Insight into the Distribution of Arabinoxylans, Endoxylanases, and Endoxylanase Inhibitors in Industrial Wheat Roller Mill Streams

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To gain insight into the distribution of arabinoxylans (AX), endoxylanases, and endoxylanase inhibitors in industrial wheat roller milling, all streams, that is, 54 flour fractions, 4 bran fractions, and the germ, were analyzed for ash, starch, and protein contents, α-amylase activity levels, total (TOT-AX) and water-extractable arabinoxylan (WE-AX) contents, endoxylanase activity levels, and endoxylanase inhibitor (TAXI and XIP) contents. In general, bran fractions were significantly richer in TOT-AX and WE-AX contents, endoxylanase activity levels, and endoxylanase inhibitor contents than germ and, even more so, than flour fractions. In the 54 different flour fractions, minimal and maximal values for TOT-AX and WE-AX contents differed by ca. 2-fold, whereas they differed by ca. 15-fold for endoxylanase activity levels. The latter were positively correlated with ash and negatively correlated with starch content, suggesting that the endoxylanase activity in flour is strongly influenced by the level of bran contamination. TAXI contents in the flour fractions varied ca. 4-fold and were strongly correlated with bran-related parameters such as ash content and enzyme activity levels, whereas XIP contents varied ca. 3-fold and were not correlated with any of the parameters measured in this study. The results can be valuable in blending and optimizing wheat flour fractions to obtain flours with specific technological and nutritional benefits.

KEYWORDS: Wheat; milling; arabinoxylan; endoxylanase; endoxylanase inhibitor

INTRODUCTION

A large part of the wheat produced worldwide is converted into flour by dry wheat roller milling as an intermediate step in the processing of the wheat into bread, pastry, cookies, starch, gluten, etc. Roller milling separates the starchy rich endosperm material from the outer bran layers of the wheat kernels. In short, it is a process of grinding and separating. Wheat kernels are ground on a series of successive milling rolls, either corrugated or smooth, to break up the kernels and reduce the particle size. The resulting endosperm particles are subsequently separated from the bran and germ particles using plansifters and purifiers. At the end of the milling process, approximately 75% of the wheat is recovered as flour. The byproducts of the milling process, called millfeeds, are often used as animal feed. Wheat germ, which theoretically represents 2−3% of the wheat kernel, is a more valuable milling byproduct as it contains high levels of vitamins, lipids, and proteins and is often used for human consumption (*1*).

Milling fractions contain endosperm, aleurone, pericarp, seed coat, and germ material in different proportions. As wheat components are not uniformly distributed over the wheat kernel, the obtained milling fractions differ in composition as well as

in functional properties. The miller can combine all flour streams to produce straight-run flour. The quality of such flour depends not only on the milling process itself but also on the wheat selected for milling. To give the miller more flexibility in selecting wheat varieties and hence in minimizing production costs, a technique called split-run milling or divide milling is often used. Using this technique, different final flours can be produced by blending certain selected flour streams from the mill (*2*, *3*). In this way, it is easier to fulfill customers' specifications. To ensure that the resulting flour is of high quality, it is important to know the composition of the different mill streams that are selected for blending and the functionality of the different components.

The distribution of ash, starch, protein, and α-amylases over different wheat roller mill streams has been investigated quite extensively (*2*, *4−6*), but less information is available about the distribution of arabinoxylans (AX) (*7−9*), which are the predominant cell wall non-starch polysaccharides of the wheat kernel. It is, however, well-established that AX have a major impact on the functionality of wheat in biotechnological processes and applications such as breadmaking, gluten−starch separation, and refrigerated doughs due to their unique physicochemical properties (*10*). In addition, it becomes more and more clear that AX can have an important nutritional value as soluble and insoluble dietary fiber. Even less is known about the distribution of wheat-associated endoxylanases, the most

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important AX-hydrolyzing enzymes, and wheat proteinaceous endoxylanase inhibitors, which can inhibit the majority of microbial endoxylanases, in industrial wheat mill streams, in spite of the well-established impact and use of endoxylanases from microbial origin in breadmaking (*10*, *11*), gluten−starch separation (*12*), and animal feeds (*13*).

Therefore, the purpose of this study was to gain insight into the distribution of AX, endoxylanases, and endoxylanase inhibitors over the wheat mill streams and their correlation with ash, starch, and protein contents and α-amylase activity levels. Principal component analysis (PCA) was used as a tool to uncover the principal mechanisms underlying the distribution patterns.

EXPERIMENTAL PROCEDURES

Materials. All chemicals, bovine serum albumin (BSA), and reagents were of at least analytical grade and supplied by Sigma-Aldrich (Bornem, Belgium) unless specified otherwise. *Bacillus subtilis* glycoside hydrolase family (GHF) 11 endoxylanase (Grindamyl H640, Swissprot Accession no. P18429) was from Danisco (Brabrand, Denmark), and *Penicillium purpurogenum* GHF 10 endoxylanase (Swissprot Accession no. Q9P8J1) was kindly made available by Prof. Jaime Eyzaguirre (Laboratorio de Bioquimica, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile). Azurine-cross-linked (AZCL) arabinoxylan and amylose tablets were purchased from Megazyme (Bray, Ireland).

Milling. Wheat, consisting of 50% Belgian, 35% German, and 15% French wheat varieties (harvest 2004), was milled with an industrial roller mill (Ceres-Soufflet, Brussels, Belgium) to an extraction rate (ER) of 74.9%. Protein and ash contents of the wheat used were 12.4 and 1.64%, respectively (methods described below). The industrial roller mill had five break rolls, four coarse reduction rolls, eight fine reduction rolls, and two bran finishers. Several rolls were equipped with semolina graders to increase the capacity of the mill. Semolina grader 1 increased the capacity of the first two break rolls, whereas semolina grader 2 received material from the fourth break roll. Semolina grader 3 was supplied with material from the third coarse reduction roll and semolina grader 4 with material from the bran finishers. The obtained wheat mill streams consisted of 20 break flour fractions, 27 coarse reduction flour fractions, 14 fine reduction flour fractions, 4 bran finisher flour fractions, 4 bran fractions, and a germ fraction. Of these 70 mill streams, 11 streams could not be analyzed as they provided only very little material (in total, <1.5%).

Break flour 1−2, semolina grader flour 1, coarse reduction flour 1−2, and fine reduction flour 1−2 fractions were considered to be of first quality, whereas break flour 3, semolina grader flours 2 and 3, coarse reduction flour 3, and fine reduction flour 3−4 fractions were of second quality and break flour 4−5, semolina grader flour 4, coarse reduction flour 4−5, fine reduction flours 5−6 and 7−8, and bran finisher flour fractions were of third quality. The quality specification was based on baking trials for this specific milling setup.

Methods. *Standard Analyses.* Moisture and ash contents were measured in duplicate according to the AACC methods 44-15a and 08-21, respectively (*14*). The coefficients of variation for the determination of moisture and ash contents were typically 1%. Protein contents were determined in triplicate using the Dumas combustion method, an adaptation of the AOAC Official Method (*15*) to an automated Dumas protein analysis system (EAS, varioMax N/CN, Elt, Gouda, The Netherlands), using 5.7 as a factor for conversion from nitrogen to protein content. The coefficient of variation for the determination of protein contents was typically 3%. Ash and protein contents were expressed on a dry matter basis.

Analysis of Noncellulosic Carbohydrate Composition and Content. Noncellulosic carbohydrate composition and content of milling fractions and aqueous extracts thereof were determined in triplicate and in quadruplicate, respectively, by gas−liquid chromatography. Aqueous extracts were prepared in duplicate by suspending 1.0 g of the milling fractions in 10 mL of deionized water and shaking the suspensions for 30 min (Laboshake, VWR International, Leuven, Belgium) at 7 °C to minimize enzymic breakdown of AX during extraction. The samples were subsequently centrifuged (10 min, 10000*g*, 7 °C, Beckman J2-21 centrifuge, Fullerton, CA) and filtered (MN 615 filter, Macherey-Nagel, Düren, Germany). The milling fractions themselves (10−15 mg) were hydrolyzed with 2.0 M trifluoroacetic acid (TFA) (5.0 mL) and the aqueous extracts (2.5 mL) with 4.0 M TFA (2.5 mL) for 60 min at 110 °C. Reduction with sodium borohydride and acetylation with acetic acid anhydride of the obtained monosaccharides were executed according to the procedure of Englyst and Cummings (*16*). The formed alditol acetates (1.0 μL) were separated on a Supelco SP-2380 polar column (30 m length, 0.32 mm internal diameter, 0.2 μm film thickness) (Supelco, Bellefonte, PA) in an Agilent chromatograph (Agilent 6890 series, Wilmington, DE) equipped with an autosampler, a splitter injection port (split ratio of 1:20), and a flame ionization detector. The carrier gas was helium. Separation was at 225 °C, whereas the injection and detection were at 270 °C. AX content was calculated as 0.88 times the sum of xylose and arabinose contents, after correction of the arabinose content for the presence of arabinogalactan-peptide (*17*). The coefficient of variation for the determination of both WE-AX and TOT-AX, consisting of both WE-AX and water-unextractable AX (WU-AX), was typically 2%. Starch content was calculated as 0.90 times glucose content. Calculations were not corrected for β-glucan, as the levels of β-glucan, ca. 0.67% (*18*), in wheat are very low compared to starch. The coefficient of variation for the determination of starch contents was typically 1%. AX and starch contents were expressed on a dry matter basis.

Analysis of α-Amylase Activity Levels. α-Amylase activity levels were determined in triplicate with the Amylazyme method (Megazyme). Samples (2.0 g) were suspended in sodium maleate buffer (10.0 mL, 100 mM, pH 6.0) containing 5 mM CaCl₂. These suspensions were shaken for 30 min at room temperature, centrifuged for 10 min at 10000*g* in a Beckman J2-21 centrifuge, and filtered through an MN 615 filter. Extracts (1.0 mL) were equilibrated for 10 min at 40 °C before the addition of an AZCL-amylose tablet. The reaction was stopped after 30 min by adding 10.0 mL of TRIS solution (2% w/v) and by vigorous vortex stirring. After filtration through an MN 615 filter, the extinction of the filtrates at 590 nm (E_{590}) [Ultraspec III UV−visible spectrophotometer (Pharmacia Biotech, Uppsala, Sweden)] was measured against a control, prepared by incubating the extracts without the AZCL-amylose tablet. Extinction values were corrected for nonenzymic color release by the AZCL-amylose tablets. Activities were expressed in amylase units (AU) per gram. One AU is the amount of enzyme needed to increase the E_{590} value by 1.0 per 60 min of incubation, under the conditions of the assay. The coefficient of variation for the determination of α-amylase activity levels was typically 3%.

Analysis of Endoxylanase Activity Levels. Endoxylanase activity levels were determined in triplicate with the Xylazyme AX method (Megazyme). Samples (2.0 g) were suspended in sodium acetate buffer (10.0 mL, 25 mM, pH 5.0) and shaken for 30 min at room temperature. After centrifugation for 10 min at 10000*g* in a Beckman J2-21 centrifuge, the suspensions were filtered through an MN 615 filter. Extracts (1.0 mL) were equilibrated for 10 min at 40 °C before the addition of an AZCL-AX tablet. After appropriate incubation times, the reaction was stopped by adding 10.0 mL of 1.0% (w/v) TRIS solution and by vigorous vortex stirring. After 10 min at room temperature, the solutions were filtered and the extinction values at 590 nm (E_{590}) (Ultraspec III UV−visible spectrophotometer) were measured against a control, prepared by incubating the extracts without the AZCL-AX tablet. Correction was made for nonenzymic color release by the AZCL-AX tablets. Activity levels were expressed in enzyme units (EU) per gram. One EU is the amount of endoxylanase needed to yield an E_{590} value of 1.0 per 60 min of incubation, under the conditions of the assay. In view of the results by Dornez et al. (*19*) it is clear that the endoxylanase activity levels measured by this assay must be regarded as apparent activity levels because of the presence of endoxylanase inhibitors in the milling fractions and the inhibition of a variable proportion of wheat-associated microbial endoxylanases during aqueous extraction by these proteins. The coefficient of variation for the determination of endoxylanase activity levels was typically 1%.

Analysis of Endoxylanase Inhibitor Contents. Endoxylanase inhibitor

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Table 1. Calculated Weight Average Values for Yield, Ash, Starch, and Protein Contents, α-Amylase Activity Levels, TOT-AX and WE-AX Contents, Endoxylanase Activity Levels, and TAXI and XIP Contents in Flour Fractions from the First to the Later Passages for Each Milling Step, Bran Fractions, and Germ. Breadmaking Quality of the Flour Is Also Indicated

		yield (%)	ash (%)	starch (%)	protein (%)	α-amylase (AU/g)	TOT-AX (%)	WE-AX (%)	endo-xylanase (EU/g)	TAXI (ppm)	XIP (ppm)	quality
break rolls	1–2	8.44	0.53	80.2	11.1	2.19	1.96	0.41	0.05	65	225	first
	3	3.27	0.60	78.0	13.3	1.72	2.04	0.37	0.05	72	237	second
	4	1.34	0.87	73.7	16.2	1.97	2.40	0.36	0.07	86	236	third
	5	0.44	1.16	71.4	16.8	2.14	2.53	0.35	0.11	97	151	third
	all	13.49	0.60	78.7	12.3	2.05	2.04	0.39	0.06	70	227	
coarse reduction rolls	1	19.36	0.39	81.7	10.3	1.18	1.95	0.42	0.02	60	227	first
	2	8.02	0.44	81.2	9.8	1.16	1.83	0.44	0.02	61	223	first
	3	3.06	0.58	79.8	11.1	1.59	2.20	0.46	0.04	75	194	second
	4	0.55	0.83	77.4	10.9	1.99	2.43	0.39	0.09	82	248	third
	all	30.99	0.43	81.3	10.2	1.23	1.95	0.43	0.02	62	223	
fine reduction rolls	1–2	17.62	0.43	80.8	10.6	1.20	2.19	0.48	0.02	66	201	first
	3–4	3.78	0.52	80.1	10.8	1.44	2.38	0.54	0.03	78	181	second
	5–6	1.37	0.75	77.2	12.6	1.98	2.90	0.51	0.07	88	195	third
	7–8	1.68	1.02	74.2	12.6	2.22	3.38	0.53	0.10	139	269	third
	all	24.46	0.51	80.0	10.9	1.35	2.34	0.50	0.03	74	202	
bran finishers	1	2.44	0.66	77.4	12.6	2.44	2.29	0.42	0.09	74	238	third
	2	1.28	1.22	71.5	13.8	2.81	2.82	0.38	0.15	94	276	third
	all	4.49	0.87	75.2	13.2	2.57	2.53	0.40	0.11	83	246	
bran fractions	CW1[a]	2.49	4.85	31.3	18.4	8.19	18.40	0.90	0.59	335	603	
	FW[a]	3.45	2.76	54.0	16.0	6.68	10.77	1.01	0.43	238	501	
	CW2	10.15	5.30	25.5	17.5	6.85	22.45	0.66	0.63	205	413	
	bran	7.86	6.16	24.9	16.7	8.11	24.48	0.62	0.43	175	339	
germ		0.09	4.67	28.2	34.2	1.83	5.61	0.37	0.14	91	336	

[a] CW, coarse weatings; FW, fine weatings.

activities were measured in triplicate in the sodium acetate extracts described above with a variant of the Xylazyme AX method. *Triticum aestivum* xylanase inhibitor (TAXI) and xylanase-inhibiting protein (XIP) activities were measured with *Bacillus subtilis* and *Penicillium purpurogenum* endoxylanases, respectively, as these enzymes are inhibited by only TAXI and XIP, respectively (*20*, *21*). The *B. subtilis* and *P. purpurogenum* enzyme solutions (2.0 EU/mL) were prepared in sodium acetate buffer (25 mM, pH 5.0) containing BSA (0.5 g/L). The extracts were diluted to ensure a linear response between the concentration of inhibitor and the inhibition activity measured with the assay below.

Endoxylanase solutions (0.5 mL) were preincubated for 30 min at room temperature with an equal volume of buffer (reference) or (diluted) sample to allow formation of enzyme−inhibitor complexes in the latter case. After preincubation, the mixtures were equilibrated for 10 min at 30 °C. An AZCL-AX tablet was then added. The reaction was stopped after 60 min of incubation by adding 1.0% (w/v) TRIS solution (10.0 mL) and vortex mixing. After 10 min at room temperature, the tubes were shaken vigorously and the contents were filtered. The E_{590} values of the reference and the sample were measured against controls, prepared by incubation with buffer instead of enzyme solution. Although both controls allowed for correction for nonenzymic color release by the AZCL-AX tablets, the control of the sample allowed for correction for the presence of endoxylanase activity. The percentage inhibition was calculated from the difference between the E_{590} values of the reference and the sample. The coefficient of variation for the determination of both TAXI- and XIP-type inhibitor contents was typically 6%.

For conversion of endoxylanase inhibitor activities into inhibitor contents, dose−response curves with pure inhibitors, prepared as described first by Bonnin et al. (*22*), were determined, allowing expression of the endoxylanase inhibitor contents in wheat in parts per million (ppm). Due to the presence of endoxylanases in the milling fractions, the inhibitor contents must be theoretically regarded as apparent contents. However, as endoxylanase inhibitors occur in much higher concentrations than endoxylanases in wheat, the difference between apparent and total endoxylanase inhibitor contents is negligible (*19*).

Statistical Analysis. The variations in the composition of the different mill streams were examined by PCA with the Statistical Analysis System software 8.1 (SAS Institute, Cary, NC). PCA allows a large number of observable variables to be reduced to a few orthogonal factors or principal components (PC). These PC describe the greatest covariance in the data analyzed and often represent underlying principles that cannot be measured directly (*23*). Pearson's correlation coefficients analyses were also performed with the Statistical Analysis System software 8.1.

RESULTS AND DISCUSSION

Differences in Composition of Germ, Flour, and Bran Fractions. Table 1 shows the evolution of the different parameters going from head-end to tail-end streams. The represented values are calculated weight averages of the different flour fractions. **Table 1** also shows the yield and composition of bran and germ fractions. In **Figure 1**, all of the different parameters measured are cumulatively plotted as a function of ER. These cumulative curves were obtained by classifying all of the wheat mill streams in ascending order of ash content, multiplying the amount of the parameter measured by the yield of that specific passage, and finally adding up those values fraction per fraction. It is well-established that the composition of the flour fractions gradually changes from head-end to tail-end milling fractions.

Ash and Starch Contents. For ash content, a cumulative plot as a function of ER is often constructed to evaluate the efficiency of the milling process. Ash content is a well-established index of flour refinement as it is predominantly present in the outer layers of the wheat kernel (*24*). In the present case, ash content varied from 2.76 to 6.16% in bran fractions (**Table 1**) and from 0.32 to 1.22% in flour fractions (results not shown). The germ fraction also had a high ash content (4.67%) (**Table 1**). In a good milling process, the curve of ash content plotted cumulatively as a function of ER should remain horizontal and as
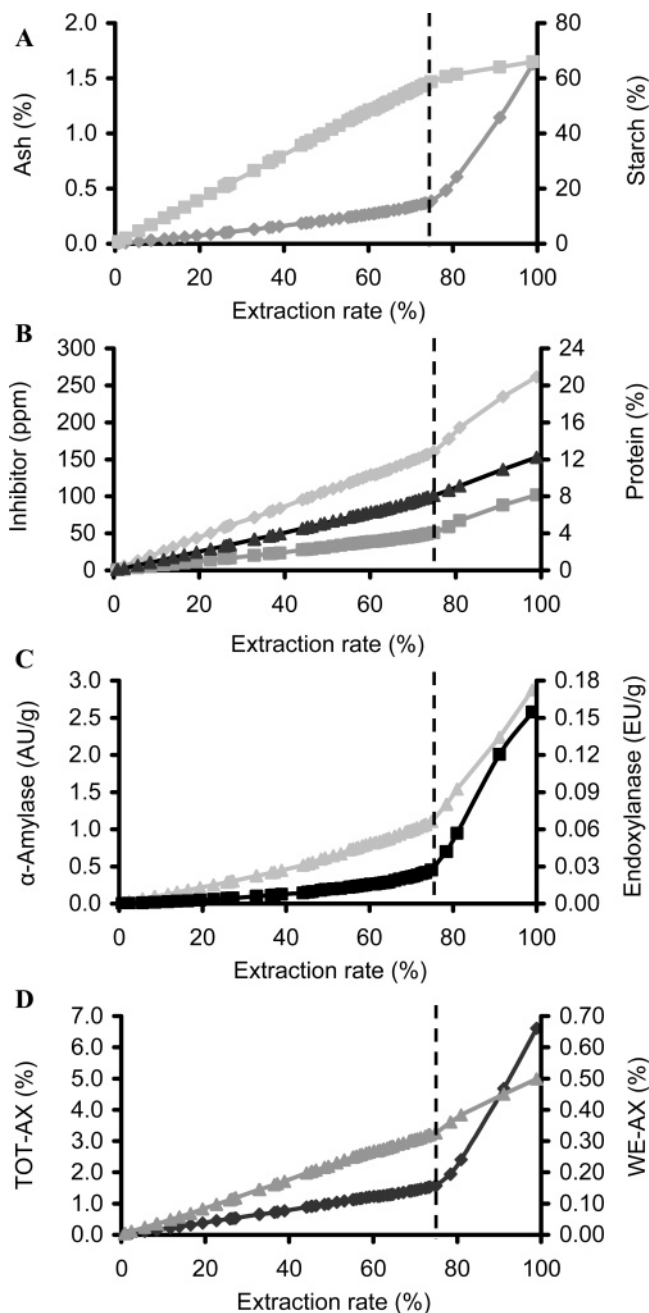
Figure 1. Cumulative ash (gray diamond) and starch (gray square) contents (**A**), cumulative protein (black triangle), TAXI (gray square), and XIP (gray diamond) contents (**B**), cumulative α-amylase (gray triangle) and endoxylanase (black square) activity levels (**C**), and cumulative TOT-AX (black diamond) and WE-AX (gray triangle) contents (**D**) as a function of extraction rate (ER). The dotted line at an ER of 75% represents straight-run flour.

low as possible up to the point of approximately 75−80% as this indicates a low bran contamination of the wheat flour (*2*). From **Figure 1A** it is clear that ash increased slowly with increasing ER up to approximately 75%, above which the increase in ash content was much stronger. Ash content in wholemeal was about 3.3 times higher than in flour (**Table 2**).

In contrast to ash, starch is known to be mainly located in the central part of the wheat kernel, more specifically in the starchy rich endosperm. Indeed, starch contents in flour fractions were around 80%, whereas germ and bran fractions had starch contents of approximately 30% (**Table 1**). Starch content hence gradually increased with increasing ER, but the curve leveled off above 75% (**Figure 1A**). Starch content in flour was 1.2 times higher than in wholemeal (**Table 2**).

From **Table 1**, it is also obvious that ash contents always increased, whereas starch contents always decreased going from head-end to tail-end flour fractions. The lower ash values of the head-end streams are due to the extraction of central endosperm with a minimum contamination of bran and germ, whereas the tail-end streams contain more peripheral endosperm and are contaminated with aleurone and pericarp material with high ash contents (*2*).

Protein Contents. Protein contents ranged from 9.3 to 16.8% in the flour (results not shown) and from 16.0 to 18.4% in the bran fractions (**Table 1**). The germ fraction had an extremely high protein content of 34.2% (**Table 1**). Protein contents increased linearly with increasing ER up to 75% (**Figure 1B**). Thereafter, the increase became stronger, but was much less outspoken than the increase observed for ash content. The protein contents in wholemeal and flour varied by only a factor 1.1 (**Table 2**). Protein contents increased from head-end to tail-end fractions, but for coarse reduction rolls, no trend could be observed (**Table 1**). This is in agreement with the results obtained by Prabhasankar et al. (*4*), who also reported an increase in protein content with increasing sequence number of the rolls for break rolls, but who were unable to find a trend for reduction rolls. In contrast, Gebruers et al. (*25*) found an increase in protein content from the first to the later passages for both break and reduction rolls. As the differences in protein content are much smaller for reduction than for break rolls, it is logical that the increasing trend is often not that clear for reduction rolls.

α*-Amylase Activity Levels.* α-Amylase activity levels ranged from 0.98 to 2.81 AU/g in flour (results not shown) and from 6.68 to 8.19 AU/g in bran fractions (**Table 1**). The α-amylase activity level in germ was 1.83 AU/g (**Table 1**). α-Amylase activity levels increased gradually with increasing ER below an ER of 75%, but still showed a sharp increase at an ER of 75% (**Figure 1C**). The similarity between α-amylase activity and ash distribution was previously reported (*5, 6*), and a moderate correlation between these parameters in mill stream

Table 2. Calculated Weight Average Values for Ash, Starch, and Protein Contents, α-Amylase Activity Levels, TOT-AX and WE-AX Contents, Endoxylanase Activity Levels, and TAXI and XIP Contents for the Different Qualities of Flour, Straight-Run Flour, Bran Material, and Wholemeal[a]

	yield (%)	ash (%)	starch (%)	protein (%)	α-amylase (AU/g)	TOT-AX (%)	WE-AX (%)	endoxylanase (EU/g)	TAXI (ppm)	XIP (ppm)
first-quality flour	53.45	0.43	81.1	10.4	1.34	2.01	0.44	0.03	63	218
second-quality flour	10.11	0.56	79.4	11.7	1.57	2.22	0.46	0.04	75	203
third-quality flour	9.87	0.89	75.1	13.5	2.30	2.70	0.43	0.10	94	237
straight-run flour	73.43[b]	0.51	80.0	11.0	1.50	2.13	0.44	0.04	69	218
bran material	23.95	5.17	30.0	11.2	7.38	21.01	0.72	0.53	213	421
wholemeal	97.47[b]	1.66	67.7	12.6	2.95	6.78	0.51	0.16	105	268

[a] First-quality flour consists of break flour 1–2, semolina grader flour 1, coarse reduction flour 1–2, and fine reduction flour 1–2. Second-quality flour consists of break flour 3, semolina grader flours 2 and 3, coarse reduction flour 3, and fine reduction flour 3–4. Third-quality flour consists of break flour 4–5, semolina grader flour 4, coarse reduction flour 4–5, fine reduction flour 5–8, and bran finisher flour. [b] Calculated yield is lower than the obtained yield due to missing fractions.

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flours was found (*6*). α-Amylase activity levels in wholemeal were 2.0 times higher than in flour (**Table 2**). Earlier studies indicated that α-amylase activity levels increase going from head-end to tail-end flour fractions (*5*, *6*). Gys et al. (*26*) found a reduction of α-amylase activity with more than 15% in wholemeal when wheat was debranned by only 7%. In the present study, this was indeed the case for reduction and bran finisher flour fractions, but not for break flour fractions (**Table 1**). Every et al. (*6*) also explained the higher α-amylase activity in the later flour fractions by contamination of the flour with small germ and/or bran particles and concluded that most α-amylase activity in flour originates from contamination with bran.

Arabinoxylan Contents. TOT-AX and WE-AX contents in flour fractions ranged from 1.79 to 3.98% and from 0.31 to 0.57%, respectively (results not shown), whereas the contents in the bran fractions ranged from 10.77 to 24.48% and from 0.62 to 1.01%, respectively (**Table 1**). The germ fraction had a TOT-AX content of 5.61% and a WE-AX content of 0.37% (**Table 1**). TOT-AX contents increased going from head-end to tail-end flour fractions, whereas WE-AX did not (**Table 1**). Water extractability of AX is hence lower in tail-end flour fractions. Ranges and trends are in good agreement with the results of Hartunian-Sowa (*7*), Delcour et al. (*8*), and Wang et al. (*9*), although, in the first study, WE-AX contents were not determined. In addition, TOT-AX content showed a sharp increase at an ER of 75%, whereas WE-AX did not (**Figure 1D**), again indicating that TOT-AX was much more concentrated in the outer wheat kernel layers than in the starchy endosperm and that the water extractability of the AX is significantly lower in the outer layers of the wheat kernel. This is in line with the findings of Maes and Delcour (*27*), who found that only 6% of the bran AX is water extractable. This is much lower than the 25−30% water extractability of flour AX (*28*). These findings also confirm the results of Delcour et al. (*8*) and Wang et al. (*9*), who investigated the distribution of AX in common wheat mill streams, and of Nyman et al. (*29*), who observed that soluble fiber content is independent of ER, whereas insoluble fiber levels increase rapidly at ER exceeding 80%. Furthermore, Wang et al. (*9*) studied the distribution of TOT-AX and WE-AX in mill streams of six different wheat varieties and observed that the AX distribution patterns among these wheat varieties were very similar. Wholemeal contained approximately 3.2 times the level of TOT-AX and 1.2 times the level of WE-AX of the flour (**Table 2**), which again corresponds well with the results of Delcour et al. (*8*) and Wang et al. (*9*).

Endoxylanase Activity Levels. Endoxylanase activity levels in the 54 different flour fractions and the 4 bran fractions ranged from 0.01 to 0.15 EU/g (results not shown) and from 0.43 to 0.63 EU/g (**Table 1**), respectively. The endoxylanase activity level in the germ fraction was 0.14 EU/g (**Table 1**). Endoxylanases were hence more concentrated in the bran than in the flour fractions, and, for a number of samples, the differences between flour and bran were far more apparent for endoxylanases than for α-amylases. In toto, endoxylanase activity levels in wholemeal were a factor of 4.3 higher than in flour (**Table 2**). The cumulative endoxylanase curve was similar to those of ash and α-amylase (**Figure 1C**), with endoxylanase activities increasing slowly up to an ER of 75% and from then onward much more steeply. These results confirm that the largest part of the endoxylanases is located in the outer wheat kernel layers (*19*, *30*, *31*) and are consistent with the results of Gys et al. (*26*), who showed that small scale debranning of wheat kernels

by only 7% led to a reduction in endoxylanase activity of >80% in wholemeal, whereas α-amylase activity was only reduced by 15%. This also confirms that endoxylanases are more associated with the outer wheat kernel layers than α-amylases. As most wheat-kernel-associated endoxylanases are from microbial origin (*19*) and most α-amylases are produced by the wheat itself, this is not surprising. The gradual increase with increasing sequence number of the rolls for all roll types (**Table 1**) and the large variation observed for endoxylanase activity levels in flour fractions can most likely be explained by the amount of bran contamination. Indeed, taking into account the extremely large difference (ca. 60-fold) between the levels in bran and some flour fractions, it is obvious that small bran contamination in flour can seriously affect the endoxylanase activity level and consequently the functional properties of the flour.

Endoxylanase Inhibitor Contents. TAXI and XIP contents in flour ranged from 49 to 197 ppm and from 130 to 338 ppm, respectively (results not shown), and those in bran from 175 to 335 ppm and from 339 to 603 ppm, respectively (**Table 1**). The inhibitor contents in the germ fraction were in the range of the contents found for flour fractions (**Table 1**). Inhibitor contents increased linearly with increasing ER up to 75% (**Figure 1B**) and from thereafter more steeply. The ratios of the contents in wholemeal to those in flour were factors of 1.5 and 1.2 for TAXI and XIP, respectively (**Table 2**). TAXI hence seemed to be more concentrated in the outer wheat kernel layers than XIP. TAXI contents increased from head-end to tail-end flour fractions, but for XIP contents, no trend could be observed. Gebruers et al. (*25*) also found that the inhibition activities against *Bacillus subtilis* (TAXI) and *Aspergillus niger* (TAXI and XIP) endoxylanases were higher in tail-end flour fractions.

From the results, it is clear that flour fractions from consecutive rolls largely differ in composition. Insight into the different compositions can be used to the advantage of tailor-made flour production. We calculated the compositions of first-, second-, and third-quality flours using the compositions and yields of the individual fractions. **Table 2** shows the calculated composition of these first-, second-, and third-quality flours. As expected, ash, protein, TOT-AX, and TAXI contents and endoxylanase and α-amylase activity levels increased, whereas starch content decreased from first- to third-quality flour. For XIP and WE-AX contents, the differences between first- and third-quality flours were not that apparent. In a similar way, the compositions of straight-run flour, bran material, and wholemeal were also calculated (**Table 2**). The composition of the straight-run flour was quite similar to that of the second-quality flour.

PCA and Correlations between the Different Parameters. To obtain an integrated view of the occurrence of different components over the wheat milling fractions, PCA was executed (**Figure 2**) and Pearson's correlation coefficients between the different components were calculated (**Table 3**).

Analysis of All Milling Fractions except Germ. Initially, statistical analyses were performed for all 59 milling fractions. As could be expected from its composition, the germ fraction often appeared as an outlier, especially for correlations between enzyme activity levels and inhibitor and AX contents on the one hand and starch, protein, and ash contents on the other. In comparison with the flour fractions, the germ fraction had very low starch content and very high ash and protein contents. At the same time, it had similar to slightly higher AX contents, enzyme activity levels, and inhibitor contents. Therefore, the germ fraction was omitted in PCA (**Figure 2A**) and in the
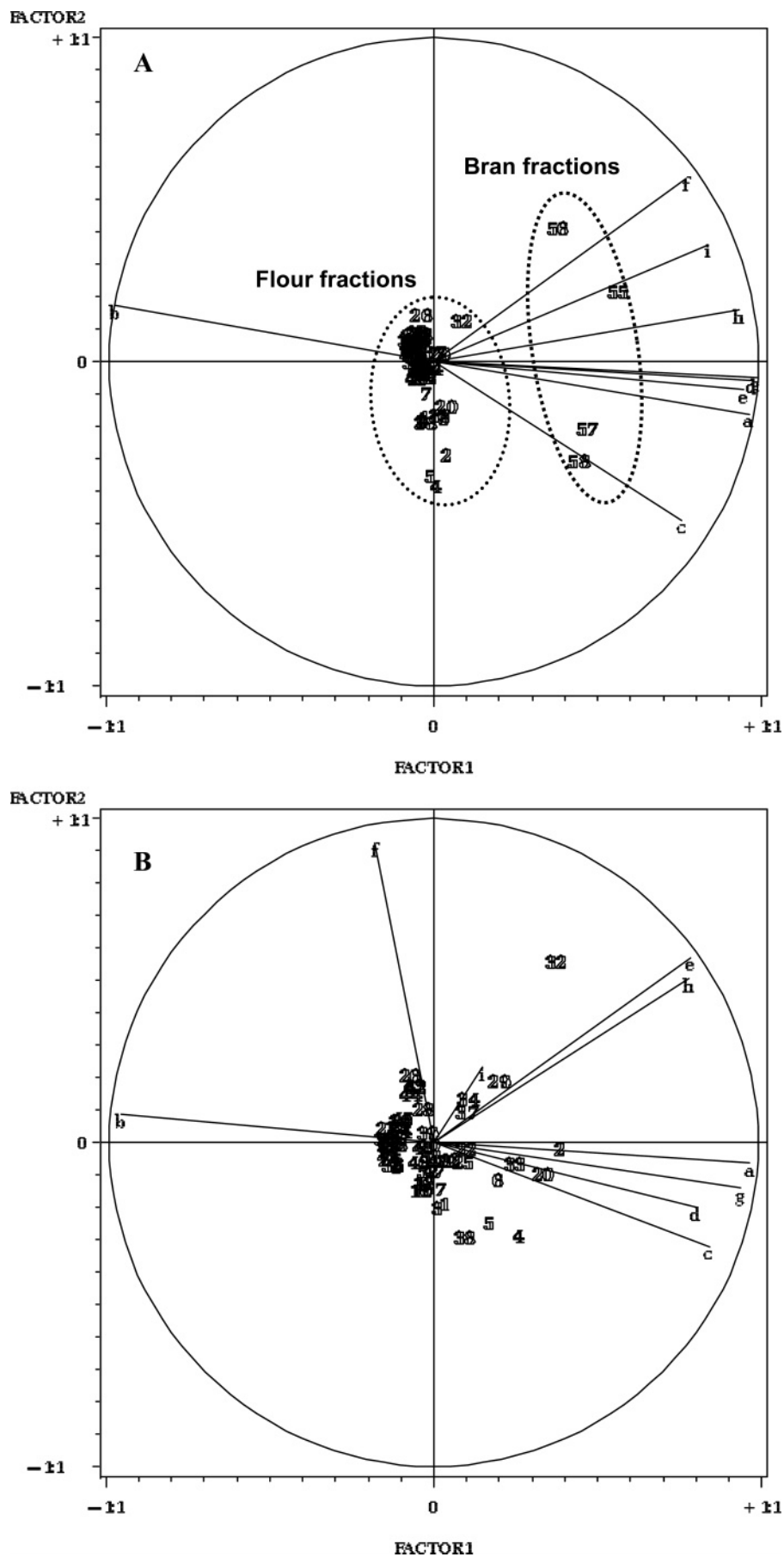
Figure 2. PCA plots for all milling fractions except germ (**A**) and for flour fractions (**B**) showing ash (a), starch (b), protein (c), α-amylase (d), TOT-AX (e), WE-AX (f), endoxylanase (g), TAXI (h), and XIP (i).

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Table 3. Pearson's Correlation Coefficients for the Correlations between Ash, Starch, and Protein Contents, α-Amylase Activity Levels, TOT-AX and WE-AX Contents, Endoxylanase Activity Levels, and TAXI and XIP Contents for All Milling Fractions except Germ (**A**) and for Flour Fractions (**B**)[a]

	ash	starch	protein	α-amylase	TOT-AX	WE-AX	endoxylanase	TAXI	XIP
				A. All Milling Fractions except Germ					
ash	1	−0.99***	0.72***	0.95***	0.99***	0.64***	0.95***	0.82***	0.71***
starch		1	−0.76***	−0.95***	−0.98***	−0.64***	−0.97***	−0.85***	−0.73***
protein			1	0.74***	0.64***	0.35*	0.75***	0.72***	0.50***
α-amylase				1	0.93***	0.71***	0.96***	0.88***	0.79***
TOT-AX					1	0.67***	0.93***	0.80***	0.71***
WE-AX						1	0.72***	0.81***	0.75***
endoxylanase							1	0.89***	0.81***
TAXI								1	0.84***
XIP									1
				B. Flour Fractions					
ash	1	−0.93***	0.81***	0.73***	0.71***	−0.21	0.94***	0.70***	0.04
starch		1	−0.87***	−0.67***	−0.71***	0.25	−0.88***	−0.68***	−0.10
protein			1	0.60***	0.48**	0.42*	0.74***	0.53***	0.06
α-amylase				1	0.48**	−0.29	0.84***	0.50***	0.15
TOT-AX					1	0.38*	0.65***	0.86***	0.18
WE-AX						1	−0.26	0.26	−0.03
endoxylanase							1	0.59***	0.13
TAXI								1	0.24
XIP									1

[a] ***, P value < 0.0001; **, P value < 0.001; *, P value < 0.01.

calculation of Pearson's correlation coefficients (**Table 3A**) for all milling fractions.

The first two principal components (PC) obtained by PCA explained 91% of the total variance in the chemical variables measured in all milling fractions except germ. The equation of the first PC, PC 1, which accounted for 90% of the explained variance, was

$$PC\ 1 = 0.96\ \text{ash} - 0.97\ \text{starch} + 0.76\ \text{protein} +$$
$$0.97\ \alpha\text{-amylase} + 0.95\ \text{TOT-AX} + 0.77\ \text{WE-AX} +$$
$$0.98\ \text{endoxylanase} + 0.93\ \text{TAXI} + 0.84\ \text{XIP}$$

where all parameters are expressed in the concentration and activity units used in **Table 1**. The equation of the second PC, PC 2, which accounted for the remaining 10% of the explained variation in the biplot, was

$$PC\ 2 = -0.16\ \text{ash} + 0.17\ \text{starch} - 0.49\ \text{protein} -$$
$$0.05\ \alpha\text{-amylase} - 0.09\ \text{TOT-AX} + 0.56\ \text{WE-AX} -$$
$$0.05\ \text{endoxylanase} + 0.16\ \text{TAXI} + 0.36\ \text{XIP}$$

In the loading plot of the first two PC, there was a clear distinction between the flour and the bran fractions (**Figure 2A**). The 54 flour fractions were all located closely together in the center of the plot. The 4 bran fractions were located more to the right of the plot as they had much higher ash, protein, AX, and endoxylanase inhibitor contents and α-amylase and endoxylanase activity levels and much lower starch contents than the flour fractions. PC 1 was hence strongly positively determined by bran-associated parameters such as ash content, enzyme activity levels, TOT-AX, and TAXI contents and strongly negatively by endosperm-related parameters such as starch content. The underlying principle of PC 1 is therefore most likely the proportion of pericarp/aleurone material present in the milling fraction. PC 2 accounted for only the remaining 10% of the variation explained in the biplot and was mainly positively influenced by WE-AX and XIP content and negatively influenced by protein content. However, it is difficult to assign a physical meaning to this factor.

The loading plot equally represents all variables measured. Variables that lie close to each other in this plot are positively correlated, whereas variables found on the opposite sides of a diagonal are negatively correlated. Variables found in orthogonal direction are independent of each other. **Figure 2A** shows that the variables ash content, α-amylase and endoxylanase activity levels, and TOT-AX content were located close to each other, which indicates that they are positively correlated. Starch content was found in the opposite direction and was hence negatively correlated with these variables. These correlations had very high Pearson's correlation coefficients ($r > 0.90$) (**Table 3A**). TAXI, XIP, protein, and WE-AX were located somewhat farther away from these components, illustrating that the correlations were less strong (**Table 3A**). WE-AX and protein were located the farthest away from each other and almost in orthogonal direction, which indicated a weak correlation ($r = 0.35$) (**Table 3A**).

Analysis of All Flour Fractions. Apart from the variation between flour and bran fractions, there was also a large one between the different flour fractions. **Figure 2B** and **Table 3B** show the results of PCA and Pearson's correlation coefficients for the 54 flour fractions. **Figure 2B** shows the loading plot of the first two PC, which described 78% of the total variance in the variables measured for all flour fractions. The first and second PC represented 76 and 24% of this variance, respectively. The equation (units and concentrations as above) of the first PC was

$$PC\ 1 = 0.96\ \text{ash} - 0.95\ \text{starch} + 0.84\ \text{protein} +$$
$$0.80\ \alpha\text{-amylase} + 0.78\ \text{TOT-AX} - 0.18\ \text{WE-AX} +$$
$$0.94\ \text{endoxylanase} + 0.78\ \text{TAXI} + 0.15\ \text{XIP}$$

This factor was hence positively determined by enzyme activity levels and protein and ash contents and negatively influenced by starch content. The equation of second PC was

$$PC\ 2 = -0.06\ \text{ash} + 0.09\ \text{starch} - 0.32\ \text{protein} -$$
$$0.20\ \alpha\text{-amylase} + 0.57\ \text{TOT-AX} + 0.92\ \text{WE-AX} -$$
$$0.14\ \text{endoxylanase} + 0.50\ \text{TAXI} + 0.23\ \text{XIP}$$

PC 2 hence strongly depended on WE-AX content and also to a lesser extent on TAXI and TOT-AX contents of the flour samples. Endoxylanase and α-amylase activity levels and protein and ash contents were closely located in the plot, illustrating their strong positive correlations, whereas starch content was

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situated opposite these variables and hence negatively correlated with them. Indeed, from **Table 3B**, it is clear that starch content was strongly negatively correlated with ash ($r = -0.93$) and protein content ($r = -0.87$). Endoxylanase activity levels in the flour fractions were strongly positively correlated with ash content ($r = 0.94$) and α-amylase activity levels ($r = 0.84$) and strongly negatively correlated with starch content ($r = -0.88$). As PC 1 was largely positively determined by ash and negatively by starch content, the underlying principle represented by PC 1 is most likely the level of bran contamination in the flour fractions. The rationale behind PC 2 is again less obvious. PC 2 was mainly determined by WE-AX and to a lesser extent by TOT-AX and TAXI contents. TOT-AX and TAXI contents were located close to one another, confirming their strong correlation ($r = 0.86$) (**Table 3B**). WE-AX content was found in orthogonal direction of all other parameters, indicating there were no or only weak correlations between this and the other parameters. XIP was found in the center of the plot, far from the other variables, indicating that neither PC 1 nor PC 2 could explain the variability in the XIP contents of the flour fractions. From **Table 3B**, it was also clear that XIP contents in flour were not correlated with the other variables (*P* value > 0.01).

Relevance of the Present Findings. From the results presented above, it is clear that flour fractions from consecutive rolls largely differ in composition and hence in nutritional value and technological functionality. Insight into the different compositions can be used to the advantage of tailor-made flour production and can help to predict the consequences of including particular milling streams in final flour products.

From a nutritional point of view, WE-AX and WU-AX are important components as they make up a considerable part of the soluble and insoluble cereal dietary fiber fractions, respectively, both of which have often been associated with health promotion. Soluble dietary fiber slows intestinal transit by increasing viscosity and thereby reduces glucose and sterol absorption by the intestine. It decreases serum cholesterol in the human body and hence reduces the risk of coronary heart disease and also decreases postprandial blood glucose and insulin contents, which is beneficial for diabetics (*32*). Insoluble dietary fiber increases and softens fecal bulk and reduces transit time due to its high water-holding capacity (*33*). From our results, it is clear that fine reduction flour fractions generally contain higher WE-AX contents than coarse reduction, break, and semolina grader flour fractions (**Tables 1** and **2**) and could hence be used as a preferred source of soluble dietary fiber. Flour fractions derived from the later milling rolls and especially bran fractions are rich in WU-AX and could hence be used as a source of insoluble dietary fiber (**Table 1**).

The different milling fractions not only differ in nutritional value but also behave differently in biotechnological applications. AX are generally considered to have a significant bearing on wheat functionality and, taking into account the impact of endoxylanases and endoxylanase inhibitors on the molecular weight and physicochemical properties of AX, it is clear that these components also largely determine the suitability of flours or flour fractions for certain applications. In this respect, it is important to realize that using third- or fourth-passage flour fractions in blending can seriously affect flour quality (**Tables 1** and **2**). Indeed, leaving other important quality determining characteristics such as protein content and composition aside, it is clear that adding flour fractions of such passages to first- and second-passage flours will increase AX contents, endoxylanase activity levels, and endoxylanase inhibitor contents and decrease the WE-AX/TOT-AX ratio (**Table 2**). For dough and bread characteristics, a high WE-AX/TOT-AX ratio is preferable (*34*), whereas for gluten−starch separation, an increase in both WE-AX and WU-AX content is detrimental for gluten agglomeration (*35*). High levels of endoxylanase activity in wheat flour should be avoided as they can cause uncontrolled degradation of AX during bread dough processing, gluten−starch separation, or refrigerated dough storage. High endoxylanase inhibitor contents can reduce AX solubilization by added microbial endoxylanases in breadmaking (*36*) or gluten−starch separation (*37*), thereby impairing processing or product quality. For refrigerated dough production, on the contrary, high inhibitor levels can increase the shelf life of the refrigerated product (*38*). Flour fractions with low endoxylanase activities and high inhibitor levels are, however, not easily found as endoxylanases and TAXI-type inhibitors are positively correlated with each other (**Table 3**).

Conclusion. This study showed that, in general, bran fractions were significantly richer in TOT-AX and WE-AX contents, endoxylanase activity levels, and endoxylanase inhibitor contents than germ and, even more so, than flour fractions. TOT-AX contents were much higher in bran than in flour, whereas WE-AX contents did not differ so drastically, indicating a much lower water extractability of AX in the outer wheat kernel layers. Endoxylanase activity levels varied enormously between the different flour fractions and were strongly positively correlated with ash and negatively correlated with starch content. This indicates that the endoxylanase activity level in wheat flour largely depends on the level of bran contamination and that microbial endoxylanases, situated on the outer wheat kernel layers, end up as a contamination in wheat flour. TAXI contents in flour fractions varied ca. 4-fold, whereas XIP contents varied ca. 3-fold. TAXI contents seemed to be higher in the outer wheat kernel layers than XIP contents and were, in contrast to XIP contents, well correlated with bran-related parameters such as ash content and enzyme activity levels.

Although the results in this study were obtained using a mix of wheat varieties and one milling scheme, it can be expected that the correlations found in this study will also be valid for other wheat varieties or other milling schemes and hence can be helpful in optimizing blending of wheat flour fractions to obtain flours suitable for specific applications in which AX, endoxylanase, and endoxylanase inhibitors play a crucial role or to obtain flours with specific nutritional qualities with respect to dietary fiber composition.

ABBREVIATIONS USED

AU, amylase unit; AX, arabinoxylan; AZCL, azurine-cross-linked; BSA, bovine serum albumin; CW, coarse weatings; E_{590}, extinction at 590 nm; ER, extraction rate; EU, endoxylanase unit; FW, fine weatings; GHF, glycoside hydrolase family; PC, principal component; PCA, principal component analysis; TAXI, *Triticum aestivum* xylanase inhibitor; TFA, trifluoroacetic acid; TOT-AX, total arabinoxylan; WE-AX, water-extractable arabinoxylan; WU-AX, water-unextractable arabinoxylan; XIP, xylanase-inhibiting protein.

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