

# Distribution of Glutathione in Millstreams and Relationships to Chemical and Baking Properties of Flour

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## ABSTRACT

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Fourteen millstream flours, a straight-run flour, bran, pollard, and germ were prepared separately from two Australian and two New Zealand wheat cultivars using a 650 kg/hr pilot roller mill. Glutathione (GSH) and oxidized glutathione (GSSG) were measured in all samples. The Australian cultivars had higher levels of GSH and GSSG than the New Zealand cultivars, and in all cultivars the levels in pollard and germ were considerably higher than in flour samples. Generally, the early break flours and early reduction flours had lower GSSH/GSSG levels than the tail-end break and reduction flours. There was a strong correlation between GSH/GSSG and ash content in millstream flours, which indicated that

much of the GSH/GSSG in the flour was likely to have derived from contamination by bran, aleurone (pollard), and germ. There were also moderate to strong correlations between GSH/GSSG and the cysteine content of all proteins in flour. GSH/GSSG correlated strongly with the albumin and globulin content of flour but not with gliadin and glutenin. The volume and crumb texture properties of bread made with millstream flours in the absence of ascorbic acid (AA) were negatively correlated with GSH/GSSG. The change in bread volume and texture properties when AA was added to dough (baking improver effect of AA), however, were poorly correlated with GSH/GSSG.

The germ was the first fraction of wheat in which glutathione (GSH) was detected (Sullivan et al 1936). Subsequently, GSH and oxidized glutathione (GSSG) have been measured quantitatively in germ (Fahey et al 1980) and flour (Hird et al 1968; Archer 1972; Grosch 1986; Schofield and Chen 1995). Chen (1994) measured GSH and GSSG in the following Bühler milling fractions of wheat: break flour streams, reduction streams, bran fraction, and germ plus shorts fraction. To our knowledge, however, GSH and GSSG have not been measured on wheat fractions from a commercial mill.

GSH has been associated with dough and bread quality ever since 1936 (Balls and Hale 1936; Jorgensen 1936; Sullivan et al 1936; Ziegler 1940; Grosch and Wieser 1999). The high levels of GSH in wheat germ and millstream flours contaminated with germ were considered to be injurious to bread texture when these wheat fractions were included in the flour mixture (Sullivan et al 1936; Ford and Maiden 1938; Hullet and Stern 1941). It has also been claimed that the baking improver effect of oxidizing agents, such as potassium bromate and ascorbic acid (AA)/dehydroascorbic acid (DHA), is mediated through rapid removal of GSH from dough by oxidation to GSSG (Sullivan et al 1936; Ziegler 1940; Hullet and Stern 1941; Grosch and Wieser 1999). It is clear from these and many other reports that, in the absence of oxidizing agents, addition of GSH to dough is harmful to breadbaking. However, it has been shown that, in the presence of AA, addition of GSH to dough up to three times the levels found naturally in flour produces better quality bread than bread that is made from flour with natural levels of GSH (Chen 1994; Every et al 2000). These and other results (Every et al 1999, 2000) indicate that GSH is indeed involved in the AA improver effect, not because oxidative removal of GSH is necessary but because both GSH and AA are somehow necessary in the sulfhydryl/disulfide interchange reactions that produce optimal dough and bread quality.

In an attempt to shed further light on the role of GSH and AA in breadmaking and possibly improve optimization of the millstream flour blending process, our objective was to investigate the distribution of GSH and GSSG in all of the milling fractions of four wheat cultivars, and examine the relationship of GSH and GSSG content in flour to various chemical and breadmaking properties of flour.

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## MATERIALS AND METHODS

### Chemicals and Wheat Samples

GSSG (98%) and 1,10-phenanthroline were purchased from Sigma-Aldrich (St. Louis, MO). GSH (>99%) and 1-fluoro-2,4-dinitrobenzene (>99%) were purchased from Merck (Darmstadt, Germany). All other chemicals were AnalaR products purchased from Merck (Palmerston North, New Zealand).

Two New Zealand wheat cultivars (Sapphire and Monad) were grown in New Zealand, and two Australian cultivars (Janz and Frame) were grown in Australia. Two tonne lots of each cultivar were milled by established procedures at 75% extraction on a 650 kg/hr pilot roller mill (BRI pilot mill, Sydney) into 14 separate millstream flours: 1st, 2nd, 3rd, and 4th break, break middlings (BM), sizings (SZ), and A, B, C, B2, D, E, F, and PF reduction flours. In addition, a straight-run flour was collected, consisting of all the flour streams in proportion to their production on the mill. After milling, samples (20–50 kg) of each millstream flour were stored at 10°C until analysis for total protein, ash, and bake tests. Duplicate subsamples (300 g) were stored at –18°C until analysis for cysteine content of protein and protein composition. Duplicate subsamples (10 g) were stored at –80°C until analysis for GSH and GSSG. Duplicate subsamples (10 g) of bran, pollard, and germ fractions were also collected and stored at –80°C until analysis for GSH and GSSG. Individual bran and pollard fractions for cultivar Janz were not examined because these fractions were accidentally mixed at the mill.

### Analytical Methods

All analytical tests were made on each of two samples for each millstream fraction and cultivar. Total protein was determined using an analyzer (Leco CNS-2000, Leco Corporation, St. Joseph, MI) and a nitrogen to protein conversion factor of 5.7. Ash determinations were made using Approved Methods (AACC International 2000).

SDS-soluble proteins (sol-protein) and SDS-insoluble proteins (insol-protein) were determined by size-exclusion HPLC (Gupta et al 1993; Sutton et al 2003), and glutenins, gliadins, albumins, and globulins were determined by RP-HPLC analysis as described by Sutton et al (2003). Cysteine content of sol-proteins (sol-cysteine) and insol-proteins (insol-cysteine) was measured as  $\mu\text{mol}$  of cysteine/g of protein using bromobimane labeling of cysteine and SE-HPLC (Sutton et al 2003).

GSH and GSSG were measured using the derivatization and RP-HPLC methods of Schofield and Chen (1995) and Bollecker et al (2000). To prevent oxidation of GSH during the procedure, the

method was modified by adding a 50 mM solution of 1,10-phenanthroline in ethanol (0.1 mL) to the GSH extract (1.0 mL) before treatment with 0.1M iodoacetic acid and derivatization with 1-fluoro-2,4-dinitrobenzene (Livesey and Reed 1984; Every et al 1999).

### Breadbaking Tests

All baking tests were made on each of two samples for each millstream flour and cultivar. Bread was made by a mechanical dough development (MDD) system using 125 g of flour as described by Swallow and Baruch (1986) and Larsen and Greenwood (1991), except potassium bromate was not used and each flour sample was baked with or without 100 ppm of AA. Water absorption and work input values were determined for individual samples on a 125-g MDD Mitchell mixer. Loaf volumes were measured by rapeseed displacement and crumb grain was scored subjectively by experienced judges on a scale of 1–14 (highest quality) at 16–20 hr after removal from the oven. In this study, the baking response to AA was measured by the difference in volume or texture between loaves baked with and without AA.

### Statistical Analysis

Means for all analytical and baking tests on each millstream fraction and cultivar were calculated. The various measurements were compared by calculating correlation coefficients between these means. Data presented graphically was analyzed with analysis of variance to provide estimates of variability (least significant differences). The GSH and GSSG measurements were logarithmically transformed before the analysis to make the variance more homogeneous across the range of data.

## RESULTS AND DISCUSSION

### GSH and GSSG Distribution in Milling Fractions

Figures 1 and 2 show the distribution of GSH and GSSG in milling fractions and straight-run flour of four wheat cultivars. The two Australian cultivars, Frame and Janz, had higher levels of GSH in all millstreams than the two New Zealand cultivars, Sapphire and Monad. Except for the break millstreams, the Australian cultivars also had higher levels of GSSG than the New Zealand cultivars, although the differences were generally not as great as with GSH. Again, the levels of GSH and GSSG in straight-run flours were higher in the Australian cultivars, but all cultivars were within the same range as European, UK, and North American straight-run flours (Sarwin et al 1992; Schofield and Chen 1995). The GSH/GSSG differences between Australian and New Zealand

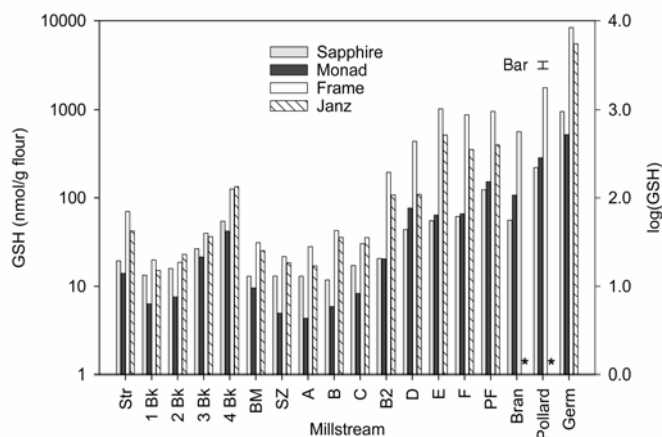
wheat may be caused by an environmental effect such as the level of trace elements in the soil; New Zealand soils and wheat grain are known to be deficient in selenium and marginally deficient in copper. Also, the pH values of soil in the Canterbury region of New Zealand ( $\approx$ pH 6) are higher than the pH of soils in Australia ( $\leq$ pH 5), and because minerals are more soluble at low pH there would be more uptake of minerals into wheat in Australia than New Zealand. The uptake of certain levels of copper in Arabidopsis plants increases the levels of GSH and GSSG (Drazkiewicz et al 2003).

Chen (1994) reported slightly higher levels of GSH and GSSG in break flour streams on Buhler milling than in reduction flour streams. On the commercial mill used in this study, the distribution patterns of GSH and GSSG were more varied. Generally, the early break flour streams (1Bk and 2Bk), BM stream, SZ stream, and early reduction flour streams (A–C) all had similar low levels of GSH and GSSG. The late break flours (3Bk and 4Bk) and reduction flours (B2–PF) had increasingly higher levels of GSH and GSSG. This probably reflects increasing contamination of these millstream flours with aleurone (enriched in the pollard) and germ, which generally contain much higher levels of GSH and GSSG than do millstream flours (Figs. 1 and 2).

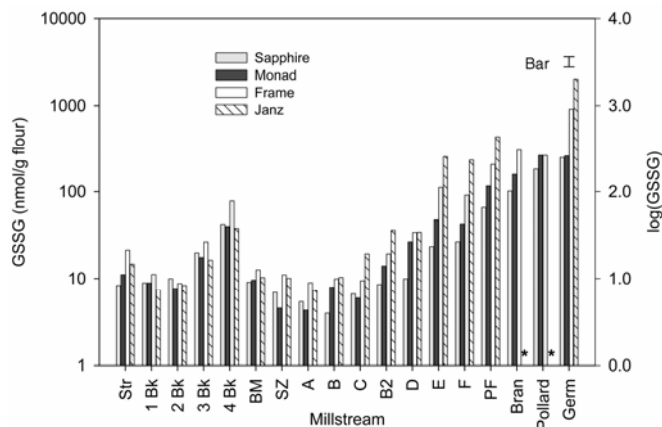
Sarwin et al (1992) found that the levels of GSH and total glutathione (GSH plus GSSG) in straight-run flour increased strongly with the ash content, which corresponds to the extraction grade of the flours. They suggest that as GSH and GSSG are preferentially located in the germ and aleurone cells of the wheat grain, their concentration in flour may increase with increasing extraction grade. Every et al (2002) reported the distribution of ash in the same millstream flours studied here. The patterns of ash distribution in millstream flours are very similar to the pattern of GSH and GSSG distribution (Figs. 1 and 2), and Table I shows good correlations between ash content in millstream flours and GSH and GSSG content ( $r = 0.84\text{--}0.98$ ) for all cultivars. This supports the suggestion that the higher GSH and GSSG levels in higher extraction grade flours are due to contamination with the germ and aleurone components of wheat.

### Relationships of GSH and GSSG to Other Chemical Properties of Flour

Apart from the GSH and GSSG data presented here, the quantitative data for other chemical properties of millstream flours used in this study have been presented previously (Every et al 2002; Sutton and Simmons 2006). The correlation coefficients for chemical properties of flour that generally showed moderate to strong correlations are shown in Table I. Other chemical properties such



**Fig. 1.** Distribution of GSH in millstream fractions and straight-run flour of four wheat cultivars. Bar indicates least significant difference at the 5% level (df 70) between two means on the log scale derived from analysis of variance. Locations marked \* have no data for bran or pollard of Janz.



**Fig. 2.** Distribution of GSSG in millstream fractions and straight-run flour of four wheat cultivars. Bar indicates significant difference at the 5% level (df 70) between two means on the log scale derived from analysis of variance. Locations marked \* have no data for bran or pollard of Janz.

as total protein, SDS-soluble protein, SDS-insoluble protein, gliadin, and glutenin protein subunit content generally showed weak or no correlation to GSH/GSSG content of millstream flours (correlation data not shown).

As discussed above, the strong correlations between ash and GSH/GSSG indicated that much of the GSH/GSSG in millstream flours is likely to be derived from contamination with bran, aleurone, and germ. The strong correlation between the early-eluting albumin/ globulin from RP-HPLC and GSH/GSSG (Table I) may also indicate that some of this albumin/globulin in the millstream flours derives from contamination with bran, aleurone, and germ. This view is supported by the demonstration of a very strong correlation between early-eluting albumin/globulin and ash ( $r = 0.984-0.995$ ). The SE-HPLC peak 4 proteins consist mostly of low molecular weight albumins and globulins and, as discussed above, the moderate to strong correlations of peak 4 cysteine with GSH/GSSG may be a consequence of contamination of flour with bran, aleurone, and germ.

On the other hand, the general lack of correlation between GSH/GSSG and the gliadin and glutenin fractions measured by RP-HPLC is consistent with the fact that these proteins are derived from the endosperm. Also, SE-HPLC peaks 1-3 mostly consist of gliadin and glutenin which, unlike GSH, GSSG, and ash, do not arise through contamination of flour by bran, aleurone, and germ. Thus, the moderate to strong correlation between GSH/GSSG and the cysteine content of soluble-protein peaks 1-3 cannot be explained by contamination of flour with nonendosperm components of grain. These correlations may indicate that the higher the level of GSH in millstream flours, the greater the protection of protein cysteine from oxidation. As indicated by the correlations in Table I, millstream flours with high levels of GSH/GSSG also have high levels of albumin/globulin, particularly the early-eluting

albumin/globulin from RP-HPLC. As suggested for GSH, certain components of these albumin/globulin fractions, of flour may prevent oxidation of cysteine in protein and may indeed promote reduction of protein disulfides to cysteine in protein. Three albumin/globulin components found in cereal grain that may serve this role are thioredoxin (Wong et al 1993; Jarraud et al 2000), glutathione reductase (Sha et al 1997), and glutathione reductase (Jarraud et al 2000). The  $x$ -fraction of flour albumins described by Every et al (1999) may also promote the high protein cysteine content in the millstream flours that contain high albumin/globulin and GSH/GSSG content.

### Relationship of GSH and GSSG to Breadmaking Properties

Figures 3 and 4 show the baking properties of millstream flours without AA and the change in baking properties when AA is added to the dough. Bread made without AA from the tail-end reduction flours (particularly E, F, and PF) had the lowest loaf volumes and crumb textures of all millstream flours for all cultivars. Also, bread made without AA from the tail-end 4 Bk flour had the lowest loaf volumes and crumb textures of all the break flours for all cultivars. These relatively low baking properties of tail-end break and reduction flours in the absence of AA were in spite of the 4 Bk flour having the highest protein content of all millstream flours and the E, F, and PF reduction flours having the highest proteins of all the reduction flours (Every et al 2002; Sutton et al 2006). The poorer baking properties of these tail-end millstream flours coincided with higher GSH and GSSG levels in these flours (Figs. 1-4), and Table II showed negative correlation coefficients between these parameters. Even when AA was used in baking there were still negative correlations between baking quality parameters and GSH/GSSG content in flour, although some correlations were small (Table II). These results,

TABLE I  
Correlations of Reduced Glutathione (GSH) and Oxidized Glutathione (GSSG) with Other Chemical Properties of Millstream Flours<sup>a</sup>

Flour Property	HPLC Fraction	Sapphire		Monad		Frame		Janz	
		GSH	GSSG	GSH	GSSG	GSH	GSSG	GSH	GSSG
Ash		<b>0.94</b>	<b>0.98</b>	<b>0.94</b>	<b>0.97</b>	<b>0.91</b>	<b>0.98</b>	<b>0.84</b>	<b>0.91</b>
Albumin/globulin	Early-eluting <sup>b</sup>	<b>0.93</b>	<b>0.96</b>	<b>0.95</b>	<b>0.97</b>	<b>0.94</b>	<b>0.96</b>	<b>0.88</b>	<b>0.94</b>
	Mid-eluting <sup>b</sup>	<b>0.60</b>	<b>0.68</b>	<b>0.60</b>	<b>0.68</b>	0.23	<b>0.55</b>	0.41	0.41
	Late-eluting <sup>b</sup>	<b>0.86</b>	<b>0.88</b>	<b>0.86</b>	<b>0.84</b>	<b>0.82</b>	<b>0.95</b>	<b>0.82</b>	<b>0.86</b>
Cysteine of soluble-protein	Peak 1 <sup>c</sup>	<b>0.94</b>	<b>0.84</b>	<b>0.87</b>	<b>0.79</b>	<b>0.96</b>	<b>0.81</b>	<b>0.97</b>	<b>0.91</b>
	Peak 2 <sup>c</sup>	<b>0.91</b>	<b>0.78</b>	<b>0.85</b>	<b>0.76</b>	<b>0.95</b>	<b>0.75</b>	<b>0.97</b>	<b>0.89</b>
	Peak 3 <sup>c</sup>	<b>0.94</b>	<b>0.82</b>	<b>0.88</b>	<b>0.81</b>	<b>0.98</b>	<b>0.77</b>	<b>0.95</b>	<b>0.97</b>
	Peak 4 <sup>c</sup>	<b>0.96</b>	<b>0.85</b>	<b>0.92</b>	<b>0.86</b>	<b>0.97</b>	<b>0.82</b>	<b>0.96</b>	<b>0.97</b>
Cysteine of insoluble-protein	Peak 1 <sup>c</sup>	<b>0.75</b>	<b>0.63</b>	0.28	0.14	0.31	0.01	<b>0.77</b>	<b>0.87</b>
	Peak 2 <sup>c</sup>	<b>0.90</b>	<b>0.83</b>	<b>0.79</b>	<b>0.73</b>	<b>0.79</b>	<b>0.57</b>	<b>0.87</b>	<b>0.95</b>
	Peak 3 <sup>c</sup>	<b>0.84</b>	<b>0.77</b>	<b>0.71</b>	<b>0.65</b>	<b>0.74</b>	<b>0.57</b>	<b>0.73</b>	<b>0.78</b>
	Peak 4 <sup>c</sup>	<b>0.83</b>	<b>0.75</b>	<b>0.84</b>	<b>0.78</b>	<b>0.84</b>	<b>0.62</b>	<b>0.82</b>	<b>0.85</b>

<sup>a</sup> Correlation coefficients (between means for 15 millstream flours)  $>0.52$  or  $<-0.52$  are in bold and considered weak, moderate, or strong. Lesser positive correlations or greater negative correlations than these values are considered to be poor.

<sup>b</sup> Fractions from RP-HPLC

<sup>c</sup> Fraction descriptions from SE-HPLC were similar to those described by Gupta et al (1993). Peak 1 = polymeric proteins (MW  $> 300,000$ ); Peak 2 = polymeric proteins (MW  $< 300,000$ ); Peak 3 = gliadins; Peak 4 = albumins and globulins.

TABLE II  
Correlations of Reduced Glutathione (GSH) and Oxidized Glutathione (GSSG) with Breadmaking Properties of Millstream Flours<sup>a</sup>

	Sapphire		Monad		Frame		Janz	
	GSH	GSSG	GSH	GSSG	GSH	GSSG	GSH	GSSG
Vol no AA	<b>-0.88</b>	<b>-0.77</b>	<b>-0.74</b>	<b>-0.66</b>	<b>-0.92</b>	<b>-0.79</b>	<b>-0.89</b>	<b>-0.89</b>
Vol with AA	<b>-0.74</b>	<b>-0.62</b>	<b>-0.60</b>	-0.51	<b>-0.93</b>	<b>-0.86</b>	<b>-0.85</b>	<b>-0.88</b>
Vol change <sup>b</sup>	-0.16	-0.09	-0.01	0.06	-0.07	-0.23	-0.30	-0.43
Tex no AA	<b>-0.63</b>	<b>-0.58</b>	<b>-0.88</b>	<b>-0.82</b>	<b>-0.65</b>	<b>-0.66</b>	<b>-0.75</b>	<b>-0.68</b>
Tex with AA	<b>-0.77</b>	<b>-0.70</b>	-0.40	-0.27	<b>-0.89</b>	<b>-0.81</b>	<b>-0.66</b>	<b>-0.83</b>
Tex change <sup>b</sup>	-0.39	-0.35	<b>0.57</b>	<b>0.64</b>	-0.36	-0.27	-0.06	-0.32

<sup>a</sup> Correlation coefficients (between means for 15 millstream flours)  $>0.52$  or  $<-0.52$  are in bold and considered weak, moderate, or strong. Lesser positive correlations or greater negative correlations than these values are considered to be poor.

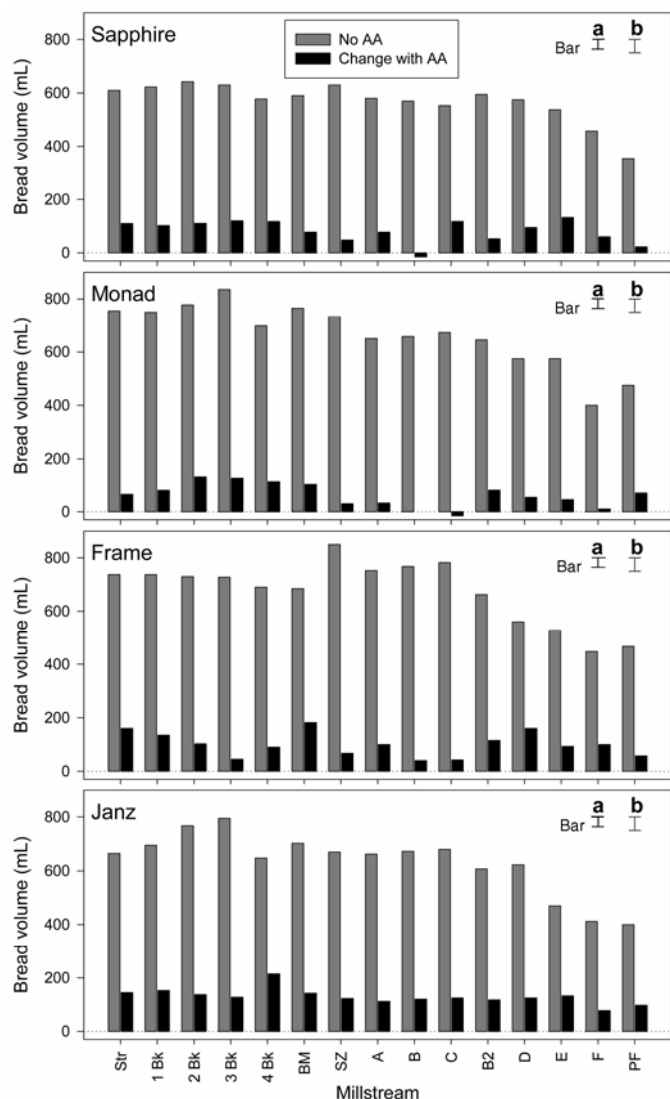
<sup>b</sup> Vol or Tex change are determined as volume or texture with ascorbic acid (AA) minus volume or texture without AA.

which are based on endogenous levels of GSH/GSSG in flour, are consistent with well-established results using straight-run flour samples in which high levels of exogenous GSH/GSSG (added either as germ or pure compounds) are harmful to bread quality. On the other hand, the results with millstream flours are inconsistent with the results of Schofield and Chen (1995) and Bollacker et al (2000) that suggest the volume and quality of bread made with a variety of straight-run flours has no relation to the levels of endogenous GSH/GSSG in the flour. For the millstream flours, GSH and GSSG may also contribute only a small detrimental effect to bread quality. Millstreams containing high GSH and GSSG also contain high ash content, which could indicate high levels of contamination with trichomes or brush hairs of the outer pericarp in bran, and trichomes cause loss in bread quality (Gan et al 1988). These trichomes may be causing more loss in bread quality than the high GSH and GSSG in the high ash millstreams.

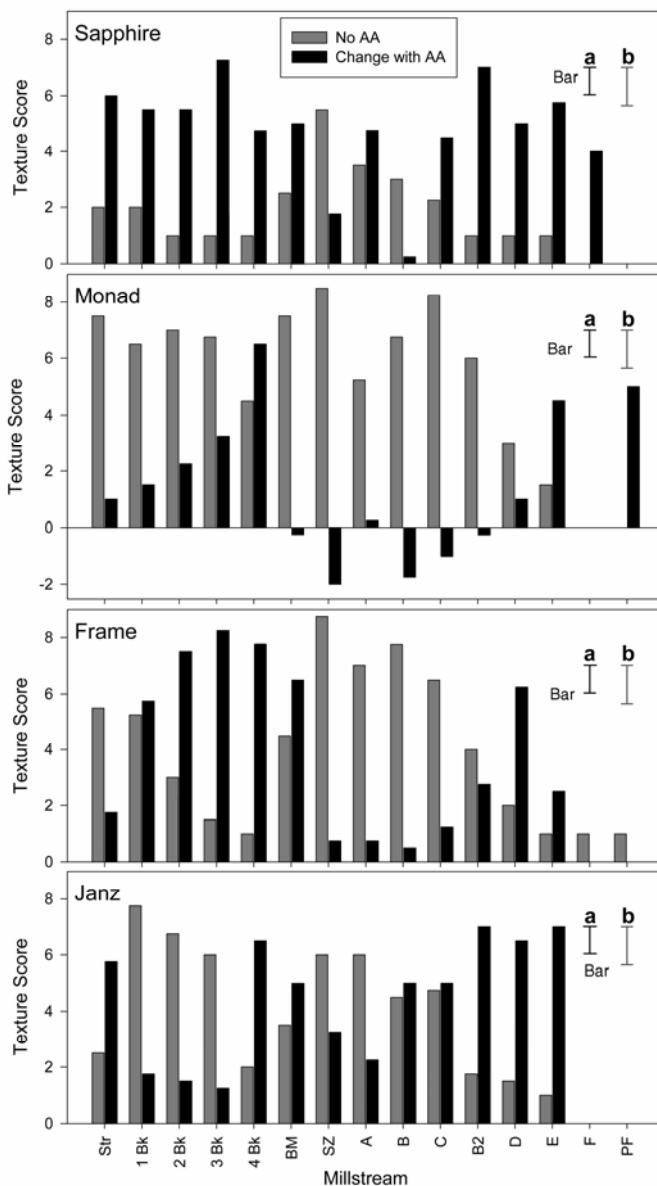
The volume and texture changes due to AA tended to be least in some early reduction flours (A–C) and the SZ, F, and PF millstreams (Figs. 3 and 4), but the overall pattern of these changes generally bore little relationship to the pattern of GSH/GSSG in millstream flours (Figs. 1 and 2). Table II demonstrates poor corre-

lation between bread quality changes with AA and GSH/GSSG levels, except a weak positive correlation of texture and GSH/GSSG for Monad. As for GSH/GSSG, relationships between baking properties and cysteine content of both SDS-soluble and SDS-insoluble protein showed moderate to strong negative correlation coefficients when bread was made without AA but poor correlation between protein cysteine and baking response to AA (data not shown). Also, the correlation between the quantity of early- and late-eluting albumin/globulin fractions from RP-HPLC and baking data were similar to the correlation between GSH/GSSG and baking data (data not shown).

Thus, with regard to millstream flours, levels of GSH/GSSG do not appear to be related to the AA improver effect in baking. This is in contrast to experiments using single straight-run flour samples in which Chen (1994) and Every et al (2000) showed that addition of exogenous GSH actually improved the quality of bread made with AA and enhanced the AA improver effect. There was an optimum level of GSH (endogenous plus exogenous) for



**Fig. 3.** Volume of bread made from millstream flours of four wheat cultivars. Loaves made without any oxidative improver (no AA) (grey bars). Loaves made with AA and the consequent increase or decrease in volume (change with AA) (solid bars). Least significant differences (a no AA; b, change with AA) at the 5% level (df 119) derived from analysis of variance.



**Fig. 4.** Crumb texture of bread made from millstream flours of four wheat cultivars. Loaves made without any oxidative improver (no AA) (grey bars). Loaves made with AA and the consequent increase or decrease in crumb texture (change with AA) (solid bars). Least significant differences (a no AA; b, change with AA) at the 5% level (df 117) derived from analysis of variance.

bread quality baked with AA of  $\approx 100$ – $200$  nmol of GSH/g of flour; higher levels of GSH (400 nmol/g of flour) reduced the baking quality, even in the presence of AA. This relationship between GSH and bread quality when using a single flour sample appears to be obscured when correlating these parameters using many different millstream flours or straight-run flour samples. Bread quality and the effect of AA on a wide variety of flour types is likely to be determined by many variables besides GSH/GSSG content in flour such as protein composition, protein cysteine content, and redox enzyme composition.

It is likely that both reducing and oxidizing systems are necessary in dough to achieve optimal dough and bread properties. In addition to the above GSH/GSSG/AA/DHA redox system, a NADP/thioredoxin redox system added to dough has strengthened dough (Wong et al 1993). The results of Jarraud and Kobrehel (2000) suggest that free sulfhydryl groups, formed through reduction by the NADP/thioredoxin system and a glutathione system, are essential in forming gluten aggregates. As mentioned before, the thioredoxin protein and other low molecular weight disulfide reducing proteins (Sha et al 1997; Every et al 1999; Jarraud et al 2000) may be part of the early- or late-eluting albumin/globulin fractions and together with AA/DHA redox system in dough may produce optimal dough and bread properties.

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