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Determining the Water Absorption and Rheological Properties of Rye Dough Made Using the Planetary Mixer P 600

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Abstract In comparison to processed wheat flour products, there is no established method for determining the water absorption of rye flour. The aim of this study was to work out a method using the Planetary mixer P 600 for the determination of water absorption (WA) and the rheological properties in rye dough made from rye flours of 4 different types (I-IV). In the correlation analysis showed that WA had positive correlation with ash, beginning of gelatinization, 125-160 μm particles, sedimentation values at 20 and 25 min, bread yield, pH, and total titratable acidity, but had negative correlation with initial viscosity, gelatinization maximum, viscosities on swelling at 25, 28, and 31^{o}C. The WA determined by the Planetary mixer P 600 agreed well with the experimental baking tests.

Keywords: rye flour, water absorption, rheological property, Planetary mixer P 600, rye dough

Introduction

Just as for wheat, rye is used for making bread in Germany. The use of rye is one reason for the variety in German breads. The principal difference between wheat flour and rye flour is that rye proteins cannot form gluten after being mixed with water, which is the basis of wheat bread production. The pentosans in rye, however, can bind water during mixing to produce dough that can be baked into bread (1). Rye dough is stiffer and more rigid than the tensile-elastic wheat dough, probably due to the absence of viscoelastic gluten and the presence of pentosan molecules, which are viscous but not stretchable and short (not elastic) when torn. Water appears to be the plasticizing agent, increasing the softening of the dough (2). The water binding in rye dough and the resulting dough yield is an important characteristic for evaluating flour. Determining the water absorption ability based on a certain dough consistency has, therefore, important calculatory, technological, and qualitative aspects.

Water absorption of rye flours is affected by many physical and compositional factors. It increases with increasing fineness of the flour. The pentosans of the soluble fraction affect water absorption much more than the proteins (3). Water absorption of rye dough does not remain constant but changes with time at a rate that depends on temperature, pH, and salt content. In the dough stage, the proportion of insoluble and soluble constituents continuously changes because of enzymatic action. As the insoluble constituents gets little, their contribution to water absorption rapidly diminishes. If enzymatic action is extensive, it eventually destroys the water-binding properties of water-extractable constituents. The consistency of rye dough is extremely important to baking quality. This differs from wheat dough, which must have an optimal balance between elastic and viscous properties for best baking performance. Viscosity of rye dough determines dough yield, stability, and volume, as well as bread loaf volume. Dough of higher consistency yield more dough by retaining more water; they also have better stability. However, they yield lower loaf volumes (1). The pentosans play the key role in dough consistency. Proteins are important but not to the same extent as in wheat dough, mainly because of the difference in the solubility between wheat and rye proteins after the flours are mixed into dough. Only about 10% of wheat flour protein is soluble in water, whereas about 80% of the rye protein becomes soluble in the sourdough (4).

In comparison to processed wheat flour products there does not exist an established method for determining the water absorption of rye flour. The baker has to rely on his experience when producing rye dough which leads quite often to the result that the rye dough is too firm. Since 1987 there is a proposal from Brümmer (5) for the determination of the water absorption of rye flours, using the Farinograph® mixer which was developed for wheat flours. Generally speaking this test was the right step and own tests showed that e.g., the firmness of rye dough could be compared by using this method. The use of the Farinograph® mixer for determining the water absorption of milled rye products does not provide results with the same exactness, because the Farinograph® mixer was designed for viscoelastic dough but not for plastic rye dough (6). Many tests showed that when checking the water absorption with the Farinograph® mixer the tendency for bridging is very high and therefore deviations within the reproducibility are given. The use of the Farinograph® mixer which is well suited for wheat dough was definitely a right step also considering missing alternatives. There, however, will always be the basic difference to wheat dough, because rye dough act plastic

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and therefore the consistency should be also determined with an appropriate measuring system.

A measuring system for plastic materials was found with the Farinograph®-E as a drive unit and instead of the wheat mixer the Planetary mixer P 600 was attached. The planetary mixer originates from the measuring programme for testing plastics and it is used for the quality assurance for some years (6). Therefore, to develop an analyzer, capable of registering both the current flow characteristics and changes due to mechanical influence during a period, we cooperated with important manufacturer of measuring instruments. With this method, standards characterizing, for example, the water binding of rye dough, could be set much like for wheat dough.

The objective of this study was to characterize the effects of rye flours from 4 different types (I, II, III, and IV) on the determination of water absorption and the rheological properties of rye doughs using the Planetary P 600.

Materials and Methods

Analysis of physiochemical properties of rye flours The physiochemical properties of rye flours from 4 different types based on content (mg) of ash (flour I: Type 997, flour II: Type 1150, flour III: Type 1370, flour IV: wholemeal; Rolandmühle, Bremen, Germany) were analyzed using International Association of Cereal Science and Technology (ICC, 7) standard methods (moisture, No. 110/1; ash, No. 104/1; falling number, No. 107/1; amylogram, No. 126/1) described in Table 1. The ash contents ranged from 910 to 1,100 mg/100 g dry weight at the flour I; from 1,110 to 1,300 mg at the II; from 1,310 to 1,600 mg at the III; from 1,610 to 1,800 mg at the IV (wholemeal).

Particle size analysis The particle size distribution of 100 g of rye flour was determined by sieve analysis on a sieve-shaker (Type ADEB 56N4 R3; J. Engelsmann AG, Ludwigshafen, Germany) using 71-, 100-, 125-, and 160-μm sieves of DIN (Deutsches Institut für Normung e.V.) 4188.

Zeleny sedimentation test Sedimentation value (3.2 g of rye flour on a 14% moisture basis) was determined by ICC Standard No. 116/1 (7), using the Zeleny test with a shaker (Type M 300; Apparatebau Max Egger, St. Blasen, Austria).

Swelling test of rye flours In the swelling test, a modified method from Stephan and Exner (8), viscosity of rye flour suspension (80 g flour on a 14% moisture basis in 450 mL distilled water) was measured in an amylograph (Brabender GmbH & Co., KG, Duisburg, Germany). The detected parameter in BU was initial viscosity at 25°C and then viscosity at 28, 31, 34, 37, 40, and 43°C.

pH and total titratable acidity (TTA) of sourdough and bread crumb The pH and TTA were measured by titrating 10 g of sample (sourdough and bread crumb) with 0.1 N NaOH to pH 8.5, according to the standard methods of the Association of Cereal Research (ACR, Arbeitsgemeinschaft Getreideforschung e.V., 9) with pH meter (Type CG 701; Schott AG, Mainz, Germany).

Rheological measurements of rye dough The rheological properties of dough and determination of the water absorption of each rye flour were assessed using a new analytical method developed by the Planetary mixer P 600 with Kenwood-kneading hook (Brabender GmbH & Co.) under operating conditions without and with ingredients of sourdough, yeast, and salt.

The operating conditions of P 600 for rye dough without ingredients are tempering (1 min)→adding water (2 min) →hydration (1 min)→scraping (1 min)→mixing (10 min). The final consistency after mixing and kneading for 15 min was 6 Nm±0.15 (6).

The operating conditions of P 600 for rye dough with ingredients are tempering and premixing (1 min)→adding water (1 min)→hydration (1 min)→scraping (1 min)→mixing (10 min). Final consistency after mixing and kneading for 14 min was 6 Nm±0.15. Rheological properties obtained from the Planetary mixer P 600 curve were dough development time (DDT), consistency decrease (CD), water absorption (WA), and energy during the mixing and kneading (Fig. 1). WA must be calculated from the deviations from 6 Nm. The 0.15 Nm corresponds to WA of 1%.

Baking test Rye bread was produced by the Detmold one-stage sourdough process. Rye flour (1,000 g), water (1,000 g), and sourdough starter (100 g) was mixed for 3.5 min in a paddle mixer (Garbe, Lahmeyer & Co. AG., Aachen, Germany) and ripened at 24°C for 20 hr. All rye bread formulations contained total rye flour (2,500 g), sourdough (1,500 g), yeast (50 g), and salt (50 g). The

Table 1. Physiochemical properties of rye flours

Component	I	II	III	IV	Pr>F
Moisture (%)	13.0±0.06^{a}[1)]	13.1±0.10^{a}	13.0±0.06^{a}	12.6±0.10^{b}	0.0004
Ash (%)	1.089±0.012^{d}	1.188±0.018^{c}	1.259±0.025^{b}	1.666±0.022^{a}	0.0001
Falling number (sec)	203±1.53^{a}	197±1.15^{b}	160±4.73^{c}	196±3.46^{b}	0.0001
Amylograph					
Initial viscosity (BU)	73±6.11^{a}	73±2.31^{a}	.60±8.50^{b}	53±3.00^{b}	0.0049
Beginning of gelatinization (°C)	54.4±0.45^{b}	54.3±0.64^{b}	54.9±0.69^{b}	57.0±0.66^{a}	0.0020
Gelatinixation temperature (°C)	70.3±0.20^{b}	70.1±0.20^{b}	67.8±0.21^{c}	71.8±0.47^{a}	0.0001
Gelatinization maximum (BU)[2)]	528±2.52^{a}	493±1.00^{b}	342±10.26^{c}	324±4.04^{d}	0.0001

[1)]Mean±SD (*n*=3); Means in a row sharing different superscript letter(s) are significantly different ($p<0.01$).
[2)]BU, Brabender unit.

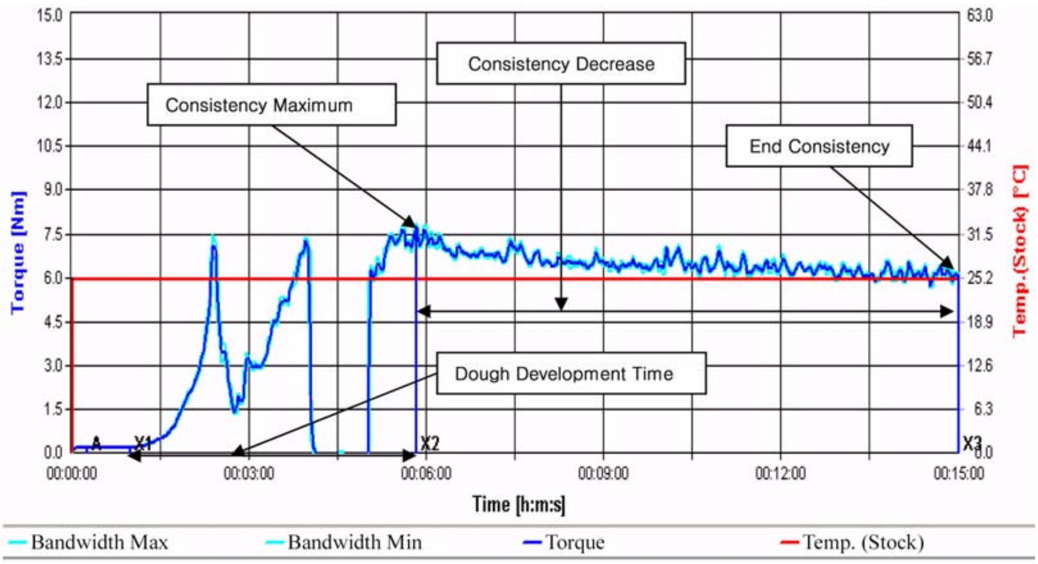

Fig. 1. Exemplar for the results of rheological properties of rye dough made using the Planetary mixer P 600.

amount of water (I: 1,750 g; II: 1,850 g; III: 1,900 g; IV: 1,950 g) was adjusted according to the WA with only rye flours determined by the Planetary mixer P 600. Rye dough was mixed for 10 min in a paddle mixer. After dough maturing (15 min at room temperature), dough pieces (1,150 g) were molded by hand and proofed (50 min at 32°C and 80% relative humidity) in a proofing basket. The breads were baked at upper heat 260°C and under heat 240°C for 50 min. After storage (20 hr at room temperature) the pH and TTA of bread crumb, bread weight (Industrial Scale Type SB8001; Mettler-Toledo GmbH, Giessen, Germany), and bread loaf volume, as determined by rapeseed displacement, were measured. Experienced panelists did the sensory evaluation of the rye bread according to criteria for the sensory evaluation of ACR such as form, browning, crumb loosening, pore uniformity, crumb elasticity, and flavor.

Statistical analysis All statistical analyses were performed using SAS (Statistical Analysis System, 10). Data were analyzed by a one-way analysis of variance (ANOVA). Differences among mean values were tested for significance by means of Duncan's multiple-range tests. Tests were considered significant at $p<0.01$ and $p<0.05$. Pearson's correlation analysis was used to find a correlation between the WA with only rye flours and physiochemical properties of flours, rheological properties of doughs, and baking quality characteristics of breads.

Results and Discussion

Particle size distributions of rye flours These 4 rye flours showed different particle size distribution (PSD, Fig. 2). The particles of rye flour II were significantly coarser than the particles of rye flour I, III, and IV.

This result does not agree with type numbers of rye flour. Many researchers (2,11-17) have reported the importance of particle size and structural state of the cell walls on dough rheology. According to Drews and Seibel (3), WA increases with increasing fineness of the flour.

Sedimentation values of rye flours In the Zeleny sedimentation test, the suspensions prepared from the 4 rye flours in a lactic acid solution in the presence of bromophenol blue showed different sedimentation values that decreased with rising test times and increased with rising rye flour numbers (Table 2). The effect of rye flour IV on the sedimentation value was significantly higher than the effects of rye flour I, II, and III after 20 and 25 min of test times.

These results agree with protein contents (rye flour I, 11.1%; II, 12.5%; III, 13.8%; IV, 14.9%) and ash contents (rye flour I, 1.089%; II, 1.188%; III, 1.259%; IV, 1.666%). No information on the sedimentation value of rye flour has so far been obtained.

Viscosities on swelling of rye flours The studies of the slurries prepared from the 4 rye flours in the swelling test showed different IV that decreased with rising temperature and rye flour numbers (Table 3). The effects of the rye flour I and II on viscosity were significantly higher than the effect of rye flour III and IV.

Information from amylograph on the correlation of WA and initial viscosity is limited. Stephan and Exner (9) suggested that the swelling ability of the rye flours, based on the aqueous suspension in an amylogram, does not always agree with the WA ability during dough preparation. The constant consistency of the rye doughs with 500 BU consistency units is not determined only by the swelling characteristics, but largely, by the tenacity and cohesiveness of the water binding components. Milling-technical differences during flour production also influence the level of the initial viscosity; this applies particularly to the different compositions of milling grooves. Huber (18) reported that the differences in the water binding capacity of rye flour correlate highly with the milling technique. The swelling-substance-rich components (proteins, hemicelluloses, pentosans, and β-glucans) lie in the endosperm: bran. With

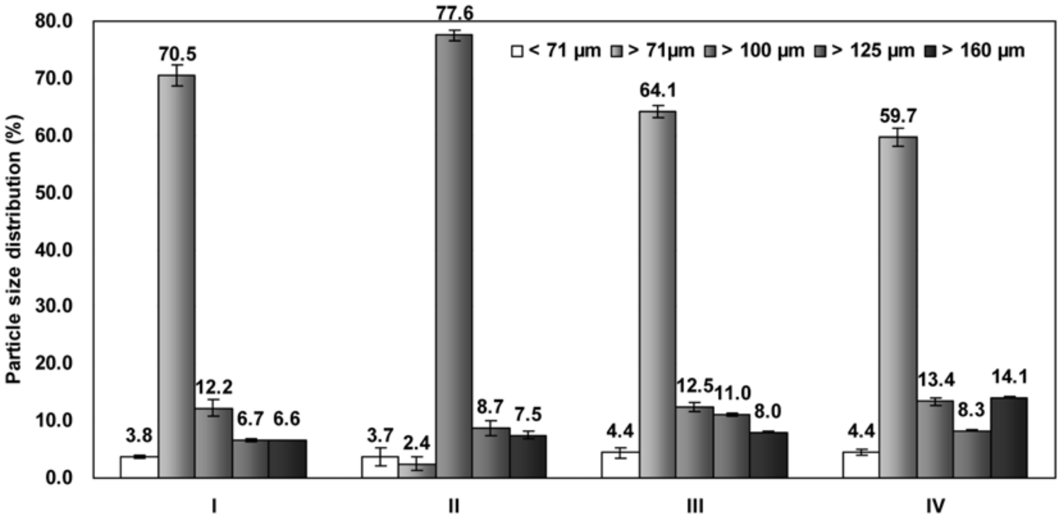

Fig. 2. Particle size distributions of rye flours.

the increase of the swelling substances, the water binding capacity increases, becoming stronger in after-swelling. The rye flour I and II were higher viscosities on swelling than the rye flour III and IV because lower number of flours contain higher percentages of soluble pentosan. Brümmer (19) noted that higher percentages of soluble pentosans seem to affect the peak values of the amylogram.

Effects of rye flours without ingredients on the rheological properties The doughs prepared from the 4 rye flours without ingredients showed different rheological properties in DDT, CD, WA, and energy that increased with rising rye flour type numbers (Table 4). The ranges of the analytical data for the samples were from 4:27 to 4:53 min for the DDT, from 1.30 to 2.28 Nm for the CD, from 70.2 to 78.3% for the WA, and from 28.5 to 29.5 kNm for energy. The effects of all rye flours on the DDT and energy were not significant. Rye flour I affected the CD significantly more than rye flour II, III, and IV. The effect of the rye flour IV on WA was significantly higher than the effect of flour I, II, and III (p<0.01).

The WA increased with rising rye flour numbers because higher number of flour contains more percentage of water-binding-strong components such as pentosan and protein. Brümmer (19) described that the behavior of swelling substances of the rye during processing (e.g., consistency of the dough) determined by the pentosan and protein content and its susceptibility.

Effects of rye flours with ingredients on the rhelogical properties The doughs prepared from the 4 rye flours with ingredients showed different rheological properties for DDT, CD, WA, and energy (Table 5). The ranges of the analytical data for the samples were from 4:20 to 5:19 min

Table 2. Sedimentation values of rye flours (mL)

Component (min) \ Sample	I	II	III	IV	Pr>F
15	unreadable	unreadable	unreadable	unreadable	-
20	19.8±1.00[d1)]	22.8±1.00[c]	25.7±1.00[b]	39.4±1.00[a]	0.0001
25	16.8±1.00[d]	19.1±0.58[c]	20.8±0.95[b]	27.9±0.52[a]	0.0001

[1)]Mean±SD (n=3); Means in a row sharing different superscript letter(s) are significantly different (p<0.01).

Table 3. Viscosities on swelling of rye flours

Temp. (°C) \ Sample	I	II	III	IV	Pr>F
25	85±7.81[ab1)]	88±4.00[a]	76±2.65[b]	64±2.89[c]	0.0013
28	85±6.66[a]	87±3.51[a]	75±2.08[b]	64±2.89[c]	0.0006
31	81±4.16[a]	82±4.04[a]	71±4.04[b]	61±2.65[c]	0.0004
34	75±2.65[a]	76±2.52[a]	65±1.73[b]	57±0.58[c]	0.0001
37	68±2.08[a]	70±2.08[a]	60±3.51[b]	53±2.89[c]	0.0002
40	64±2.00[a]	65±2.65[a]	54±1.53[b]	47±1.73[c]	0.0001
43	58±4.00[a]	58±3.46[a]	50±2.52[b]	41±0.58[c]	0.0002

[1)]Mean±SD (n=3); Means in a row sharing different superscript letter(s) are significantly different (p<0.001).

Table 4. Effects of rye flours without ingredients on the rheological properties

Component \ Sample	I	II	III	IV	Pr>F
Dough development time (min)	4:27±13:32[a1)]	4:53±36:46[a]	4:45±7:57[a]	4:27±19:01[a]	0.4095
Consistency decrease (Nm)	2.28±0.29[a]	1.30±0.14[c]	1.73±0.08[b]	1.35±0.20[c]	0.0009
Water absorption (%)	70.2±0.79[c]	74.3±0.67[b]	75.7±1.10[b]	78.3±0.21[a]	0.0001
Energy (kNm)	28.7±0.55[a]	28.5±0.12[a]	29.5±0.80[a]	28.9±0.78[a]	0.3208

[1)]Mean±SD (n=3). Means in a row sharing a common superscript letter(s) are not significantly different (p<0.01).

Table 5. Effects of rye flours with ingredients on the rheological properties

Component \ Sample	I	II	III	IV	Pr>F
Dough development time (min)	4:20±15:62[b1)]	4:30±12:49[b]	4:55±18:48[ab]	5:19±23:44[a]	0.0159
Consistency decrease (Nm)	2.73±0.25[ab]	2.25±0.36[b]	3.01±0.28[a]	2.53±0.37[ab]	0.0940
Water absorption (%)	62.6±0.76[b]	64.0±1.39[ab]	62.7±1.40[b]	65.3±0.70[a]	0.0486
Energy (kNm)	29.9±0.87[b]	30.7±0.67[b]	32.4±0.31[a]	30.9±0.25[b]	0.0052

[1)]Mean±SD (n=3). Means in a row sharing different superscript letter(s) are significantly different (p<0.05).

for DDT, from 2.25 to 3.01 Nm for CD, from 62.6 to 65.3% for WA, and from 29.9 to 32.4 kNm for energy. Rye flour IV affected DDT significantly more than rye flour I and II. The effect of the rye flour III on CD was significantly higher than the effect of flour II. Rye flour IV affected WA significantly more than rye flour I and III. The effect of the rye flour III on energy was significantly higher than the effects of rye flour I, II, and IV (p<0.05).

Dough tests of dough prepared from these 4 rye flours without and with ingredients showed different rheological properties in DDT, CD, WA, and energy. The WA of rye dough with ingredients between 62.6 and 65.3%, showing decreased WA between 7.6 and 13.0% compared to dough without ingredients because a consistency loss occurs during the sourdough ripening time.

Effects of sourdough, yeast, salt, and enzymes on dough property have been reported by a few researchers. Freund (20) described that the presence of acid during rye dough processing results in increased swelling of pentosans and proteins. At pH values under 4.9, solubility decreases, and the water binding by swelling rises. Moreover, the acid inhibits the enzymatic degradation of pentosans. The acid and salt enable a higher dough yield, the dough become malleable, and the enzymatic degradation of pentosans and starch is inhibited. The presence of salt and a pH value reduced to a certain critical point ensure full water binding from flour components (starch, proteins, mucilaginous substances), which are necessary for dough formation and stable crumb structure. In addition, baking yeast needs protein substances as food, splitting those proteins by proteases. This protein degradation leads to dough softening. According to Völker (21), salt positively affects dough formation by inhibiting the enzymatic degradation and by decreasing the solubility of the protein. The ions of the dissolved salt (Na^+ and Cl^-) existing in rye dough, endeavor to attract water molecules (22). Huber (18) reported that, because of the immediately applying enzymatic-inhibiting effect, the water binding capacity in the straight-dough process is considerably higher than during the comparative sourdough process.

pH and TTA of Detmold one-stage sourdough Detmold one-stage sourdoughs prepared from the 4 rye flours showed different pH and TTA in the baking test (Table 6). The effects of all rye flours on pH were not significantly different. Rye flour IV had significantly more effect on TTA than rye flour I, II, and III (p<0.05).

The TTA increased with rising rye flour type numbers because lactic acid bacteria in rye dough prepared from higher number of flour produces acids that are more organic such lactic acid and acetic acid during sourdough fermentation.

Sensory evaluation and baking results The studies of rye breads baked from the 4 rye flours in the baking test had similar sensory evaluations of form, browning, crumb loosening, pore uniformity, crumb elasticity, and flavor. The different baking results showed similar baking loss, bread yield, volume yield, pH, and TTA that increased with rising flour type numbers (Table 6 and Fig. 3). All rye dough resulted in bread with a good range of crumb elasticity and volume yield. The effects of all rye flours on baking loss were not significantly different. Bread made from rye flours IV showed significantly higher bread yield than bread made from rye flour I. Bread made from rye flour II and III had significantly higher volume yield than bread made from rye flour I and IV. Bread made from rye flour IV showed significantly higher pH and TTA than bread made from rye flour I, II, and III (p<0.05).

Correlation coefficient between the WA of rye flours and physiochemical properties of flours and baking qualities of breads The results of Pearson's correlation analysis are shown in Table 7. In the correlation analysis between WA with only rye flours, physiochemical properties of flours, and baking qualities of breads showed that WA had positive correlation with ash, beginning of gelatinization, 125-160 μm particle, sedimentation value at 20 and 25 min of rye dough, bread yield, pH, and TTA of rye breads, but had negative correlation with initial viscosity, gelatinization maximum, viscosity of swelling at 25, 28, and 31°C of rye flours.

Table 6. pH and acidity of Detmold one-stage sourdough, and baking characteristics of bread

Component		I	II	III	IV	Pr>F
Sour-dough	pH	3.93±0.03$^{a1)}$	3.95±0.00^{a}	3.95±0.00^{a}	3.95±0.00^{a}	0.4411
	Total titratable acidity (mL)	17.7±0.06^{d}	18.7±0.10^{c}	19.8±0.06^{b}	22.1±0.10^{a}	0.0001
Bread	Form	good	good	somewhat flat	good	-
	Browning	normal	normal	normal	normal	-
	Crumb loosening	good	good	good	still good (somewhat dense)	-
	Pore uniformity	fairly uniform	fairly uniform	fairly uniform	uniform (fine pores)	-
	Crumb elasticity	good	still good	still good	good	-
	Flavor	acceptable	acceptable	acceptable	acceptable	-
	Baking loss (%)	14.4±1.35^{a}	14.8±1.35^{a}	14.4±0.99^{a}	13.1±1.20^{a}	0.4125
	Bread yield (%)	148.9±2.35^{c}	151.7±2.41^{bc}	154.1±1.78^{ab}	158.2±2.19^{a}	0.0050
	Volume yield (mL/ 100 g of flour)	282.9±3.00^{b}	292.1±2.35^{a}	294.7±2.39^{a}	281.6±4.45^{b}	0.0008
	pH	4.47±0.03^{c}	4.50±0.00^{b}	4.50±0.00^{b}	4.60±0.00^{a}	0.0001
	Total titratable acidity (mL)	9.3±0.06^{d}	9.6±0.06^{c}	10.2±0.10^{b}	11.5±0.14^{a}	0.0001

[1)]Mean±SD (n=3). Means in a row sharing different superscript letter(s) are significantly different (p<0.05).

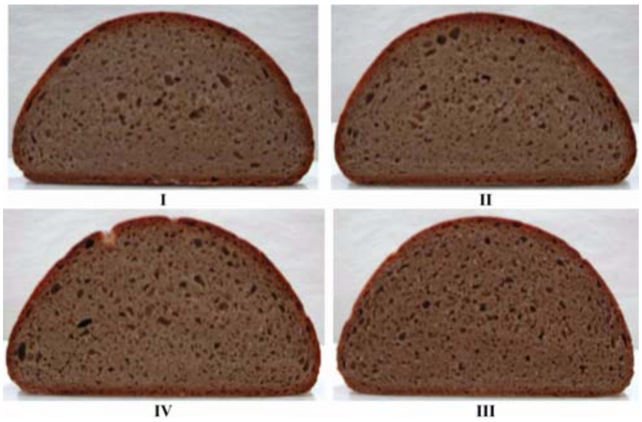

Fig. 3. The sliced bread crumbs of rye breads.

WA increased with increasing ash content, beginning temperature of gelatinisation, 125-160 μm particle content, sedimentation value at 20 and 25 min of rye flours, whereas decreased as initial viscosity, gelatinization maximum, and viscosity of swelling at 25, 28, and 31°C of rye flours increased. As WA increased, bread yield, pH, and TTA of rye breads increased.

The WA of rye doughs are affected by ash content, amylogram, particle size distribution, sedimentation value, viscosity of swelling, and baking quality. Seibel and Weipert (1), and Lorenz (23) described that the WA and consistency of rye dough are affected by many physical and compositional factors, such as particle size (fineness), α-amylase activity (measured by amylograph and falling number), temperature, pH, salt content, and water-soluble fraction (pentosans, dextrins, sugars, and proteins), extremely important and strongly interrelated properties related to baking quality.

To manufacture high-quality rye breads, the mixing and kneading procedure must always produce a dough with an average consistency of 6.0 Nm. Using the P 600, it is possible to determine the required WA. Moreover, the WA determined with the P 600 could be used as a basis for producing high-grade rye breads. Based on these analyses, rye breads can be manufactured to meet particular quality criteria like an even and well-loosened crumb with sufficient loaf volume using the P 600 to determine the dough yield of rye dough. This new testing method for WA showed good correlation between mechanical testing and sensory testing by specialists as shown in the baking tests.

Table 7. Correlation coefficient between the WA of rye flours and physiochemical properties of rye dough and bread[1)]

	Ash (%)	Amylogram			Particle size distribution	Sedimentation value		Viscosities of swelling			Baking qualities		
		IV (BU)	BOG (°C)	GM (BU)	>125 (μm)	20 (min)	25 (min)	25 (°C)	28 (°C)	31 (°C)	BY (%)	pH	TTA (mL)
WA	0.87 ***	-0.73 **	0.69 *	-0.87 ***	0.92 ***	0.85 ***	0.87 ***	-0.70 *	-0.72 **	-0.75 **	0.79 **	0.82 **	0.88 ***

[1)]IV, initial viscosity; BOG, beginning of gelatinization; GM, gelatinization maximum; BY, bread yield; TTA, total titratable acidity; WA, water absorption; *p<0.05, **p<0.01, and ***p<0.001.

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