolysis to MBOA. The detection of DIBOA in the portal blood (from the same two pigs in which MBOA was found) might have some biological significance because of its anticancer properties.[15,16]

We surmised that dietary DIMBOA-glc (Figure 1) was reduced to HMBOA-glc. Therefore, similar to the DIBOA-glc → HBOA-glc mechanism, reduction was the main mechanism of conversion. In aphids, however, DIMBOA-glc can be passively absorbed and excreted without being metabolized.[38] Overall, a substantial portion of the hydroxamic acid benzoxazinoids suffered N-reduction to lactams before subsequent absorption. The overall concentration of individual benzoxazinoids was highest in the portal vein, while the levels of benzoxazolinones (BOA and HBOA) were drastically reduced in the hepatic blood compared to the portal blood (Table 6). This observation indicates that a substantial portion of the benzoxazinoids entered the entero-hepatic circulation or is converted to other metabolites, similar to a previous report for lignans.[32] The circulation of BOA (12 to 83 nmol/L) in blood could be important because of its potential healthful effects.[13]

BOA and HBOA can be biologically transformed into APO in soil, which is considered to be a detoxification process.[3,28] APO was discovered as a new antibiotic compound in 1960 and was named Questiomycin-B.[18] Therefore, we hypothesize that BOA and HBOA, which are highly bioactive,[13,14] are converted to APO in the liver of the rye-fed pigs by a similar detoxification mechanism prior to re-excretion into the intestine through the biliary route (Figure 2). However, the bile of wheat-fed pigs

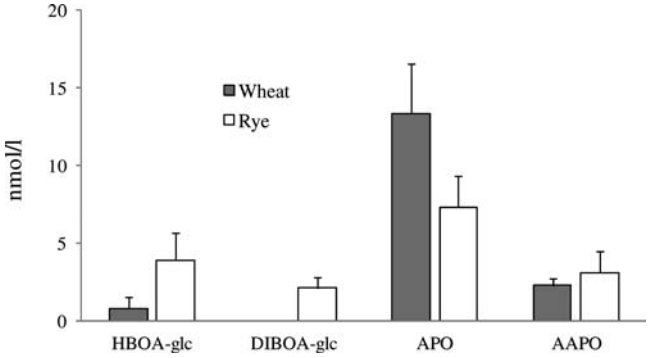


Figure 2. Concentration of benzoxazinoids and their transformation products in bile obtained 3 h postprandially from the gall bladder of pigs fed the wheat and rye diets.

contained higher concentrations of APO (13 nmol/L) than the rye-fed pigs (7 nmol/L), indicating that a completely unknown origin for the formation of APO exists in wheat-fed pigs. Compounds other than the benzoxazinoids reported in this study could be present only in the wheat or in both diets that could lead to the formation of APO. However, it is obvious that BOA and HBOA could be detoxified in the liver by an unknown mechanism because the reduction in their concentrations from the portal vein to the hepatic vein is substantial.

Various flavonoid and isoflavonoid glycosides are deglycosylated by liver β-glucosidase.[52] In the present study, this mechanism was not clearly detected by the liver metabolism of benzoxazinoids because the concentrations of glucosylated benzoxazinoids were not significantly different in the portal vein and the hepatic vein (Table 6). The presence of HBOA-glc (4 nmol/g) and DIBOA-glc (2.1 nmol/L) in the bile (Figure 2) could represent their systemic elimination due to their higher proportions in the portal blood compared with other blood vessels, as suggested by Lærke et al.[32] for plant lignans. Their highly polar nature also contributes to their elimination. It has previously been shown that compounds containing two or more rings, a highly polar anionic group, and a minimum

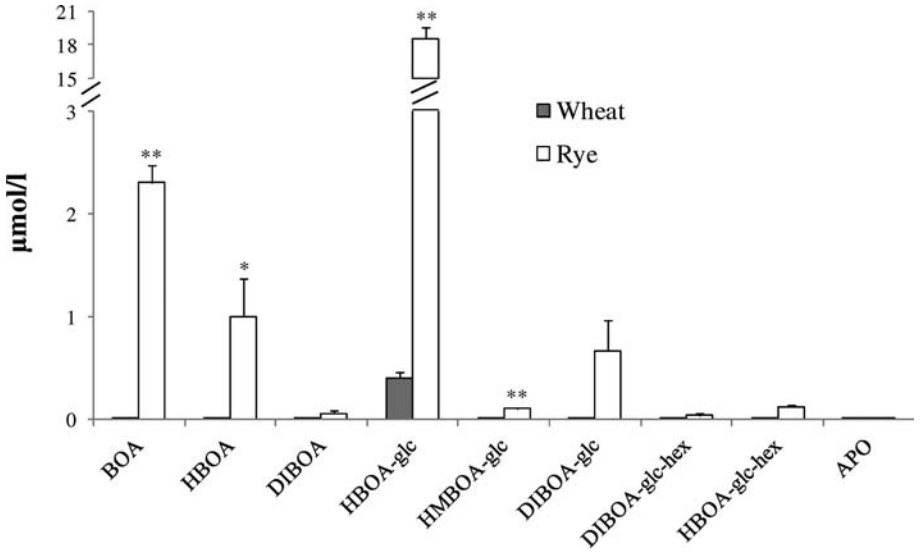


Figure 3. Concentration of benzoxazinoids and their transformation products in urine collected during a 7 day balance period from pigs fed the wheat or rye diets.

Table 7. Intake, Fecal Excretion, and Digestibility of Benzoxazinoids, and Their Transformation Products from Pigs Fed the Experimental Wheat or Rye Diets[a]

	intake (µmol/d)		excretion (µmol/d)				digestibility			
			wheat		rye		wheat		rye	
	wheat	rye	LSMean	SEM	LSMean	SEM	LSMean	SEM	LSMean	SEM
BOA	-	125.30	-		0.94	0.43			0.99	0.0034
HBOA-glc	1.57	81.07	-		0.29	0.03	1		0.99	0.0008
HMBOA-glc	-	3.18	-		-				1	
DIBOA	-	9.04	-		0.21	0.13			0.97	0.0181
DIBOA-glc	7.82	473.63	0.02	0.01	0.19	0.09	0.99	0.0013	0.99	0.0001
DIMBOA-glc	-	7.79	-		-				1	
DIBOA-glc-hex	2.32	263.13	0.05	0.00	0.14	0.06	0.97	0.0009	0.99	0.0002
HBOA-glc-hex	1.11	63.89	0.06	0.01	0.1*	0.01	0.94	0.0095	0.99	0.0001
HBOA	-	-	-		4.8	1.16				
APO	-	-	-		0.31	0.1				
AAPO	-	-	0.01	0.01	0.03	0.01				
total benzoxazinoids	12.82	1027.03	0.14	0.02	7.01**	0.73	0.98	0.0032	0.99	0.0013

[a]The values are the least squares means (LSMean) with standard errors of the means; $n = 6$. -, not detectable. *$P < 0.05$ and **$P < 0.001$ compared with the wheat diet.

molecular weight of 325 kDa are effectively excreted.[53] Alternatively, HBOA is biologically active[14] and may be selectively taken up by the liver cells and partly excreted into the bile (Figure 2) in a less bioactive form after glycosylation (HBOA-glc) and oxidation/hydroxylation (DIBOA-glc).

Benzoxazinoids and Their Metabolites in Urine. Benzoxazinoids were excreted as urinary metabolites in a manner that is similar to that of other dietary phytochemicals.[32,54−56] The concentration of total benzoxazinoids in urine collected over a 7 days period was significantly higher in rye-fed pigs compared to that of wheat-fed pigs, as expected by dietary intake (Figure 3). Hence, there was a much higher concentration of HBOA-glc in rye-fed pigs (18.6 µmol/L) compared with that in wheat-fed pigs (0.4 µmol/L). The concentrations of urinary benzoxazinoids and their transformation products in rye-fed pigs were determined to be in the following order: HBOA-glc > BOA > HBOA > DIBOA-glc > HBOA-glc-hex > HMBOA-glc > DIBOA > DIBOA-glc-hex > APO. HMBOA and very little AAPO were detected in two of the rye-fed pigs. However, compared to their blood concentrations, proportionally higher urinary concentrations were detected for BOA, HBOA, and HMBOA-glc than for other metabolites (Table 6 and Figure 3). These results indicated that HBOA-glc was partly *O*-methoxylated and partly deglucosylated in the kidney, similar to the renal metabolism of isoproterenol[57] and aminopyrine.[58] Likewise, DIBOA was not present in the arteries and jugular veins, but it was detected in the urine as a renal deglycosylated product of DIBOA-glc or DIBOA-glc-hex (Table 6 and Figure 3). Alternatively, there could be oxidative hydroxylation of HBOA, thereby transforming it to DIBOA, as catalyzed by the cytochrome P450 systems.[59] The renal metabolism of benzoxazinoids was also supported by the detection of urinary APO, a benzoxazinoid transformation product.

Fecal Excretion of Benzoxazinoids and Their Metabolites. The higher concentration of benzoxazinoids in the rye diet was reflected in a 50-fold higher total fecal excretion of benzoxazinoids compared with the wheat diet (Table 7). Fecal

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HBOA, despite its absence in the diet, accounted for 68% of the total benzoxazinoid metabolites, followed by BOA > APO > HBOA-glc > DIBOA > DIBOA-glc > DIBOA-glc-hex > HBOA-glc-hex > AAPO. A substantial part of dietary DIBOA, which was not directly absorbed in the intestine (DIBOA was not detected in blood, except in two pigs), may have been reduced to HBOA by intestinal microbes and excreted through the feces. Alternatively, it could be a deglycosylated product of HBOA-glc originating from the diet or the entero-hepatic circulation. Some flavonoid glucosides are deglycosylated in the intestinal brush border by β-glucosidase[36] or intestinal microbes.[50] Fecal DIBOA was detected in 2 of the rye-fed pigs, and BOA was detected in 3 of the 6 rye-fed pigs. The feces of 3 wheat-fed pigs contained only DIBOA-glc and AAPO, and none of the studied compounds was detected in the feces of the 3 other wheat-fed pigs. Small amounts of APO and AAPO were detected as benzoxazinoid transformation products in the feces of rye-fed pigs, which could have originated from the entero-hepatic circulation or by microbial breakdown of BOA. In soil, BOA is microbially degraded into APO and acetylated to AAPO.[27] However, biliary APO of wheat-fed pigs was not detected in the feces, indicating its microbial breakdown in the intestine.

In conclusion, the present study provides the first evidence that dietary benzoxazinoids are absorbed and metabolized in pigs. Possible mechanisms of benzoxazinoids metabolism that were discussed that merit closer examination include previously unreported routes of transformation such as reduction and the regular transformation processes such as oxidation, demethoxylation, and deglycosylation. Certain benzoxazinoids circulated in the bloodstream and were excreted in various forms via the bile and particularly in the urine. This evidence of the bioavailability of benzoxazinoids in vivo may support some biological activities in animals as reported in some previous studies. Our results supplement the healthful effects of other secondary metabolites, such as phenolic acids and lignans, in whole grain diets. Further studies are needed to elucidate the transformation and metabolic pathways of dietary benzoxazinoids. The unequivocal metabolic pathway could be established with experiments in which ^{13}C-labeled individual compounds are administered separately, or individual compounds are fed along with the blank diet. A more feasible and rapid method for assessing transformation is the microsomal metabolism of the individual benzoxazinoids in vitro. The extent to which these benzoxazinoids accumulate in the tissue deserves further research to fully understand their possible pharmacological usage. The results from our ongoing research concerning the transformation of specific benzoxazinoids by microsomes in vitro and the bioavailability of benzoxazinoids in rats and humans will be published in the near future.

AUTHOR INFORMATION

Corresponding Author

*Tel: +45 87158212. Fax: +45 87156082. E-mail: inge.fomsgaard@agrsci.dk.

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Notes

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ABBREVIATIONS USED

AP, 2-aminophenol; BOA, 2-benzoxazolinone; MBOA, 6-methoxy-benzoxazolin-2-one; HBOA, 2-hydroxy-1,4-benzoxazin-3-one; HMBOA, 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one; HBOA-glc, 2-β-D-glucopyranosyloxy-1,4-benzoxazin-3-one; HMBOA-glc, 2-β-D-glucopyranosyloxy-7-methoxy-1,4-benzoxazin-3-one; HBOA-glc-hex, double-hexose derivative of HBOA; DIBOA, 2,4-dihydroxy-1,4-benzoxazin-3-one; DIMBOA, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one; DIBOA-glc, 2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one; DIMBOA-glc, 2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one; DIBOA-glc-hex, double-hexose derivative of DIBOA; APO, 2-amino-3*H*-phenoxazin-3-one; AMPO, 2-amino-7-methoxy-3*H*-phenoxazin-3-one; AAPO, 2-acetylamino-3*H*-phenoxazin-3-one; AAMPO, 2-acetylamino-7-methoxy-3*H*-phenoxazin-3-one; PV, portal vein; HV, hepatic vein; LA, lateral auricular artery; JV, jugular vein

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