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Shore Agricultural Sustainability Program

Final Report

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EXECUTIVE SUMMARY

This report encompasses findings from a two-year integrated study that began April 1, 2002. The overall project has four distinct stages: crop production (stage 1), laboratory analysis (stage 2), crop enhancements/changes (stage 3), and feasibility studies (stage 4). It was scheduled to run for a minimum of three years.

Funding was provided by the Maryland Center for Agro-Ecology, Inc. for the first two years, within which the efforts were focused on the production of wheat and the quality assessment of the bread product so that a model could be developed for establishing application indicators for other grains such as corn and soybeans.

A spectrum of intensive management practices, as well as optimum seeding rate, year and location agronomic performance, were tested to provide recommendations to achieve top performance. By integrating with the new gradient elution techniques that offered desirable retention factor and resolution, a novel method was developed as a quality assessment tool for detecting key amino acids in wheat flour.

The addition of proline and glutamine to winter wheat flour was proven to affect the functional properties of wheat dough as well as the bread. Addition of proline or glutamine individually did not provide significant improvements in soft wheat dough and bread properties. Combination of glutamine and proline could synergistically enhance the viscous properties of dough and bread attributes such as loaf volume in soft wheat. Addition of glutamine to hard wheat increased the glutenin subunit while enhancing the development of gluten during mixing. Conversely, despite the increase in glutenin subunit, proline hindered gluten formation.

The findings in this study provide a foundation for characterizing the properties of dough and bread based on the contribution of key constituents and will be of interest to researchers and product developers for the development of specialty bread products. Additionally, should the degree of dependence of breadmaking properties on these two amino acids be elucidated, a better control of dough quality would be within reach, enabling selection criteria to be set for wheat breeders.

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INTRODUCTION

The Upper Eastern Shore of Maryland, also known as the corn belt of the Eastern Seaboard, produces corn, soybeans, and wheat as its principal farm products. Currently, almost all of these grains are sold to a commodity market, with most being used for animal feed. In a rapidly changing market, losses in local buying competition and other factors have reduced the premiums received by farmers. Data collected by a consulting firm working for the Chesapeake Fields Institute indicate that Upper Eastern Shore farmers are experiencing negative cash flows, and many farmers are leaving the industry altogether. The traditional focus by farmers on production quantity, as opposed to quality, must be reversed to ensure a sustainable and more profitable agricultural industry. Commodity grain prices have little bullish news (as in the stock market) in the foreseeable future. Compounding this is the fact that international markets continue to provide formidable competition, thereby driving prices even lower. Farm Credit data drawn from area farmers also paint a dismal picture, indicating that cash grains have shown a negative cash flow for 6 out of the past nine years. Our area grain marketing group calculates the break-even cost of production for 2001 to be as follows: Wheat: $2.91/70 bushel yield, Corn: $2.52/110 bushel yield, and Soybeans $5.25/ bushel yield. None of these price objectives were reached. Without USDA Loan Deficiency Payments (LDP), 2001 would have been devastating.

Protein content, usually constituting 7-15% of common flour on a 14% moisture basis, is an important criterion for marketing and purchasing wheat and, as such, is included in almost every flour specification. Research has shown that grain protein content affects the flour yield and breadmaking characteristics such as gluten strength of wheat (Metho et al., 1999). With climatic factors significantly influencing protein levels in wheat, suitable agronomic and fertility management practices are needed to ameliorate the climatic effects (Rao et al., 1993). Cultivar differences in breadmaking, quality characteristics and soil fertility status were found to affect grain protein yield, protein content, flour yield, loaf volume potential, and water absorption but not peak mixing time and dough characteristics (Metho et al., 1999). Johansson et al. (2001) reported that increased nitrogen supply correlated significantly to an increase in all protein components containing gliadins and glutenins, but not to those containing albumins and globulins. Other researchers later supported with evidence that grain protein concentration and grain protein yield increased consistently with increasing nitrogen fertilizer and with split nitrogen application (Ayoub et al., 1995; Dalal et al., 1997).

In general, for hard wheat, the higher the protein content, the better the breadmaking characteristics of the flour. High-protein flour is also likely to require more water and mixing time to reach an optimum consistency for processing or product purposes. Bread dough with high protein levels is generally more resistant to over-working during mixing. The increase in protein components containing gliadins and glutenins were correlated significantly with an increase in protein concentration and bread volume (Johansson et al., 2001; Hubik, 2000). Total protein content and the ratio of glutenin to gliadin, two major fractions of gluten, have been identified to independently affect dough and baking properties. Enhancements of important breadmaking parameters, including mixing time, maximum resistance to extension, extensibility, and loaf volume, were achieved with increased protein content or glutenin-to-gliadin ratio (Uthayakumaran et al., 2000). Preston et al. (1995) reported that baking water absorption and loaf volume showed significant positive correlations with protein content. However, flour starch

damage decreases in response to protein content. Baking strength index, a measure of loaf volume on a constant protein basis, also decreased with increasing protein content (Preston et al., 1995). Therefore, it is vital to develop a spectrum of analyses to identify specified protein characteristics that would facilitate optimization of quality attributes and product uniformity to meet specific customer requirements.

Hard Red Winter Wheat (HRWW) is primarily produced in the area of the United States designated as the Wheat Belt. Soft Red Winter Wheat (SRWW) has been the class of wheat primarily produced throughout the eastern United States. The milling of all-purpose flour by eastern flour mills requires a mixture of hard and soft wheat flour. In addition, many products that were exclusively made with SRWW flour previously are now requiring the addition of HRWW flour. This is most evident in packaged mixes that can be baked in a microwave oven. The inclusion of some hard wheat flour in these micro-wavable mixes yields products that have better consistency compared to when only soft wheat flour is used for microwave baking situations. The demand of flour mixes has required eastern flourmills to import and mill HRWW. This importation of HRWW to the east is facing ever-increasing transportation costs. One solution to this increasing expense has been to produce HRWW in the east. A program (now in its third year) to do just that was established by ConAgra and AgriPro. This program is currently centered in southeastern Pennsylvania and is attempting to attract producers in Maryland. The program has a goal of 70,000 acres of HRWW production within the next couple years with the demand potential even greater.

One primary limitation to the production of HRWW in this region is information about production practices necessary to produce high yielding, high quality HRWW. Protein is one of the two primary quality traits that has been identified as important to successful production of HRWW in this region. ConAgra will pay premiums for wheat that has protein contents eleven percent or greater. Protein content can be influenced by nitrogen management practices. Excessive use of nitrogen and the ultimate loss of that nitrogen from the farmland on the DelMarva have been cited as one of the key elements contributing to the degradation of the Chesapeake Bay.

One of the main objectives of this project will be to assess various nitrogen management strategies for HRWW production and the relationship to the production of both acceptable quantity and quality of the grain. This must be accomplished within the context of the current nutrient management regulations with special emphasis upon proper management of nitrogen so that potential field loss is kept to an absolute minimum.

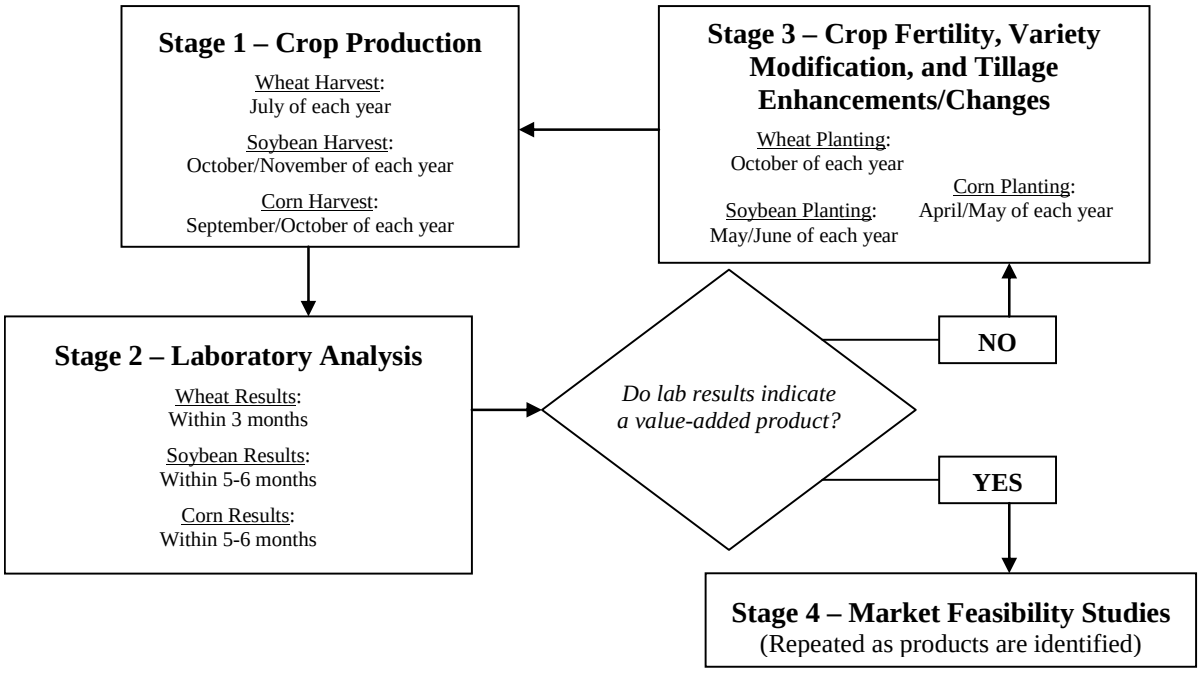
Other objectives of this project will be to assess other production management practices and identify which practices will produce high yielding, high quality HRWW economically and in an environmentally friendly manner. In addition, since this ConAgra/AgriPro program is still in its youth, the number of varieties that are currently available to producers is very small (two). This limited number of varieties jeopardizes the success of the program in light of the rapid genetic changes that disease organisms undergo in the more humid environments found in this region. An excellent example is powdery mildew. This fungal disease undergoes race changes regularly. It is not uncommon for a wheat variety that is initially marketed as resistant to powdery mildew to be susceptible to this disease within five years after it is first introduced.

This limited gene pool that exists with only two varieties warrants an agronomic performance evaluation of some other publically derived HRWW varieties.

The long-term objective of this research is to enable Eastern Shore farmers to engage in an alternative crop production process through which value-added enhancements are achieved and, subsequently, a more sustainable and profitable agricultural community emerges. Achievement of the long-term objective will result in preservation of the land through profitable and environmentally sound farming practices. The short-term objectives are to: *(1) develop an integrated quality assessment methodology; (2) incorporate the quality assessment methodology into production practices; and (3) establish a baseline against which food quality may be measured.* These objectives support the potential for biological and economic viability of wheat, corn, and soybeans in Maryland. In this particular project, our efforts were focused on the production of wheat and its applications so that a model could be established for the evaluation of other grains such as corn and soybeans.

Hypothesis: The utilization of baseline food value data and other quality controls as the driving forces behind production practices will result in a quality-oriented agricultural production system that ensures higher premiums for farmers. Chesapeake Fields Institute (CFI) was developed to research opportunities to increase farm profitability by looking at producing value-added food products instead of commodity grains. It is CFI's hope that baseline data will serve as a catalyst for future production field research.

Value-Added Research Model



MATERIALS AND METHODS

Crop Production and Changes

HRWW performance using intensive management practices (foliar fungicide use):

Two hard red winter wheat varieties (Agripro brands 'Hondo' and 'Charter') that can be locally grown under contract via the ConAgra/Agripro program were compared to a commonly grown, high yielding SRWW variety (Agripro brand 'Patton') during 2002. A variety of nitrogen fertilizer treatments were evaluated with and without the use of a foliar fungicide.

HRWW performance using intensive management practices (nitrogen rates and timing):

Agripro brands 'Charter' and 'Hondo' were evaluated at two locations per year for two years (2002 and 2003) using different nitrogen management strategies. Nitrogen treatments were 1) the use of fall nitrogen; 2) the timing [greenup (Feekes growth stage 2) and jointing (Feekes growth stage 6)] and rates for spring nitrogen applications; and 3) the effect on yield and protein of late [boot (Feekes growth stage 8) and/or heading (Feekes growth stage 10.1)] nitrogen applications. This study included an evaluation of the cost-effectiveness of these various nitrogen management practices.

Optimum seeding rate and agronomic performance

Both hard (3 and 2 for 2002 and 2003, respectively) and soft (2 and 3 for 2002 and 2003, respectively) wheat varieties planted over a range of seeding rates (750,000 to 1,750,000 seeds $acre^{-1}$) at two locations per year for two years were evaluated. Wheat Belt derived hard red winter wheat varieties were evaluated for the agronomic performance when grown under Maryland conditions. Multi-location testing was conducted during three years (2001-2003) comparing hard red winter wheat varieties to elite soft red winter wheat varieties. During the third year (2003), a select number of varieties (7) were tested by Dr. Martin Lo, Associate Professor for Food Sciences at the University of Maryland, for characteristics deemed important for production of specialty wheat products.

Analytical Procedures

Wheat samples, standards, and reagents

Samples of wheat flour were prepared by grinding the grains of Karl 92, a cultivar of hard red winter wheat grown regionally on the Eastern Shore of Maryland, using a laboratory disc mill (Model 3600, Perten Instruments, Huddinge, Sweden), and then stored at -18°C prior to analysis. Anhydrous ethanol, hydrochloric acid (HCl), triethylamine (TEA), anhydrous sodium phosphate dibasic (Na_2HPO_4), and sodium acetate trihydrate (HPLC-grade) were purchased from Fisher Scientific (Fair Lawn, NJ). Amino acid standards, as well as crystalline phenol and reagents for HPLC analysis, including water, methanol, and acetonitrile were supplied by Sigma Aldrich (St. Louis, MO). Individual standards of proline, glutamine, and glutamic acid (10 μmol/mL) were prepared with 0.1 N HCl. The use of glutamic acid standard was necessary,

since in the case of glutamine, conversion to glutamic acid happens after acid hydrolysis so that quantitative analysis necessitates that both the acid and amide forms be taken into consideration (White and Hart 1992; Molnar-Perl 1994; Tyler 2001). The derivatizing agent phenylisothiocyanate (PITC) was obtained from Pierce Chemicals (Rockford, IL).

Hydrolysis

To release key amino acids from wheat proteins, a standard hydrolysis procedure modified from Bidlingmeyer et al. (1987) and Albin et al. (2000) was used. All glassware used in hydrolysis were first washed with 6N HCl, rinsed with deionized water, and dried. Triplicate samples of each type of wheat flour were accurately weighed (ca. 200 mg each) into 15 mL screw-capped test tubes. Twelve milliliters of 6N HCl and 0.5 mg crystalline phenol were also added to the tubes (Cohen and others 1984; Albin and others 2000). The tubes were thoroughly flushed with nitrogen gas, capped, mixed, and then hydrolyzed in an oven at 110°C for 24 h. After hydrolysis, the samples were allowed to cool to room temperature before 0.5 mL of norleucine (50 μmol/mL) was added as the internal standard. The tubes were vortexed and centrifuged to remove debris. A 200-μL aliquot of the hydrolysate was transferred to a 1.5 mL microcentrifuge tube (Fisher Scientific, Pittsburgh, PA), and diluted with distilled, deionized water in a 1:10 ratio.

Pre-column derivatization

Standard solutions of proline, glutamine, and glutamic acid were prepared by dissolving 0.1 mole of each amino acid in 100 mL 0.1 N HCl. Amino acid concentrations of 10.00 μmol/mL, 7.50 μmol/mL, 5.00 μmol/mL, 2.50 μmol//mL, and 1.25 μmol/mL of each amino acid were prepared by diluting amino acid solutions with the redrying solution composed of ethanol:water:triethylamine (2:2:1), which is required in this two-step drying process to complete derivatization. To optimize the pre-column derivatization process, 20 μL of the diluted standards transferred into 1.5 mL microcentrifuge tubes were subjected to three different drying methods. Method A used a freezedrier (Dura-Top Bulk Tray Dryer, FTS System Inc, Stone Ridge, NY) to dry the standards under vacuum for 24 h at -40°C (Albin and others 2000). After adding 10 μL the redrying agent (2:2:1 ethanol:water:triethylamine), the dried standards were vortexed to resolubilize amino acids and then vacuum-dried for 6-8 h. The redried standards were derivatized with 10 μL of 7:1:1:1 ethanol:water:triethylamine:PITC (Heinrikson and Meredith 1984; Inglis and others 1988; White and Hart 1992; Molnar-Perl 1994) and allowed to stand at room temperature for 20 min. After derivatization, the standards were vacuum-dried for another 6-8 h and then resuspended with 400 mL of diluent, which consisted of a 95:5 (v/v) phosphate buffer (5mM sodium phosphate dibasic, pH 7.4):acetonitrile (White and Hart 1992). The reconstituted standards were vortexed and then filtered through a 0.25 μm filter into HPLC vials for analysis.

Methods B and C both used a SpeedVac vacuum concentrator (Model SPD121, Thermo Savant, Marietta, OH) capable of rapid dehydration under vacuum. The same reagent compositions were used for the redrying and derivatization steps as in Method A. The drying temperatures, however, were different; 60°C-65°C for Method B and 45ºC-50ºC for Method C. The concentrations of proline, glutamine, and glutamic acid were calculated using respective

standard curves with corresponding R^2 values at 0.9806, 0.9967, and 0.9864. The drying time was determined based on the percentage of amino acids recovered. After the optimum method for pre-column derivatization was identified, the best method was applied to hydrolyzed wheat samples following the aforementioned hydrolysis procedure.

Chromatography and data analysis

PTC-amino acids were separated by RP-HPLC using a Shimadzu LC2010A (Columbia, MD) equipped with serial dual plunger pumps, an oven, an automated sampling injection unit, and a UV-VIS detector (D_2 lamp light source) with a wavelength range of 190 to 600 nm. The column used was a Pico-Tag 3.9 × 300 mm reverse-phase column (Waters, Milford, MA). Sample injection volume was 20 μL. A variety of mobile phase and gradient compositions were tested in order to identify the most suitable gradient elution techniques for analyzing proline and glutamine in wheat flour. The chromatograms were acquired and the peaks with a Gaussian (symmetrical) distribution were analyzed for the retention factor (formerly called capacity factor), *k'*, a measure of the time the sample component resides in the stationary phase relative to the time it resides in the mobile phase (Bidlingmeyer 1992; Siouffi 2000):

$$k' = \frac{t_r - t_o}{t_o} \quad (1)$$

where t_r is retention time of a retained solute and t_o retention time of the unretained (inert) solute. The resolution (R_s), which is defined as the peak separation divided by the mean peak width (Siouffi 2000), of the resulted peaks were calculated based on the following equation using the Class VP 6.0 software that came with the equipment:

$$R_s = \frac{t_{r2} - t_{r1}}{(\omega_2 + \omega_1)/2} \quad (2)$$

where t_{r2} and t_{r1} represent respectively the retention time of peaks 1 and 2 and ω is the peak width.

Application Feasibility Studies

Wheat samples, standards, and reagents

Samples of wheat flour were prepared from the grains of Karl 92, a hard red winter wheat variety, and Sisson 36, a soft red winter wheat variety, both grown regionally on the Eastern Shore of Maryland. After cleaning, the wheat was moistened by holding in tempering bins for 20 h to reach 14% moisture content (wet basis) before milling (Yuan and others 2003). Such a tempering process is known to make the endosperm less susceptible to starch damage. The moisture content of the grains was checked by a PM-400 Grain Moisture Tester (Kett US, Villa Park, Calif., U.S.A.). The wheat was milled on a laboratory disc mill (Model 3600, Perten Instruments, Huddinge, Sweden) equipped with a Type 1 disk, which yielded similar ($P < 0.05$) whole-wheat flour extraction rates of 99.2% and 99.7% for Karl 92 and Sisson 36, respectively. The average feed rate of wheat grains to the mill was 150 g/min. The damaged starch was assessed using the AACC Method 76-31 (AACC 1995).

Anhydrous ethanol, hydrochloric acid (HCl), triethylamine (TEA), anhydrous sodium phosphate dibasic (Na_2HPO_4), and sodium acetate trihydrate (HPLC-grade) were purchased from Fisher Scientific (Fair Lawn, N.J., U.S.A.). Amino acid standards for proline, glutamine, glutamic acid and norleucine, as well as crystalline phenol and reagents for High Performance Liquid Chromatography (HPLC) analysis, including water, methanol, and acetonitrile were supplied by Sigma Aldrich (St. Louis, Mo., U.S.A.). Individual standards of proline, glutamine, and glutamic acid (10 μmol/mL) were prepared with 0.1 N HCl. The use of glutamic acid standard was necessary, since in the case of glutamine, conversion to glutamic acid happens after acid hydrolysis so that quantitative analysis necessitates that both the acid and amide forms be taken into consideration (White and Hart 1992; Molnar-Perl 1994; Tyler 2001). The derivatizing agent phenylisothiocyanate (PITC) was obtained from Pierce Chemicals (Rockford, Ill., U.S.A.).

Dough preparation and breadmaking

Dough samples for texture testing were prepared using a modified procedure from Tseng and Lai (2002). Two hundred grams of each type of wheat flour were mixed with the optimum % of water added (Pratt 1971) using a KitchenAid Model K45 bench-top mixer equipped with a 4.5 Qt. stainless steel mixing bowl (Hobart Manufacturing Co., Troy, Ohio, U.S.A.) at medium speed (setting #2) for 6 min, the optimum mixing time determined during mixing based on the feel and appearance of the dough. The optimum % of water absorption was determined based on farinographic absorption, which gives an indication of how much water (mL) must be added for 100 g of flour (14% moisture) to get a 500 Brabender Units (BU) consistency in the farinograph (Calderón-Dominguez and others 2004). The optimum water absorption was determined to be 60% and 57.5% for Karl 92 and Sisson 36, respectively, to fit the value of flour farinographic absorption (in mL water/100 g flour), which corresponds to visual appearance as smooth and neither too sticky nor too dry (Tseng and Lai 2002).

Bread samples were prepared using dry yeast (5.3% flour basis) following the optimized straight-dough bread-making method from AACC Method 10-10B without addition of optional ingredients such as dough oxidant to assess the natural breadmaking potential of each flour (AACC 1995). Each flour was baked in triplicate. One hundred gram pup loaves were produced based on a 90-min fermentation time (Tilley and others 2001) and allowed to stand for one day at room temperature prior to texture analyses.

Texture analyses of dough and bread samples

Dough textural properties were measured in triplicate with four trials per replicate using a TA.XT2*i* Texture Analyzer (Texture Technologies Corp., Scarsdale, N.Y., U.S.A.). Dough stickiness, cohesion/dough strength, and adhesion properties were determined using the SMS/Chen-Hoseney Dough Stickiness cell coupled with a 25 mm perspex cylinder probe (5 kg load cell). Dough stickiness, which measures the force required to release the probe from the dough, and cohesion/dough strength, which is the quantity to simulate the strength of the internal bonds making up the body of the dough, were conducted using a force of 40g through a distance of 4 mm, at a pre-test speed of 0.5 mm/s, a test speed of 0.5 mm/s, and a post-test speed of 10 mm/s (Chen and Hoseney 1995a). Dough extensibility was determined using the Kieffer dough and gluten extensibility rig probe. The dough was pressed into strips by a special dough presser

provided by Texture Technologies and extended until it reached its elastic limit. The test was conducted through a distance of 75 mm, at a pre-test speed of 2.0 mm/s, a test speed of 3.3 mm/s, and a post-test speed of 10 mm/s. For this particular test, the maximum distance that the dough reached without breaking was used as a measure of extensibility. To minimize variations due to moisture loss while waiting in line for analysis, only the measurements of the four strips most similar in surface dryness and elastic limit were recorded.

The loaf volume of the bread (100 g) was determined following AACC Method 10-10 by means of rapeseed displacement (AACC 1995). The texture properties (firmness, gumminess, springiness, resilience, and cohesiveness) of sliced bread samples were determined through AACC Method 74-09 using a 36 mm cylinder probe (AACC 1995). All samples were measured in triplicate with four trials per replicate. Firmness is the maximum force required while cutting under specific conditions. Gumminess is the quantity to simulate the energy required to disintegrate a semi-solid sample to a steady state of swallowing. Springiness is the rate at which a deformed sample goes back to its undeformed condition after the deforming force is removed. Resilience is a measurement of how the sample recovers from deformation both in terms of speed and forces derived. The moisture content (%) of the bread samples after standing at room temperature for one day, the same time as texture measurements, was also measured.

Effects of proline and glutamine

The effects of proline and glutamine on the functional properties of wheat dough were investigated using two approaches. First, the wheat variety determined to have lower concentrations of proline or glutamine was treated with the required amount of proline or glutamine dissolved in distilled water, reaching total volume 11.55 mL, to bring amino acid concentrations to the same level as the variety that has higher proline or glutamine contents. Second, a fixed known amount (5% by weight of wheat flour) of proline or glutamine aqueous solution was added to Karl 92 to compare the differences before and after treatment. Addition of proline or glutamine at 5% (w/w) was chosen because it represents a concentration increase of flour proline by five-folds, while doubling the glutamine concentration in the flour. Dough samples prepared from proline- or glutamine-treated wheat flours were subjected to textural analyses (Chen and Hoseney 1995a).

The effects of proline and glutamine on gluten formation were determined by isolating glutenin and gliadin fractions after dough mixing and after baking to evaluate the changes brought about by the addition. Glutenin and gliadin fractions were isolated using the low-cross-contamination protocols (Suchy and others 2003) with minor modifications. By using this method, protein fractions in the form of dry residue can be stored long-term at room temperature without adverse changes in nitrogen content. Bread samples were freeze-dried at -40ºC using a Dura-Top Bulk Tray Dryer (FTS System Inc, Stone Ridge, N.Y., U.S.A.). After freeze-drying, the samples were ground with dry ice using a Wiley Mill (Thomas Scientific, Swedesboro, N.J., U.S.A.). Four hundred mg of ground samples were hydrolyzed in 12 mL 6N HCl at 110ºC for 24 h. The extractable amount of proline and glutamine were analyzed by an improved HPLC method (Fermin and others 2003).

Statistical analyses

Data were analyzed using the Univariate and Mixed Procedures of the Statistical Analysis System version 6.02 (SAS Institute Inc., Cary, N.C., U.S.A.). Pairwise mean differences were evaluated using the Tukey's test ($\alpha = 0.05$), which operates with 3 or more samples and their means and tests the mean of each population against the mean of the other populations (Croarkin and Tobias 2002).

RESULTS AND DISCUSSION

Crop Production and Changes

Little fungal disease was present during 2002 so there was no positive benefit attained with the use of a foliar fungicide. Studies conducted prior to 2002 had evaluated 'Charter' and 'Hondo' for their fungal disease susceptibility and response to foliar fungicide use. Those studies had determined that 'Charter' and 'Hondo' are not highly susceptible to the primary fungal diseases (powdery mildew, septoria leaf spot, glume blotch, and leaf rust) that generally cause significant problems for Maryland wheat production. The combination of using resistant varieties along with an intensive, integrated pest management (IPM) scouting program, similar to a scouting program designed for soft red wheat, should be used for hard red winter wheat production. The application of a fungicide should be made only when it is determined necessary. This will avoid costly applications of unneeded chemicals. 'Charter' produced equivalent to the SRWW variety, 'Patton' with both varieties producing approximately 30% more wheat than 'Hondo'. 'Charter' clearly is the better performing variety that is currently available to producers through the ConAgra/Agripro program. These results were obtained using intensive management practices but the yield difference between 'Hondo' and the other two varieties in the study is similar to that observed in the Maryland Wheat Variety Performance Tests. Those tests have also determined that 'Charter' can consistently yield as well as many of the top-producing soft red winter wheat varieties that are currently available. Both hard red winter wheat varieties had test weight that met the minimum contract standard established by the ConAgra/Agripro program, 60 lb bu^{-1}. 'Hondo' had approximately 1% greater protein than both 'Charter' and 'Patton'.

In the study where HRWW performance was evaluated using intensive management practices (nitrogen rates and timing) it was found that there was no benefit for crop yield, wheat protein content or test weight attained with a fall application of nitrogen. This finding is not surprising considering that the benefits attained with the use of fall nitrogen depend upon a number of factors including soil type, previous crop performance, date of wheat planting, residual nitrogen present following preceding crop, precipitation received by previous crop, and fall/winter precipitation received by the wheat crop. Positive results with the utilization of fall nitrogen for wheat production are highly variable. A more definitive mechanism to determine if it is a necessary practice would be beneficial to both producers (cost-savings) and the environment (water quality). Moreover, split applications of nitrogen during the spring (1/2 at greenup and 1/2 at jointing) produced the most wheat and are recommended as efficient, environmentally friendly practices for applying spring nitrogen on wheat. Results from the two years of this work indicated that nitrogen rates of 45 to 60 lb a^{-1} per each spring application (90-

120 lb a^{-1} total) will produce maximum economic yield. The high end of the range is more appropriate for sandier soil types and the low end of the range better suited for silt and clay soil types under normal spring precipitation patterns. Seasonal rainfall or the use of supplemental irrigation should be taken into consideration when determining the rate of spring nitrogen to use for each application. If soil conditions are dry because of drought conditions, the rates should be reduced accordingly. Since 2003 was an exceptionally wet year, this study is being repeated during 2005 to fine-tune the spring nitrogen rates. Late applications of 15 lb N a^{-1} at the boot and/or heading growth stages did increase wheat protein content. This study determined that applications at both those stages did not increase protein content above what a single application at either growth stage attained. An economical analysis using the protein premiums per the ConAgra/Agripro contracts effective during the timeframe of this study determined that a late application of nitrogen was not cost-effective. This does not preclude the potential for this practice to enhance protein as well as provide a profitable return if the protein premiums are higher. Comparable to the results for study 1, 'Charter' was the superior variety for yield. 'Hondo' proved to be the superior variety for protein content comparable to the results observed in study 1. During 2002, both varieties were able to easily attain the minimum standard test weight of 60 lb bu^{-1} required by the ConAgra/Agripro program. For 2003, the standard test weight was not attained for either variety. There was an extremely wet, rainy spring and early summer that delayed maturity and impacted harvest. Test weights during 2003 reflected the poor conditions that existed during this year and were representative of what can happen to test weight when rainy weather is prevalent during harvest.

The optimum seeding rate for currently available hard red winter wheat cultivars and comparing those rates to seeding rates for soft red winter wheat cultivars was determined. A seeding rate of 1,250,000 viable seeds $acre^{-1}$ was found to be adequate for maximum yield attainment. This equates to 16.5 to 18 viable seeds ft^{-1} of row for 7-7.5 inch drill spacing. This seeding rate was sufficient for maximizing yield for hard and soft red winter wheat varieties. To achieve an adequate plant population suitable for optimum yield with any wheat variety, a farmer should calculate the actual planting rate in total seeds $acre^{-1}$ by dividing the seeding rate goal by the percent germination for the seed lot being planted. For example, a seed lot with a germination of 90% would require an actual planting rate of approximately 1,390,000 total seeds $acre^{-1}$ to attain a plant population of 1,250,000 seedlings $acre^{-1}$. Additionally, it is important to calibrate the grain drill each time a new lot of seed wheat is planted. Year and location agronomic performance results for all the varieties tested can be found at the University of Maryland Cropping Systems web site: www.mdcrops.umd.edu. A number of hard red winter wheat varieties were observed to yield comparably to top producing soft red winter wheat varieties. These varieties were also observed to have similar agronomic characteristics (head date, plant height, lodging and disease resistance) to the soft red varieties indicating that some will perform well under Maryland conditions. More importantly, however, for specialty wheat production in this region is the performance of these hard red wheat varieties when used to make specialty wheat products.

Key Constituents in Wheat Flour

Pre-column derivatization

Figure 1 compares the effectiveness of glutamine, proline, and glutamic acid recovery by three different pre-column derivatization methods. For all three amino acids the percentages of recovery were significantly higher when the SpeedVac concentrator was used than the freezedrier. The highest recovery at 89% was reached by using SpeedVac at 45ºC. The overall time required to complete each of the derivatization methods is summarized in Table 1. It should be noted that glutamine and glutamic acid recovery using freeze-drying was extremely poor even after the total of more than 36 h to complete the process. In SpeedVac vacuum-drying the samples at 60ºC took less than half of the time required at 45ºC, however, the reduced amino acid recovery percentages with increased variations failed to justify the speed of derivatization at 60°C, due in part to the decreased conformational stability at elevated temperatures. In the present study, since lowered recovery of the standards was obtained at temperatures lower than 40°C (data not shown), the initial drying step at 45°C appears to be the most adequate temperature to effectively derivatize amino acids using the SpeedVac. As glutamine converts into glutamic acid under acidic conditions, both glutamine and glutamic acid peaks should be quantified as a measure of glutamine. However, no glutamine was recovered by freeze-drying; indicating that the freeze-drying process was not feasible for pre-column derivatization.

Figure 1—Comparison of amino acid recovery using different methods for pre-column derivatization. □, Freezedryer at -40°C; ▩, SpeedVac at 45°C; ■, SpeedVac at 60°C.

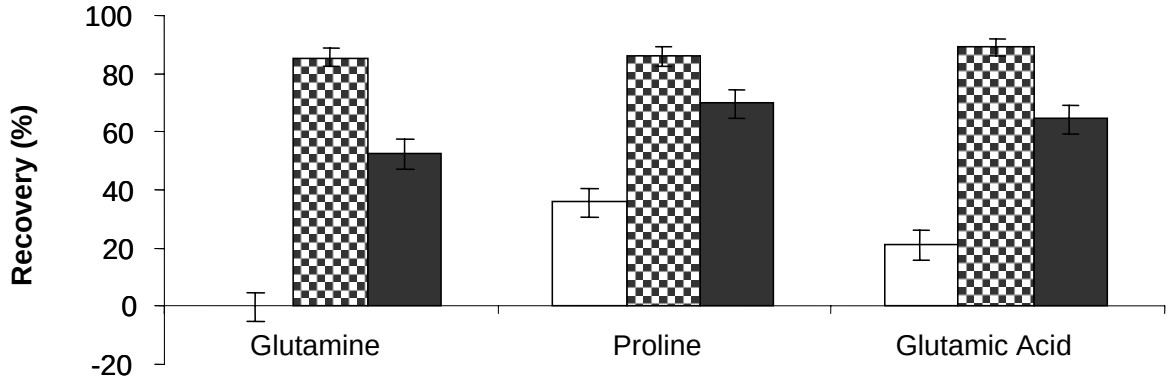


Table 1—Summary of the time required for each of the steps involved in the three pre-column derivatization methods studied

Derivatization Steps	Method A Freezedrier, -40ºC	Method B SpeedVac, 60ºC-65ºC	Method C SpeedVac, 45ºC-50ºC
Initial Drying	24 h	30-40 min	60-80 min
Redrying	6-8 h	15-20 min	20-30 min
Drying after Derivatization	6-8 h	15-20 min	20-30 min
Total Drying Time	36-40 h	60-80 min	100-140 min

The differences in the glutamine and proline concentrations detected using the three pre-derivatization methods (Figure 2) could be attributed to the two major steps involved during derivatization. Known as the redrying or coupling step, the first step is performed under alkaline conditions in order to eliminate the last traces of water and to ensure that the amino groups of the acids are in the "free" state (Molnar-Perl 1994). This is followed by vacuum removal of the liquid, resulting in deprotonated amines so that when the derivatizing agent is added, reaction proceeds rapidly to completion. Previous studies have indicated that concentrating is more preferable to drying the samples during derivatization since it prevents losses due to insolubility during reconstitution (Heinrikson and Meredith 1984; Bidlingmeyer and others 1987; Cohen and Strydom 1988; White and Hart 1992; Molnar-Perl 1994; Albin and others 2000). Extended drying has been shown to cause significant losses of the charged amino acids. Drying at extremely slow speed, as seen in the freeze-drying case, may lead to changes in retention behavior of amino acids (Cohen and others 1984).

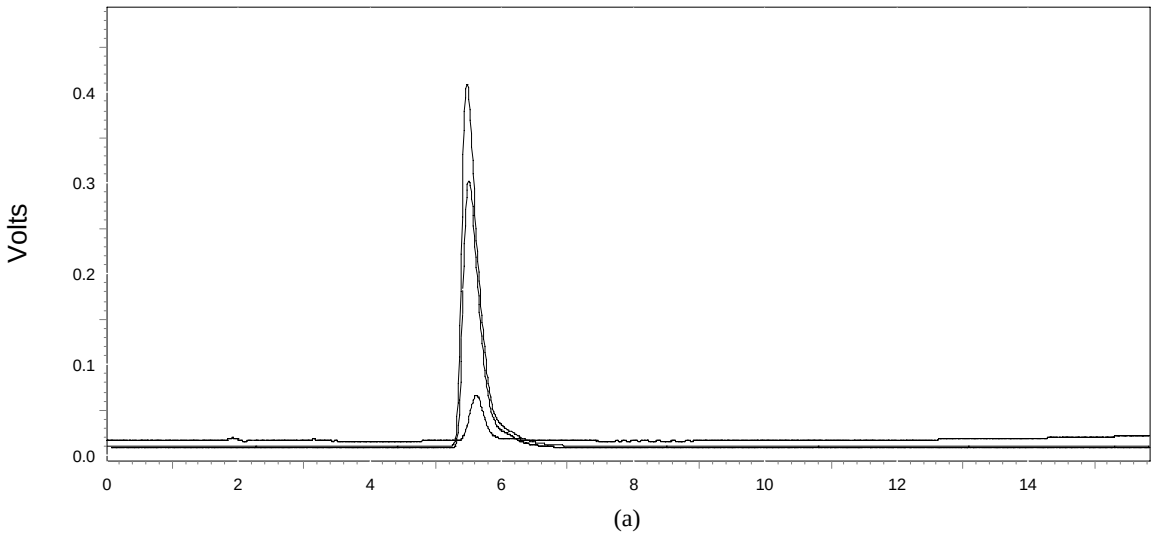
The second step is the actual derivatization reaction. It has been shown that longer reaction times in this step should not pose any problems (Morvai and others 1992). The recommended reaction time is 15-20 min to ensure completion of reaction (Cohen and Strydom 1988; White and Hart 1992). The only concern in this step is that excess derivatization reagent and its volatile byproducts need to be removed under reduced pressure after completion of the derivatization process (Heinrikson and Meredith 1984; Morvai and others 1992; White and Hart 1992); otherwise if PITC is not adequately removed, chromatography problems and early column degradation may occur.

Optimization of gradient elution

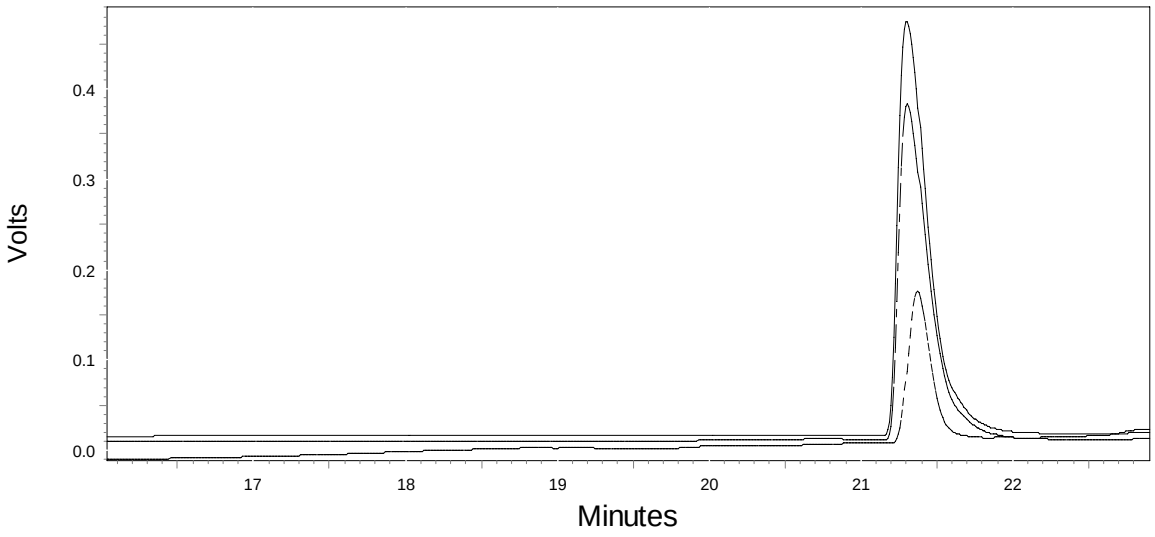
Following the derivatization steps, the derivatized PTC-amino acids were separated by RP-HPLC. The non-fluorescing nature of the derivatives limits the use of this technique to UV detection, which normally takes place at 254-269 nm (White and Hart 1992). In this study, we developed a gradient elution technique using two mobile phases: A, which consisted of 0.05 M sodium acetate buffer, pH 7.2, and B, a combination of 0.1 M sodium acetate, acetonitrile, and methanol at the ratio of 46:44:10, pH 7.2. The eluent flow pattern used in this method was: 0% to 46% B in 21 min; 100% B at 21.5 min; and then back to 0% B at 30 min. Column re-equilibration with 100% mobile phase A was done up to the 45-min mark. In this method, the gradient elution started at a flow rate of 1 mL/min and then gradually increased to 1.3 mL/min in 22 min, followed by gradually reset to 1mL/min at 45 min.

Figure 3 shows an example of a chromatogram obtained from a wheat flour sample from the Karl 92 cultivar using the optimized method. Unlike Method A, in which very poor separation was obtained, the elution times for glutamic acid, glutamine, and proline were clearly found to be 5.6 min, 17.6 min and 21.4 min, respectively. One criterion for a successful chromatogram is the retention of the components in a mixture, measured through the retention factor, *k*'. The respective value of *k'* based on the peaks of glutamic acid, glutamine, and proline was found to be 2, 8, and 10, all within the optimum range of 2-10 (Bidlingmeyer 1992). On the other hand, a good separation must have an R_s value greater than 1. The R_s values for each specific amino acid and the closest neighboring peak were found to be 1, 2, and 0.9 for glutamic acid, glutamine, and proline, respectively.

Figure 2—Chromatogram showing peaks of the (a) glutamic acid and (b) proline standards after derivatization using three different methods. ———, SpeedVac 45°C; — · · — · · —, SpeedVac 60°C; ·····, Freezedrier -40°C.

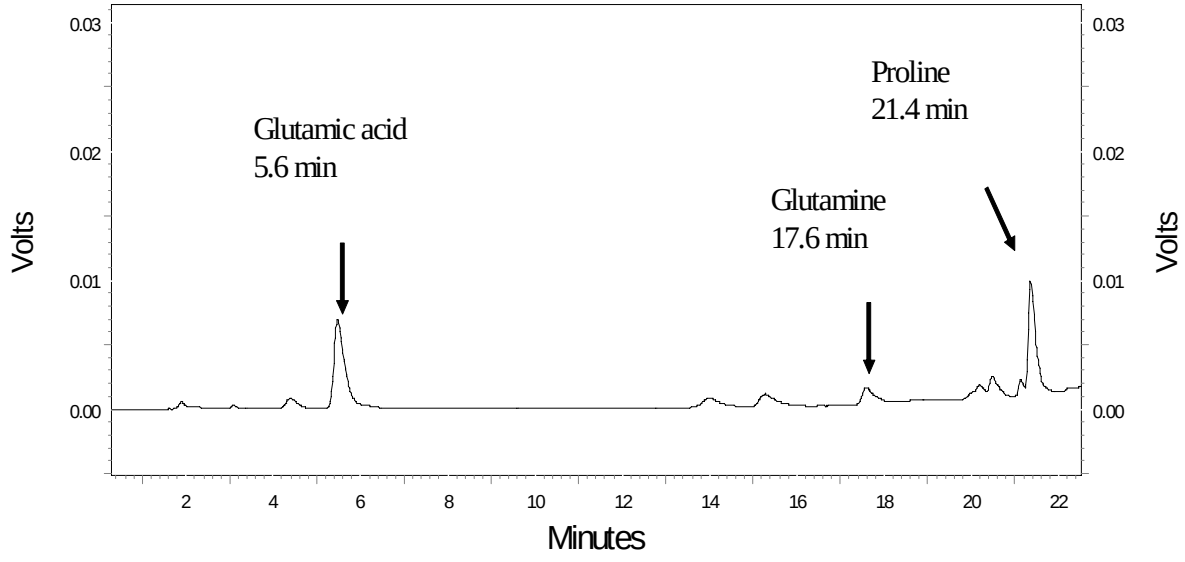


(a)



(b)

Figure 3—Example chromatogram of glutamic acid, glutamine, and proline in a flour sample from Karl 92, a cultivar of hard red winter wheat, using the rapid pre-column derivatization in combination with the modified gradient elution technique.



Application Feasibility Studies

Table 2 compares the protein content (%) and the amount of proline and glutamine in the whole-wheat flours milled from the whole grains of Karl 92, a hard red winter cultivar, and Sisson 36, a soft red winter cultivar. The whole grains of Karl 92 and Sisson 36 have average total protein content at approximately 12.50%. As expected, the flours of Sisson 36 were found to have significantly lower concentrations of both proline and glutamine than Karl 92 flours. The respective proximate content (ash and protein) of the whole-wheat flours of Karl 92 and Sisson 36 was 1.66% and 1.53% (ash) and 11.4% and 10.8% (protein). The pH values were 6.43 and 6.41, respectively. No difference was found between the flours of Karl 92 and Sisson 36 in terms of ash, protein, and pH ($P < 0.05$).

One of the factors that can cause changes in water requirements, dough mixing times, browning of crusts, and gas evolution in yeast systems is starch damage (Atwell 2001). The flours milled from Karl 92 contained damaged starch at 4.8-6.5% of the flour weight, whereas the flours from Sisson 36 showed slightly less starch damage (3.6-5.6% of flour weight). However, the difference was insignificant ($P < 0.05$). Therefore, the differences between the flours milled from Karl 92 and those from Sisson 36 in proline and glutamine concentrations were used as the bases for the addition of proline or glutamine in the following studies.

Table 2. Comparison of the protein content (%) and the key amino acids proline and glutamine (μmol per gram of wheat flour) in the flours milled from Karl 92 and Sisson 36

Wheat Variety	Protein	Glutamine	Proline
Karl 92	11.4 ± 0.4[a]	224 ± 17*,[a]	152 ± 11[a]
Sisson 36	10.8 ± 0.5[a]	181 ± 16[b]	130 ± 8[b]
Difference	0.6 ± 0.9	43 ± 33	22 ± 19

*Means ± SD, n = 15. Values in the same column with the same letter superscripts are not significantly different ($P < 0.05$).

Effect of proline and glutamine on functional properties

The effects of proline and glutamine on the functional properties of dough and bread produced from Sisson 36, the cultivar that has lower glutamine and proline than Karl 92, were investigated by adding the respective differences of proline and glutamine between the flour of Karl 92 and Sisson 36 to the latter. Proline and glutamine (22.62 and 43.14 μmol per g of flour, respectively) were dissolved in distilled water prior to addition to minimize possible competition for water during the mixing of dough. As the rheological properties of dough play important roles during several processing steps after mixing, five important physical properties of dough during breadmaking were measured: stickiness, work of adhesion, dough strength (cohesion), resistance to extension, and extensibility. No significant differences were found among the dough and bread properties of Sisson 36 flours treated with proline and the untreated Sisson 36 flours, except for dough stickiness and bread firmness (Table 3). Addition of proline significantly increased dough stickiness, a characteristic that is usually coupled with the measurements of dough strength and adhesion properties, and slightly increased dough strength and work of adhesion. Previous studies have demonstrated that the intramolecular H bonding in proline is much stronger than in other amino acids (Stepanian and others 2001). While direct evidence remains to be uncovered to fully elucidate the specific role of proline in dough, based on the changes in dough properties it is reasonable to infer that the molecular interactions in the flour were enhanced by proline addition, resulting in a dough that was more sticky and adhesive than that of Sisson 36.

In terms of bread quality, dough resistance to extension and extensibility remained unchanged, whereas the bread firmness was increased after proline addition (Table 3). Intriguingly, while a firmer loaf was produced with the addition of proline, a reduction in loaf volume and a coarser crumb structure were observed in proline-treated samples. Known to favor β sheets and similar structures that have been hypothesized to be responsible for some of gluten's elastic character (Ewart 1968; Stauffer 1998), proline was expected to increase the elastic character that would provide greater gas retention capacity, leading to increased loaf volume and a good aerated crumb structure with less firm texture. However, such effects appeared to be ineffective in the present study when only proline was added.

Table 3. Comparison of functional properties of Sisson 36 and Sisson 36 treated with the required amount of proline (Pro) and/or glutamine (Gln) to match the levels in Karl 92

Textural Properties	Sisson 36	Sisson 36 + Pro[1]	Sisson 36 + Gln[2]	Sisson 36 + Pro + Gln	Karl 92
Dough Properties					
Stickiness (g)	3.0 ± 1.3*, d	4.0 ± 0.6[c]	4.0 ± 1.3[c]	5.4 ± 1.2 [b]	8.4 ± 1.0 [a]
Work of Adhesion (g-s)	0.10 ± 0.05[c]	0.13 ± 0.03[c]	0.14 ± 0.06[c]	0.30 ± 0.09[b]	0.50 ± 0.11[a]
Cohesion/Strength (mm)	0.3 ± 0.1[b]	0.3 ± 0.1[b]	0.4 ± 0.1[b]	0.6 ± 0.1[a]	0.7 ± 0.1[a]
Resistance to Extension (g)	27.9 ± 1.6[c]	29.5 ± 1.3[c]	28.0 ± 1.3[c]	32.0 ± 2.3[b]	50.0 ± 2.1[a]
Extensibility (mm)	9.4 ± 1.5[b]	9.0 ± 1.1[b]	10.0 ± 1.2[b]	12.5 ± 1.5[a]	14.5 ± 1.5[a]
Bread Properties					
Firmness (g)	5.5 ± 0.2[b]	5.8 ± 0.7[a]	5.4 ± 0.1[b]	5.5 ± 0.4[b]	5.4 ± 0.2[b]
Chewiness (g)	1.2 ± 0.3[c]	1.1 ± 0.2[c]	1.3 ± 0.5[c]	2.1 ± 0.2[b]	2.8 ± 0.1[a]
Gumminess (g)	3.2 ± 0.7[b]	3.1 ± 0.2[b]	3.6 ± 0.4[b]	3.4 ± 0.3[b]	4.1 ± 0.4[a]
Cohesiveness	0.51 ± 0.09[c]	0.45 ± 0.06[c]	0.53 ± 0.08[c]	0.71 ± 0.03[b]	0.78 ± 0.07[a]
Resilience	0.05 ± 0.01[a]	0.05 ± 0.01[a]	0.04 ± 0.02[a]	0.05 ± 0.01[a]	0.05 ± 0.01[a]
Springiness	0.83 ± 0.07[b]	0.76 ± 0.03[b]	0.79 ± 0.05[b]	0.84 ± 0.04[b]	0.94 ± 0.02[a]
Loaf Volume† (cm^3)	759.0 ± 24.8[c]	637.2 ± 36.9[d]	764.1 ± 12.7[c]	791.0 ± 21.0[b]	805.0 ± 42.1[a]

* Means ± SD, n = 12. Values in the same row with the same superscripts are not significantly different ($P < 0.05$).

† Loaf volume of 100 g bread was determined using AACC Method 10-10.

[1] Addition of proline was based on 22.62 μmol of proline per gram of wheat flour.

[2] Addition of glutamine was based on 43.14 μmol of glutamine per gram of wheat flour.

While only the difference of glutamine was added into Sisson 36 (Table 3), the dough stickiness was increased, as seen when proline was added alone. However, no significant difference was found in bread firmness and other functional properties. Despite the changes in dough stickiness, the effect of glutamine appeared to be not as significant in the finished bread as it was in the dough, suggesting that the heating during the baking process might have hindered the ability of glutamine to bind water effectively when it stayed in the free form (Atwell 2001). Comprising one third of the amino acids in gluten, the H bonds resulting from glutamine side chains have been shown to be an important factor in determining the rheological properties of gluten (Mita and Matsumoto 1981). However in the present study, loss of glutamine's water binding capacity during heating when incorporated as a free amino acid (Crowley and others 2001) might have hindered its contribution to the functional properties of dough and bread.

When the differences of proline and glutamine between Karl 92 and Sisson 36 were both added to the latter, most dough and bread properties were increased, except bread firmness, gumminess, resilience, and springiness (Table 3). Dough stickiness was only increased slightly with addition of proline or glutamine. Further increase was observed when both amino acids were added. Calderón-Dominguez and co-workers (2004) suggested that changes in dough stickiness usually correspond to differences in the dough's resistance to extension. In this case, resistance to extension remained unchanged when only proline or glutamine was added. Significant increase in resistance to extension was observed when proline and glutamine were both added. Since resistance to extension is the degree of force required for the dough to change its shape as it resists deformation (Cauvain 1998), when only a modest force is applied, the dough that is more sticky in nature reverts faster and easier to its original shape. Similarly, a slight decrease in loaf volume was observed when Sisson 36 was treated with proline alone, whereas treatment with glutamine appeared to have no change on the volume. The loaf volume was significantly increased when both proline and glutamine were added. These observations could possibly be attributed to a synergistic effect on the stickiness of dough when both proline and glutamine were added.

As seen in Table 3, the dough and bread properties of Sisson 36 treated with both proline and glutamine were still inferior to those of Karl 92, despite the significant increases from untreated Sisson 36 or Sisson treated with one of the amino acids. It is generally agreed that soft winter wheat (in this case, Sisson 36) gives less desirable breadmaking performance than that of hard winter wheat (Karl 92), which is currently used in the industry for the making of many breads (Atwell 2001). The dough and bread properties showing synergistic increases that were unseen when either proline or glutamine was added individually suggested that both amino acids are needed to achieve the desirable texture attributes. For instance, dough extensibility, which is defined by the distance that the center of the dough piece can be stretched, is closely linked to dough elasticity that is dependent on the development of molecular network, as it is defined by the amount of stress that can be applied before the dough breaks (Cauvain 1998; Stauffer 1998).

Increasing glutamine that readily forms hydrogen bonds with electron donors such as other amides and water molecules should increase intermolecular reactions in the dough matrix. In fact, individual hydrogen bonds are relatively weak, but the presence of large numbers of them lends overall strength to the interchain reactions. Aside from interchain reactions, hydrogen bonds serve to stabilize the β-turn spirals in the central portions of glutenin molecules,

influencing solubility and viscosity properties (Ewart 1972; Mita and Matsumoto 1984; Stauffer 1998). The high proline content in glutenin provides gluten with the ability to form extended conformations, as proline residues cause bends and folds in the polypeptide chains, requiring force to unfold them. Once the internal molecular adhesion is overcome, a great capability for extension becomes possible (Ewart 1968). Proline residues favor the formation of β sheets composed of β-turn spirals (connected by hydrogen bonding) which can be slightly extended and act as springs (Tatham and others 1985; Stauffer 1998). Hence, increases of proline would result in a more rigid, more intact dough matrix with increased tolerance to environmental stress.

Dependency of functional properties on proline and glutamine

The ability of proline and glutamine to modify the functional properties of dough and bread appears to be interdependent. The known ability of glutamine to provide more intermolecular and intramolecular H bonding during the mixing of dough (Belton 1999) could be leveraged when the conformational bends created by proline offers more points of contact for H bonding to occur (Nelson and Cox 2000; Tseng and Lai 2002). To further investigate the dependency of functional properties on proline and glutamine, as well as the differences between soft wheat and hard wheat, a second approach was taken in which Karl 92, a hard red winter wheat, was used. The addition of proline and glutamine were fixed at 5% by weight of wheat flour. Initial tests were conducted where 1% of proline or glutamine was added to Karl 92 but no significant effects were noted at this level (Table 4).

Table 4. Comparison of dough and bread properties before and after respective addition of proline (Pro) or glutamine (Gln) at 5% by weight of flour to Karl 92 flours

Textural Properties	Karl 92	Karl 92 + Pro	Karl 92 + Gln
Dough Properties			
Stickiness (g)	8.4 ± 1.0*, b	12.4 ± 1.7[a]	6.9 ± 1.2[c]
Work of Adhesion (g-s)	0.50 ± 0.11[b]	0.80 ± 0.19[a]	0.30 ± 0.14[c]
Cohesion/Strength (mm)	0.7 ± 0.1[b]	0.9 ± 0.2[a]	0.7 ± 0.2[b]
Resistance to Extension (g)	50.0 ± 2.1[a]	50.1 ± 1.6[a]	46.2 ± 1.8[b]
Extensibility (mm)	14.5 ± 1.5[b]	17.1 ± 0.8[a]	18.0 ± 1.4[a]
Bread Properties			
Firmness (g)	5.4 ± 0.2[a]	5.2 ± 0.2[a]	5.3 ± 0.1[a]
Chewiness (g)	2.4 ± 0.3[a]	2.3 ± 0.6[a]	1.2 ± 0.2[b]
Gumminess (g)	4.1 ± 0.4[b]	4.8 ± 0.9[a]	5.0 ± 0.7[a]
Cohesiveness	0.78 ± 0.07[b]	0.92 ± 0.17[a]	1.06 ± 0.14[a]
Resilience	0.05 ± 0.01[b]	0.06 ± 0.01[a]	0.07 ± 0.01[a]
Springiness	0.94 ± 0.02[a]	0.96 ± 0.01[a]	0.95 ± 0.03[a]
Loaf Volume (cm^3)	805.0 ± 42.1[c]	885.0 ± 38.3[a]	831.2 ± 24.9[b]

*Means ± SD, n = 12. Values in the same row with the same superscripts are not significantly different ($P < 0.05$).

Based on these data, glutamine and proline exhibit opposite effects on the stickiness properties of the dough. Proline increased stickiness and dough cohesion/strength as well as the work of adhesion required. Glutamine, on the other hand, reduced dough stickiness, cohesion and work of adhesion, which was opposite from what was seen when it was added to soft wheat (Sisson 36). The implications of these effects are crucial in the early stage of mixing, since the quality and extent of dough and gluten development are determined at this stage in the breadmaking process (Chen and Hoseney 1995b; Stauffer 1998). Also different from soft wheat samples, while the addition of proline and glutamine both significantly increased dough extensibility to equivalent levels ($P < 0.05$), only glutamine was shown to affect resistance to extension and deformation by reducing such resistance, which could consequently reduce the elastic character of the dough.

On the other hand, bread firmness and springiness were not affected by any of the amino acid treatments. Measurements of chewiness, gumminess, cohesiveness, and resilience are designed to predict bread textural behavior when forces are exerted during the chewing process. Results showed that both proline and glutamine affected sensory texture properties of bread in terms of gumminess, cohesiveness and resilience. The loaf volume of Karl 92 bread was increased with the addition of 5% (w/w) proline, more significant than the addition of glutamine (5%, w/w), indicating the ability of proline to contribute more than glutamine to the network of bread structure. These observations were different from when glutamine and proline were added to Sisson 36 flours (Table 3). Proline caused a reduction in loaf volume and glutamine did not seem to have any effect when added alone. Therefore, it is obvious that the superior dough properties of Karl 92 flours to Sisson 36 are also dependent on other factors in addition to the proline and glutamine contents.

Effect of the breadmaking process on the retention of proline and glutamine

To understand the effect of breadmaking process on the retention of proline and glutamine, Karl 92 flour was used to take advantage of its superior breadmaking properties. Table 4 summarizes the changes in proline and glutamine, the gluten content, as well as the glutenin and gliaden fractions, before and after baking using flours with or without adding proline or glutamine. The initial glutamine content of wheat flour was 223.2 μmol/g; initial proline 152.0 μmol/g. Addition of glutamine and proline to the wheat flour increased initial glutamine and proline to 567.2 μmol/g and 589.1 μmol/g, respectively. In proteins, the number of each amino acid species and the unique sequence of its incorporation through peptide bond formation determine the conformation of a protein molecule by defining the possibilities for interaction of any residue with other residues within or outside the chain (Kasarda and others 1971). Untreated flour resulted in the greatest losses in proline and glutamine concentrations after undergoing the breadmaking process. Treatment with proline increased proline concentration after baking, and resulted in decreased glutamine losses compared to untreated flour. Treatment with proline or glutamine reduced glutamine and proline losses after the breadmaking process compared to untreated flour. Addition of either proline or glutamine appears to have a protective effect on the retention of the other amino acid.

In addition, the changes of gluten and its subunits, glutenin and gliadin were measured, before and after dough mixing (Table 5). The gluten content remained almost unchanged after

mixing in the control Karl 92. Addition of 5% glutamine increased the overall gluten percentage per dry weight of the flour, in agreement with the enhanced dough properties reported in Table 4. Contrarily, addition of 5% proline reduced the total percentage of gluten in the dough, which is in line with the reduced values of the majority of dough and bread properties (Table 4). Moreover, addition of proline and glutamine affected the composition of gluten. The glutenin subunit was increased in dough with amino acid addition, whereas the gliadin subunit was decreased after mixing ($P < 0.05$). Glutenin is a very large molecule that consists of protein subunits connected by disulfide linkages (Atwell 2001). Increasing glutamine increases amide side chains of the gluten proteins that are responsible for the cohesiveness of the dough structure because of their great H-bonding capability. It could therefore be inferred that this capacity for H-bonding in turn strengthens the gluten network and forms a more stable dough matrix which would be more tolerant to environmental stress brought about by heating, pH changes or ingredient addition and mixing. The increase of glutenin subunit after mixing when proline was added to Karl 92 could be attributed to the fact that proline is known to stabilize β-sheets, which are held together by hydrogen bonds (Stauffer 1998), and proline's cyclic ring structure theoretically constrains the conformational flexibility of proline (Stepanian and others 2001).

The decrease in gliadin, which is composed of individual protein chains and imparts the viscous nature to gluten (Atwell 2001), after mixing when glutamine was added supports the previous observation that the viscous properties (*e.g.* stickiness and resistance to extension) of dough was reduced when 5% glutamine was added to Karl 92 flour (Table 4). Conversely, the viscous properties of Karl 92 dough added with 5% proline were increased (Table 4), in spite of the reduction in gliadin percentage (Table 6), indicating that the viscous properties of dough are more dependent on the overall gluten content than on glutenin or gliadin. The increase of loaf volume (Table 4) appeared to correlate well with the concentration shift in glutenin and gliadin (Table 6), regardless of the slight decrease in overall gluten content when 5% proline was added to Karl 92 flour. This is most likely due to the ability of the increased glutenin to form disulfide bonds during baking, yielding a product with better gas holding capacity and, consequently, higher loaf volume.

CONCLUSIONS

Pre-column derivatization using the SpeedVac concentrator at 45ºC significantly reduced the time required for the process as compared to the same concentrator at 60°C or by using a freezedrier. RP-HPLC analysis has proven that the recovery of proline, glutamine, and glutamic acid could be improved with excellent consistency by this approach. Integrating with the new gradient elution techniques that offer desirable retention factor and resolution, this method could serve as a quality assessment tool for detecting key amino acids in wheat flour. The addition of proline and glutamine to winter wheat flour was proven to affect the functional properties of wheat dough as well as the bread. Addition of proline or glutamine individually did not provide significant improvements in soft wheat dough and bread properties. Combination of glutamine and proline could synergistically enhance the viscous properties of dough and bread attributes such as loaf volume in soft wheat. Addition of glutamine to hard wheat increased the glutenin subunit while enhancing the development of gluten during mixing. Conversely, despite the increase in glutenin subunit, proline hindered gluten formation. The findings in this study provide a foundation for characterizing the properties of dough and bread based on the

contribution of key constituents and will be of interest to researchers and product developers for the development of specialty bread products. Additionally, should the degree of dependence of breadmaking properties on these two amino acids be elucidated, a better control of dough quality would be within reach, enabling selection criteria to be set for wheat breeders.

Table 5. Changes in proline and glutamine contents (μmol per gram of flour) before and after the breadmaking process (AACC Method 10-10B)

Content	Before	After	% Difference
Karl 92			
Proline	152.0 ± 4.2*, a	66.1 ± 6.8^{b}	-57%
Glutamine	223.2 ± 6.4^{a}	59.6 ± 4.8^{b}	-74%
Karl 92 + Proline			
Proline	589.1 ± 4.2^{a}	530.1 ± 5.2^{b}	-10%
Glutamine	223.7 ± 6.4^{a}	205.9 ± 1.6^{b}	-8%
Karl 92 + Glutamine			
Proline	152.4 ± 4.2^{a}	147.8 ± 2.4^{a}	N/D**
Glutamine	567.2 ± 6.4^{a}	417.0 ± 9.8^{b}	-26%

* Mean ± SD, n = 3. Values in the same row with the same superscripts are not significantly different ($P < 0.05$).

** N/D: Not differentiable.

Table 6. Changes in gluten (%, dry basis), glutenin, and gliadin fractions (% per weight of gluten) of flour dough before and after mixing with optimum water absorption

	Type of Flour		
Components	**Karl 92**	**Karl 92 + 5% Gln**	**Karl 92 + 5% Pro**
Gluten			
Before Mixing	13.6 ± 3.7*, a (A)	13.2 ± 3.1^{a} (B)	13.0 ± 2.8^{a} (A)
After Mixing	14.2 ± 2.7^{b} (A)	17.3 ± 1.8^{a} (A)	11.1 ± 2.2^{c} (B)
Glutenin			
Before Mixing	61.1 ± 5.4^{a} (A)	61.4 ± 3.2^{a} (B)	60.2 ± 3.9^{a} (B)
After Mixing	59.3 ± 4.2^{c} (A)	69.1 ± 2.5^{a} (A)	64.1 ± 3.0^{b} (A)
Gliadin			
Before Mixing	38.8 ± 1.6^{a} (A)	38.5 ± 3.7^{a} (A)	39.7 ± 5.1^{a} (A)
After Mixing	40.6 ± 5.2^{a} (A)	30.8 ± 2.6^{c} (B)	35.8 ± 2.9^{b} (B)

* Mean ± SD, n = 3. Values in the same row with the same superscripts, or in the same column under the same component with the same letter in parenthesis, are not significantly different ($P < 0.05$).

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APPENDICES

1. Fermin, B.C., J.A. Radinsky, R.J. Kratochvil, J.E. Hall, and Y.M. Lo. 2003. Integration of rapid derivatization and gradient elution techniques for enhanced High Performance Liquid Chromatography analysis of key amino acids in wheat flour. *Journal of Food Science*, 68 (9): 2667-2671.
2. Fermin, B.C., T.S. Hahm, J.A. Radinsky, R.J. Kratochvil, J.E. Hall, and Y.M. Lo. 2005. Effect of proline and glutamine on the functional properties of wheat dough in winter wheat varieties. *Journal of Food Science*, 70 (4): E273-278.

Integration of Rapid Derivatization and Gradient Elution Techniques for Enhanced High-Performance Liquid Chromatography Analysis of Key Amino Acids in Wheat Flour

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ABSTRACT: A rapid pre-column derivatization process before ultraviolet chromatographic analysis using a Pico-Tag C_{18} column was developed in combination with modified gradient elution techniques for the analysis of key amino acids (proline and glutamine) in wheat flour. With use of a vacuum concentrator at 45 °C, the pre-column derivatization process was significantly accelerated before entering a reverse-phase high-performance liquid chromatography. When running at an optimized scheme—in which the gradient elution started at a flow rate of 1 mL/min and then gradually increased to 1.3 mL/min in 22 min, followed by reset to 1 mL/min at 45 min—the profiled mobile phase mixture was able to yield chromatograms with desirable retention factor and resolution.

Keywords: proline, glutamine, glutamic acid, reverse-phase HPLC, resolution

Introduction

The protein composition in mature wheat (*Triticum aestivum* L.) has been reported to play a critical role in governing wheat flour properties and uses (He and Hosency 1991; Delwiche and others 1998; Wrigley and Bekes 2001; Daniel and Triboi 2002; Thiele and others 2002). For many decades, research efforts have been focused on gliadin and glutenin contents, both known to affect the functional properties of gluten (Frater and others 1960; Mauritzen and Stewart 1963; Singh and others 1987; Graveland and Henderson 1990; Uthayakumaran and others 2000). On the basis of the interchange of disulfide bonds during dough mixing (Ewart 1968), the linear concatenation model of glutenin subunits has long been the basis for understanding elasticity, viscous flow and relaxation, dough breakdown on over-mixing, and the action of oxidants (Ewart 1972, 1979; Shewry and Tatham 1997). However, no conclusive, direct evidence is available that disulfide bond interchange actually occurs during dough mixing (Payne and others 1979, 1981; Payne 1987). Understanding the complex quaternary structure of gluten remains a challenge, especially with new evidence that formation of non-disulfide covalent intermolecular bonds among glutenin proteins might occur (Tilley and others 2001).

Among the amino acids in wheat, proline, and glutamine are the 2 most abundant (Wrigley and Bietz 1988). Although no direct correlations between these amino acids and wheat flour properties have yet been reported, the structures of both proline and glutamine suggest a possible role for each in the formation and development of gluten. The rigid cyclic ring structure of proline prevents the formation of the α helix protein conformation and is responsible for the kinks and folds in the gluten structure, thereby contributing to the elastic nature of gluten (Ewart 1968; Nelson and Cox 2000). Accounting for approximately a third of the amino acids in wheat, glutamine's amide side chain has a great capacity for hydrogen bonding. The effects of glutamine on the rheological properties of dough have been demonstrated (Mita and Matsumoto 1981, 1984), contributing to the theory that the intermolecular and intramolecular hydrogen bonding in the gluten complex is responsible for the viscoelastic nature of dough (Belton 1999). Rapid and reliable quantitative monitoring of proline and glutamine throughout the process of breadmaking should provide insights on their contributions to gluten formation and development.

Analytically, aside from expensive, specialized amino acid analyzers, separation and quantification of amino acids could be achieved by reverse-phase high-performance liquid chromatography (RP-HPLC) with high precision and specificity (Peter and Toth 1997; Pena-Valdivia and Rodriguez-Gracia 1999; Izco and others 2000; Czauderna and others 2002). However, because most underivatized amino acids do not possess suitable chromophores for detection except at very short wavelengths such as 200 to 250 nm, at which many other compounds are also absorbed, the α-amino groups in the samples have to be converted into suitable chromophores before (pre-column derivatization) or after (post-column derivatization) chromatographic separation to enable detection of amino acids with minimal risk of interferences (White and Hart 1992). Use of pre-column derivatization has become popular because it eliminates the need for cumbersome and expensive post-column reactors, allows hydrophobic amino acid derivatives to be separated on reverse-phase columns, and shows greater versatility at the instrument (White and Hart 1992, Heems and others 1998). Phenylisothiocyanate (PITC) has been widely used to derivatize amino acids because of its sensitivity and stability, as well as the characteristic specificity of phenylthiocarbamyl (PTC) derivatives (Cohen and Strydom 1988; Morvai and others 1992). Ideal for the derivatization of primary and secondary amino acids, PITC

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forms PTC derivatives that initiate the 3-step sequential Edman degradation process capable of determining the primary structure of peptides and proteins (Heinrikson and Meredith 1984; Cohen and Strydom 1988). Nonetheless, existing pre-column derivatization procedures using freeze-drying are time-consuming because they require lengthy initial drying (about 24 h), redrying (6 to 8 h), and drying after derivatization (6 to 8 h) steps to eliminate excess reagents and byproducts that cause interfering peaks, poor baseline separation, or early column degradation (Albin and others 2000).

Furthermore, although complete hydrolysis of wheat samples remains the most time-intensive (about 24 h) procedure before pre-column derivatization (Bidlingmeyer and others 1987; Albin and others 2000), the overall time involved in amino acid analysis could be greatly reduced if an automated, high-throughput HPLC system were used. However, many parameters must be determined and set before placing the wheat samples into the instrument. Therefore, the purposes of the present study were 2-fold: (1) to establish an accelerated pre-column derivatization process for enhanced amino acid recovery under accelerated vacuum at elevated temperatures, as compared with freeze-drying; (2) to evaluate the gradient elution techniques in a high-throughput RP-HPLC unit and optimized these techniques for detection and quantification of proline and glutamine.

Materials and Methods

Wheat samples, standards, and reagents

Samples of wheat flour were prepared by grinding the grains of Karl 92, a cultivar of hard red winter wheat grown regionally on the Eastern Shore of Maryland, using a laboratory disc mill (Model 3600, Perten Instruments, Huddinge, Sweden) and then stored at −18 °C before analysis. Anhydrous ethanol, hydrochloric acid (HCl), triethylamine (TEA), anhydrous sodium phosphate dibasic (Na_2HPO_4), and sodium acetate trihydrate (HPLC-grade) were purchased from Fisher Scientific (Fair Lawn, N.J., U.S.A.). Amino acid standards, as well as crystalline phenol and reagents for HPLC analysis, including water, methanol, and acetonitrile, were supplied by Sigma Aldrich (St. Louis, Mo., U.S.A.). Individual standards of proline, glutamine, and glutamic acid (10 μmol/mL) were prepared with 0.1 *N* HCl. The use of glutamic acid standard was necessary because in the case of glutamine, conversion to glutamic acid happens after acid hydrolysis so that quantitative analysis necessitates that both the acid and amide forms be taken into consideration (White and Hart 1992; Molnar-Perl 1994; Tyler 2001). The derivatizing agent (PITC) was obtained from Pierce Chemicals (Rockford, Ill., U.S.A.).

Hydrolysis

To release key amino acids from wheat proteins, a standard hydrolysis procedure was used modified from Bidlingmeyer and others (1987) and Albin and others (2000). All glassware used in hydrolysis was first washed with 6 *N* HCl, rinsed with deionized water, and dried. Triplicate samples of each type of wheat flour were accurately weighed (about 200 mg each) into 15-mL screw-capped test tubes; 12 mL of 6 *N* HCl and 0.5 mg crystalline phenol were also added to the tubes (Cohen and others 1984; Fountoulakis and Lahm 1998; Albin and others 2000). The tubes were thoroughly flushed with nitrogen gas, capped, mixed, and then hydrolyzed in an oven at 110 °C for 24 h. After hydrolysis, the samples were allowed to cool to room temperature before 0.5 mL of norleucine (50 μmol/mL) was added as the internal standard. The tubes were vortexed and centrifuged to remove debris. A 200-μL aliquot of the hydrolysate was transferred to a 1.5-mL microcentrifuge tube (Fisher Scientific, Pittsburgh, Pa., U.S.A.), and diluted with distilled, deionized water in a 1:10 ratio.

Pre-column derivatization

Standard solutions of proline, glutamine, and glutamic acid were prepared by dissolving 0.1 mole of each amino acid in 100 mL 0.1 *N* HCl. Amino acid concentrations of 10.00 μmol/mL, 7.50 μmol/mL, 5.00 μmol/mL, 2.50 μmol/mL, and 1.25 μmol/mL of each amino acid were prepared by diluting amino acid solutions with the redrying solution composed of ethanol:water:triethylamine (2:2:1), which is required in this 2-step drying process to complete derivatization. To optimize the pre-column derivatization process, 20 μL of the diluted standards transferred into 1.5-mL microcentrifuge tubes were subjected to 3 different drying methods. Method A used a freeze-dryer (Dura-Top Bulk Tray Dryer, FTS System Inc, Stone Ridge, N.Y., U.S.A.) to dry the standards under vacuum for 24 h at −40 °C (Albin and others 2000). After adding 10 μL of redrying agent (2:2:1 ethanol:water:triethylamine), the dried standards were vortexed to resolubilize amino acids and then vacuum-dried for 6 to 8 h. The redried standards were derivatized with 10 μL of 7:1:1:1 ethanol:water:triethylamine:PITC (Heinrikson and Meredith 1984; Inglis and others 1988; White and Hart 1992; Molnar-Perl 1994) and allowed to stand at room temperature for 20 min. After derivatization, the standards were vacuum-dried for another 6 to 8 h and then resuspended with 400 mL of diluent, which consisted of a 95:5 (vol/vol) phosphate buffer (5 m*M* sodium phosphate dibasic, pH 7.4):acetonitrile (White and Hart 1992). The reconstituted standards were vortexed and then filtered through a 0.25-μm filter into HPLC vials for analysis.

Methods B and C both used a SpeedVac vacuum concentrator (Model SPD121, Thermo Savant, Marietta, Ohio, U.S.A.) capable of rapid dehydration under vacuum. The same reagent compositions were used for the redrying and derivatization steps as in Method A. The drying temperatures, however, were different: 60 ° to 65 °C for Method B and 45 ° to 50 °C for Method C. The concentrations of proline, glutamine, and glutamic acid were calculated using respective standard curves with corresponding r^2 values at 0.9806, 0.9967, and 0.9864. The drying time was determined on the basis of the percentage of amino acids recovered. After the optimum method for pre-column derivatization was identified, the best method was applied to hydrolyzed wheat samples following the aforementioned hydrolysis procedure.

Chromatography and data analysis

PTC-amino acids were separated by RP-HPLC using a Shimadzu LC2010A (Columbia, Md., U.S.A.) equipped with serial dual plunger pumps, an oven, an automated sampling injection unit, and an ultraviolet-visual (UV-VIS) detector (D_2 lamp light source) with a wavelength range of 190 to 600 nm. The column used was a Pico-Tag 3.9- × 300-mm reverse-phase column (Waters, Milford, Mass., U.S.A.). Sample injection volume was 20 μL. A variety of mobile phase and gradient compositions were tested to identify the most suitable gradient elution techniques for analyzing proline and glutamine in wheat flour. Chromatograms were acquired, and the peaks with a Gaussian (symmetrical) distribution were analyzed for the retention factor (formerly called capacity factor), k', a measure of the time the sample component resides in the stationary phase relative to the time it resides in the mobile phase (Bidlingmeyer 1992; Siouffi 2000):

$$k' = \frac{t_r - t_o}{t_o} \quad (1)$$

where t_r is retention time of a retained solute and t_o retention time of the unretained (inert) solute. The resolution (R_s), which is defined as the peak separation divided by the mean peak width (Siouffi 2000), of the resulted peaks were calculated on the basis of the following equation using the Class VP 6.0 software supplied with the equipment:

$$R_s = \frac{t_{r2} - t_{r1}}{(\omega_2 + \omega_1)/2} \quad (2)$$

where t_{r2} and t_{r1} represent, respectively, the retention time of peaks 1 and 2 and ω is the peak width.

Results and Discussion

Pre-column derivatization

Figure 1 compares the effectiveness of glutamine, proline, and glutamic acid recovery by 3 different pre-column derivatization methods. For all 3 amino acids, the percentages of recovery were significantly higher when the SpeedVac concentrator was used rather than the freeze-dryer. The highest recovery at 89% was reached by using the SpeedVac at 45 °C. The overall time required to complete each of the derivatization methods is summarized in Table 1. It should be noted that glutamine and glutamic acid recovery using freeze-drying was extremely poor, even after more than 36 h to complete the process. In SpeedVac vacuum-drying, the samples at 60 °C took less than half the time required at 45 °C; however, the reduced amino acid recovery percentages with increased variations failed to justify the speed of derivatization at 60 °C, due in part to the decreased conformational stability at elevated temperatures. In the present study, because lowered recovery of the standards was obtained at temperatures lower than 40 °C (data not shown), the initial drying step at 45 °C appears to be the most adequate temperature for effective derivatization of amino acids using the SpeedVac concentrator. As glutamine converts into glutamic acid under acidic conditions, both glutamine and glutamic acid peaks should be quantified as a measure of glutamine. However, no glutamine was recovered by freeze-drying, indicating that the freeze-drying process was not feasible for pre-column derivatization.

The differences in the glutamine and proline concentrations detected using the 3 pre-derivatization methods (Figure 2) could be attributed to the 2 major steps involved during derivatization. Known as the *redrying* or *coupling* step, the first step is performed under alkaline conditions to eliminate the last traces of water and to ensure that the amino groups of the acids are in the "free" state (Molnar-Perl 1994). This is followed by vacuum removal of the liquid, resulting in deprotonated amines so that when the derivatizing agent is added, the reaction proceeds rapidly to completion. Previous studies have indicated that concentrating is preferable to drying the samples during derivatization because it prevents losses caused by insolubility during reconstitution (Heinrikson and Meredith 1984; Bidlingmeyer and others 1987; Cohen and Strydom 1988; White and Hart 1992; Molnar-Perl 1994; Albin and others 2000). Extended drying has been shown to cause significant losses of the charged amino acids. Drying at extremely slow speed, as seen in the freeze-drying case, may lead to changes in retention behavior of amino acids (Cohen and others 1984).

The second step is the actual derivatization reaction. It has been shown that longer reaction times in this step should not pose any problems (Morvai and others 1992). The recommended reaction time is 15 to 20 min to ensure completion of reaction (Cohen and Strydom 1988; White and Hart 1992). The only concern in this step is that excess derivatization reagent and its volatile byproducts need to be removed under reduced pressure after completion of the derivatization process (Heinrikson and Meredith 1984; Morvai and others 1992; White and Hart 1992); otherwise, if PITC is not

Table 1—Summary of the time required for each of the steps involved in the 3 pre-column derivatization methods studied

Derivatization steps	Method A Freeze-dryer (–40 °C)	Method B SpeedVac (60 ° to 65 °C)	Method C SpeedVac (45 ° to 50 °C)
Initial drying	24 h	30 to 40 min	60 to 80 min
Redrying	6 to 8 h	15 to 20 min	20 to 30 min
Drying after derivatization	6 to 8 h	15 to 20 min	20 to 30 min
Total drying time	36 to 40 h	60 to 80 min	100 to 140 min

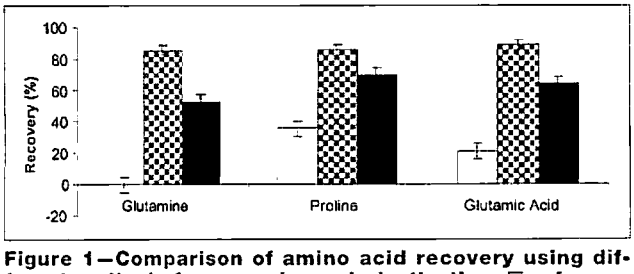

Figure 1—Comparison of amino acid recovery using different methods for pre-column derivatization. □ = freeze-dryer at –40 °C; ▯ = SpeedVac at 45 °C; ■ = SpeedVac at 60 °C.

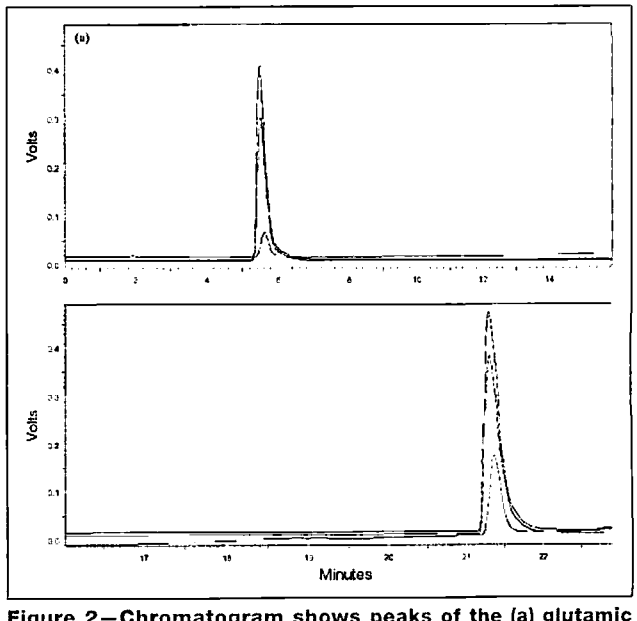

Figure 2—Chromatogram shows peaks of the (a) glutamic acid and (b) proline standards after derivatization using 3 different methods. ----- = SpeedVac 45 °C; — • • — = SpeedVac 60 °C; — — — = freeze-dryer –40 °C.

adequately removed, chromatography problems and early column degradation may occur.

Optimization of gradient elution

After the derivatization steps, the derivatized PTC-amino acids were separated by RP-HPLC. The nonfluorescing nature of the derivatives limits the use of this technique to UV detection, which normally takes place at 254 to 269 nm (White and Hart 1992). In this study, we developed a gradient elution technique using 2 mobile phases: A, which consisted of 0.05 *M* sodium acetate buffer, pH 7.2, and B, a combination of 0.1 *M* sodium acetate, acetonitrile, and methanol at the ratio of 46:44:10, pH 7.2. The eluent flow pattern used in this method was: 0% to 46% B in 21 min; 100% B at 21.5 min; and then back to 0% B at 30 min. Column re-equilibration with 100% mobile phase A was done up to the 45-min mark. In this method, the gradient elution started at a flow rate of 1 mL/min and then gradually increased to 1.3 mL/min in 22 min, and then gradually reset to 1 mL/min at 45 min.

Figure 3 shows an example of a chromatogram obtained from a wheat flour sample from the Karl 92 cultivar using the optimized method. Unlike Method A, in which very poor separation was obtained, the elution times for glutamic acid, glutamine, and proline were clearly found to be 5.6 min, 17.6 min, and 21.4 min, respectively. One criteria for a successful chromatogram is the retention of the components in a mixture, measured through the retention factor, k'. The respective value of k', based on the peaks of glutamic acid, glutamine, and praline, was found to be 2, 8, and 10, all within the optimum range of 2 to 10 (Bidlingmeyer 1992). On the other hand, a good separation must have an R_s value greater than 1. The R_s values for each specific amino acid and the closest neighboring peak were found to be 1, 2, and 0.9 for glutamic acid, glutamine, and proline, respectively.

Conclusions

Pre-column derivatization using the SpeedVac concentrator at 45 °C significantly reduced the time required for the process, compared with the same concentrator at 60 °C or by using a freeze-dryer. RP-HPLC analysis has proven that the recovery of proline, glutamine, and glutamic acid could be improved with excellent consistency by this approach. Integrating with the new gradient elution techniques that offer desirable retention factor and resolution, this method could serve as a quality assessment tool for detecting key amino acids in wheat flour.

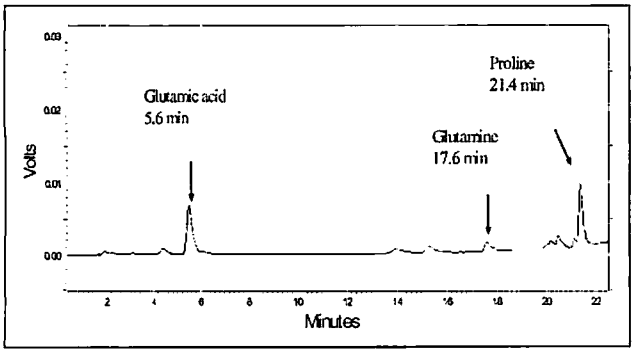

Figure 3—Example chromatogram of glutamic acid, glutamine, and proline in a flour sample from Karl 92, a cultivar of hard red winter wheat, using the rapid pre-column derivatization in combination with the modified gradient elution technique

Acknowledgments

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Effect of Proline and Glutamine on the Functional Properties of Wheat Dough in Winter Wheat Varieties

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ABSTRACT: Combinations of proline and glutamine significantly increased the functional properties of soft wheat that were unseen when added individually. To hard wheat, proline contributed more positively than glutamine on most dough and bread properties, which is different from the observations with soft wheat. Addition of glutamine to hard wheat increased the amount of gluten and glutenin after mixing, whereas proline hinders gluten formation despite the increase in glutenin. The ability of proline and glutamine to modify the functional properties of dough and bread appears to be interdependent. Addition of either proline or glutamine appears to have a protective effect on the retention of the other amino acid during breadmaking.

Keywords: dough, texture, bread, proline, glutamine

Introduction

Identification of the biochemical factors responsible for end-use quality variation of wheat is crucial for breeders, producers, grain handlers, millers, and bakers (Dobraszczyk 2001; Graybosch and others 2003). The protein composition in mature wheat has been reported to play a critical role in governing wheat flour properties and uses (He and Hoseney 1991; Delwiche and others 1998; Daniel and Triboi 2002; Thiele and others 2002). Conventionally, wheat proteins have been classified under Osborne fractionation as albumin, globulin, gliadin, and glutenin according to their solubility in different solvents (Osborne 1907). The sum of the latter 2, also known as the gluten complex, has been shown to directly impact the texture of finished products (Wrigley and Bietz 1988; Graveland and Henderson 1990). To date, however, no conclusive, direct evidence is available that disulfide bond interchange actually occurs during dough mixing (Payne and others 1979; 1981; Payne 1987; Tilley 1999), especially with new evidence that formation of non-disulfide covalent intermolecular bonds among glutenin proteins might occur (Tilley and others 2001). The current practice is that market values of harvested wheat are segregated solely based on the total protein content and, when applicable, variety identity (Rao and others 1993; Lasztity 1999; Wrigley and Bekes 2001).

The amino acid compositions of the gluten proteins gliadin and glutenin are considered remarkable in that relatively few amino acids predominate (Atwell 2001), with proline and glutamine the most abundant (Wrigley and Bietz 1988; Lasztity 1999; Dobraszczyk 2001). Glutamine, an amino acid that contains an amide side group that binds water well, constitutes more than 40% of all of the amino acids composing the gluten proteins, which ranges from 6% to 13% of flour, on a molar basis. The effects of glutamine on the rheological properties of dough have been demonstrated (Mita and Matsumoto 1981; 1984) and later linked to the theory that the intermolecular and intramolecular hydrogen bonding in the gluten complex is responsible for the viscoelastic nature of dough (Belton 1999). Proline, on the other hand, is composed of about 15% of gliadin, which constitutes 30% to 45% of gluten and 10% to 12% of glutenin (55% to 70% of gluten). The cyclic R group structure of proline puts a bend in a chain of amino acids and is responsible for the kinks and folds in the gluten structure, thereby contributing to the elastic nature of gluten (Ewart 1968; Nelson and Cox 2000). Proline is also known to inhibit the formation of secondary structures (α helix and the pleated β-sheet) in gluten (Atwell 2001). Although no direct correlations between these amino acids and wheat flour properties have yet been reported, the primary structures of both proline and glutamine suggest each one's possible roles in the formation and development of dough during the breadmaking process. With the establishment of a rapid derivatization method integrating with gradient elution techniques for enhanced reverse-phase high-performance liquid chromatography (HPLC) analysis of proline and glutamine in wheat flour (Fermin and others 2003), systematic approaches to characterize the roles of proline and glutamine during the process of breadmaking should provide valuable insights on their contributions to the functional properties of wheat dough.

Moreover, in addition to routine physiochemical analyses (for example, moisture, protein, ash, and pH), wheat flour quality assessment is normally implemented through rheological tests conducted using instruments such as the farinograph, the mixograph, the alveograph, and the extensigraph, all of which are designed to predict baking performance and processing behavior of the resulting dough (Metho and others 1999; Uthayakumaran and others 2000). In industry, flour quality is also assessed based on baking strength index, a measure of loaf volume on a constant protein basis (Preston and others 1995), and its ability to retain solvents, as described in AACC Method 56-11 (AACC 1995). Retention of solvents such as water, 50% sucrose, 5% sodium carbonate, and 5% lactic acid have been commonly used for predicting the baking performance of flour—a higher capacity to retain solvents indicates better baking quality (Atwell 2001). However, to provide a better under-

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standing on the functional attributes of wheat dough, an in-depth texture profiling during and after breadmaking is needed.

In the present study, the effect of the relative proportions of proline and glutamine in wheat dough on its functional properties critical to breadmaking quality was investigated. The amounts of proline and glutamine before and after baking were also measured to further characterize their roles in the breadmaking process.

Materials and Methods

Wheat samples, standards, and reagents

Samples of wheat flour were prepared from the grains of Karl 92, a hard red winter wheat variety, and Sisson 36, a soft red winter wheat variety, both grown regionally on the eastern shore of Maryland. After cleaning, the wheat was moistened by holding in tempering bins for 20 h to reach 14% moisture content (wet basis) before milling (Yuan and others 2003). Such a tempering process is known to make the endosperm less susceptible to starch damage. The moisture content of the grains was checked by a PM-400 Grain Moisture Tester (Kett US, Villa Park, Calif., U.S.A.). The wheat was milled on a laboratory disc mill (Model 3600, Perten Instruments, Huddinge, Sweden) equipped with a type 1 disk, which yielded similar ($P < 0.05$) whole-wheat flour extraction rates of 99.2% and 99.7% for Karl 92 and Sisson 36, respectively. The average feed rate of wheat grains to the mill was 150 g/min. The damaged starch was assessed using the AACC Method 76-31 (AACC 1995).

Anhydrous ethanol, hydrochloric acid (HCl), triethylamine (TEA), anhydrous sodium phosphate dibasic (Na_2HPO_4), and sodium acetate trihydrate (HPLC-grade) were purchased from Fisher Scientific (Fair Lawn, N.J., U.S.A.). Amino acid standards for proline, glutamine, glutamic acid, and norleucine, as well as crystalline phenol and reagents for HPLC analysis, including water, methanol, and acetonitrile, were supplied by Sigma Aldrich (St. Louis, Mo., U.S.A.). Individual standards of proline, glutamine, and glutamic acid (10 μmol/mL) were prepared with 0.1 *N* HCl. The use of glutamic acid standard was necessary because in the case of glutamine, conversion to glutamic acid happens after acid hydrolysis so that quantitative analysis necessitates that both the acid and amide forms be taken into consideration (White and Hart 1992; Molnar-Perl 1994; Tyler 2001). The derivatizing agent phenylisothiocyanate (PITC) was obtained from Pierce Chemicals (Rockford, Ill., U.S.A.).

Dough preparation and breadmaking

Dough samples for texture testing were prepared using a modified procedure from Tseng and Lai (2002). Two hundred grams of each type of wheat flour were mixed with the optimum percentage of water (Pratt 1971) using a KitchenAid Model K45 bench-top mixer equipped with a 4.5 qt. stainless-steel mixing bowl (Hobart Manufacturing Co., Troy, Ohio, U.S.A.) at medium speed (setting nr 2) for 6 min, the optimum mixing time determined during mixing based on the feel and appearance of the dough. The optimum percentage of water absorption was determined based on farinographic absorption, which gives an indication of how much water (mL) must be added for 100 g of flour (14% moisture) to get a 500 Brabender Units (BU) consistency in the farinograph (Calderón-Dominguez and others 2004). The optimum water absorption was determined to be 60% and 57.5% for Karl 92 and Sisson 36, respectively, to fit the value of flour farinographic absorption (in milliliters of water/100 g flour), which corresponds to visual appearance as smooth and neither too sticky nor too dry (Tseng and Lai 2002).

Bread samples were prepared using dry yeast (5.3% flour basis) following the optimized straight-dough breadmaking method from AACC Method 10-10B without addition of optional ingredients such as dough oxidant to assess the natural breadmaking potential of each flour (AACC 1995). Each flour was baked in triplicate. One hundred–gram pup loaves were produced based on a 90-min fermentation time (Tilley and others 2001) and allowed to stand for 1 d at room temperature before texture analyses.

Texture analyses of dough and bread samples

Dough textural properties were measured in triplicate with 4 trials per replicate using a TA.XT2*i* Texture Analyzer (Texture Technologies Corp., Scarsdale, N.Y., U.S.A.). Dough stickiness, cohesion/dough strength, and adhesion properties were determined using the SMS/Chen-Hoseney Dough Stickiness cell coupled with a 25-mm perspex cylinder probe (5-kg load cell).

Dough stickiness, which measures the force required to release the probe from the dough, and cohesion/dough strength, which is the quantity to simulate the strength of the internal bonds making up the body of the dough, were conducted using a force of $40 \times g$ through a distance of 4 mm, at a pre-test speed of 0.5 mm/s, a test speed of 0.5 mm/s, and a post-test speed of 10 mm/s (Chen and Hoseney 1995a). Dough extensibility was determined using the Kieffer dough and gluten extensibility rig probe. The dough was pressed into strips by a special dough presser provided by Texture Technologies and extended until it reached its elastic limit. The test was conducted through a distance of 75 mm, at a pre-test speed of 2.0 mm/s, a test speed of 3.3 mm/s, and a post-test speed of 10 mm/s. For this particular test, the maximum distance that the dough reached without breaking was used as a measure of extensibility. To minimize variations due to moisture loss while waiting in line for analysis, only the measurements of the 4 strips most similar in surface dryness and elastic limit were recorded.

The loaf volume of the bread (100 g) was determined following AACC Method 10-10 by means of rapeseed displacement (AACC 1995). The texture properties (firmness, gumminess, springiness, resilience, and cohesiveness) of sliced bread samples were determined through AACC Method 74-09 by using a 36-mm cylinder probe (AACC 1995). All samples were measured in triplicate with 4 trials per replicate. Firmness is the maximum force required while cutting under specific conditions. Gumminess is the quantity to simulate the energy required to disintegrate a semi-solid sample to a steady state of swallowing. Springiness is the rate at which a deformed sample goes back to its undeformed condition after the deforming force is removed. Resilience is a measurement of how the sample recovers from deformation both in terms of speed and forces derived. The moisture content (%) of the bread samples after standing at room temperature for 1 day, the same time as texture measurements, was also measured.

Effects of proline and glutamine

The effects of proline and glutamine on the functional properties of wheat dough were investigated using 2 approaches. First, the wheat variety determined to have lower concentrations of proline or glutamine was treated with the required amount of proline or glutamine dissolved in distilled water, reaching a total volume of 11.55 mL, to bring amino acid concentrations to the same level as the variety that has higher proline or glutamine contents. Second, a fixed known amount (5% by weight of wheat flour) of proline or glutamine aqueous solution was added to Karl 92 to compare the differences before and after treatment. Addition of proline or glutamine at 5% (w/w) was chosen because it represents a concentration 5-fold increase of flour proline while doubling the glutamine concentration in the flour.

Dough samples prepared from proline- or glutamine-treated

wheat flours were subjected to textural analyses (Chen and Hoseney 1995a). The effects of proline and glutamine on gluten formation were determined by isolating glutenin and gliadin fractions after dough mixing and after baking to evaluate the changes brought about by the addition. Glutenin and gliadin fractions were isolated using the low-cross-contamination protocols (Suchy and others 2003) with minor modifications. By using this method, protein fractions in the form of dry residue can be stored long-term at room temperature without adverse changes in nitrogen content.

Bread samples were freeze-dried at –40 °C using a Dura-Top Bulk Tray Dryer (FTS System Inc, Stone Ridge, N.Y., U.S.A.). After freeze-drying, the samples were ground with dry ice using a Wiley Mill (Thomas Scientific, Swedesboro, N.J., U.S.A.). Four hundred milligrams of ground samples were hydrolyzed in 12 mL of 6 *N* HCl at 110 °C for 24 h. The extractable amount of proline and glutamine were analyzed by an improved HPLC method (Fermin and others 2003).

Statistical analyses

Data were analyzed using the Univariate and Mixed Procedures of the Statistical Analysis System version 6.02 (SAS Inst. Inc., Cary, N.C., U.S.A.). Pairwise mean differences were evaluated using the Tukey's test ($\alpha = 0.05$), which operates with 3 or more samples and their means and tests the mean of each population against the mean of the other populations (Croarkin and Tobias 2002).

Results and Discussion

Table 1 compares the protein content (%) and the amount of proline and glutamine in the whole-wheat flours milled from the whole grains of Karl 92, a hard red winter cultivar, and Sisson 36, a soft red winter cultivar. The whole grains of Karl 92 and Sisson 36 have an average total protein content of approximately 12.50%. As expected, the flours of Sisson 36 were found to have significantly lower concentrations of both proline and glutamine than Karl 92 flours. The respective proximate content (ash and protein) of the whole-wheat flours of Karl 92 and Sisson 36 was 1.66% and 1.53% (ash) and 11.4% and 10.8% (protein). The pH values were 6.43 and 6.41, respectively. No difference was found between the flours of Karl 92 and Sisson 36 in terms of ash, protein, and pH ($P < 0.05$).

One of the factors that can cause changes in water requirements, dough mixing times, browning of crusts, and gas evolution in yeast systems is starch damage (Atwell 2001). The flours milled from Karl 92 contained damaged starch at 4.8% to 6.5% of the flour weight, whereas the flours from Sisson 36 showed slightly less starch damage (3.6% to 5.6% of flour weight). However, the difference was insignificant ($P < 0.05$). Therefore, the differences between the flours milled from Karl 92 and those from Sisson 36 in proline and glutamine concentrations were used as the bases for the addition of proline or glutamine in the following studies.

Effect of proline and glutamine on functional properties

The effects of proline and glutamine on the functional properties of dough and bread produced from Sisson 36, the cultivar that has lower glutamine and proline than Karl 92, were investigated by adding the respective differences of proline and glutamine between the flour of Karl 92 and Sisson 36 to the latter. Proline and glutamine (22.62 and 43.14 μmol per g of flour, respectively) were dissolved in distilled water before addition to minimize possible competition for water during the mixing of dough. Since the rheological properties of dough play important roles during several processing steps after mixing, 5 important physical properties of dough during breadmaking were measured: stickiness, work of adhesion, dough strength (cohesion), resistance to extension, and extensibility.

Table 1—Comparison of the protein content (%) and the key amino acids proline and glutamine (μmol per gram of wheat flour) in the flours milled from Karl 92 and Sisson 36[a]

Wheat variety	Protein	Glutamine	Proline
Karl 92	11.4 ± 0.4a	224 ± 17a	152 ± 11a
Sisson 36	10.8 ± 0.5a	181 ± 16b	130 ± 8b
Difference	0.6 ± 0.9	43 ± 33	22 ± 19

[a]Means ± SD, $n = 15$. Values in the same column with the same letters are not significantly different ($P < 0.05$).

No significant differences were found among the dough and bread properties of Sisson 36 flours treated with proline and the untreated Sisson 36 flours except for dough stickiness and bread firmness (Table 2). Addition of proline significantly increased dough stickiness, a characteristic that is usually coupled with the measurements of dough strength and adhesion properties, and slightly increased dough strength and work of adhesion. Previous studies have demonstrated that the intramolecular H bonding in proline is much stronger than in other amino acids (Stepanian and others 2001). Although direct evidence remains to be uncovered to fully elucidate the specific role of proline in dough, based on the changes in dough properties it is reasonable to infer that the molecular interactions in the flour were enhanced by proline addition, resulting in a dough that was more sticky and adhesive than that of Sisson 36.

In terms of bread quality, dough resistance to extension and extensibility remained unchanged, whereas the bread firmness was increased after proline addition (Table 2). Intriguingly, while a firmer loaf was produced with the addition of proline, a reduction in loaf volume and a coarser crumb structure were observed in proline-treated samples. Known to favor β-sheets and similar structures that have been hypothesized to be responsible for some of gluten's elastic character (Ewart 1968; Stauffer 1998), proline was expected to increase the elastic character that would provide greater gas retention capacity, leading to increased loaf volume and a good aerated crumb structure with less firm texture. However, such effects appeared to be ineffective in the present study when only proline was added.

While only the difference of glutamine was added into Sisson 36 (Table 2), the dough stickiness was increased, as seen when proline was added alone. However, no significant difference was found in bread firmness and other functional properties. Despite the changes in dough stickiness, the effect of glutamine appeared to be not as significant in the finished bread as it was in the dough, suggesting that the heating during the baking process might have hindered the ability of glutamine to bind water effectively when it stayed in the free form (Atwell 2001). Comprising 1/3 of the amino acids in gluten, the H bonds resulting from glutamine side chains have been shown to be an important factor in determining the rheological properties of gluten (Mita and Matsumoto 1981). However, in the present study, loss of glutamine's water-binding capacity during heating when incorporated as a free amino acid (Crowley and others 2001) might have hindered its contribution to the functional properties of dough and bread.

When the differences of proline and glutamine between Karl 92 and Sisson 36 were both added to the latter, most dough and bread properties were increased, except bread firmness, gumminess, resilience, and springiness (Table 2). Dough stickiness was only increased slightly with addition of proline or glutamine. Further increase was observed when both amino acids were added. Calderón-Dominguez and coworkers (2004) suggested that changes in dough stickiness usually correspond to differences in the dough's resistance to extension. In this case, resistance to extension remained unchanged when only proline or glutamine was added.

Table 2—Comparison of functional properties of Sisson 36 and Sisson 36 treated with the required amount of proline (Pro) and/or glutamine (Gln) to match the levels in Karl 92[a]

Textural properties	Sisson 36	Sisson 36 + Pro[b]	Sisson 36 + Gln[c]	Sisson 36 + Pro + Gln	Karl 92
Dough properties					
Stickiness (g)	3.0 ± 1.3d	4.0 ± 0.6c	4.0 ± 1.3c	5.4 ± 1.2 b	8.4 ± 1.0 a
Work of adhesion (g-s)	0.10 ± 0.05c	0.13 ± 0.03c	0.14 ± 0.06c	0.30 ± 0.09b	0.50 ± 0.11a
Cohesion/strength (mm)	0.3 ± 0.1b	0.3 ± 0.1b	0.4 ± 0.1b	0.6 ± 0.1a	0.7 ± 0.1a
Resistance to extension (g)	27.9 ± 1.6c	29.5 ± 1.3c	28.0 ± 1.3c	32.0 ± 2.3b	50.0 ± 2.1a
Extensibility (mm)	9.4 ± 1.5b	9.0 ± 1.1b	10.0 ± 1.2b	12.5 ± 1.5a	14.5 ± 1.5a
Bread properties					
Firmness (g)	5.5 ± 0.2b	5.8 ± 0.7a	5.4 ± 0.1b	5.5 ± 0.4b	5.4 ± 0.2b
Chewiness (g)	1.2 ± 0.3c	1.1 ± 0.2c	1.3 ± 0.5c	2.1 ± 0.2b	2.8 ± 0.1a
Gumminess (g)	3.2 ± 0.7b	3.1 ± 0.2b	3.6 ± 0.4b	3.4 ± 0.3b	4.1 ± 0.4a
Cohesiveness	0.51 ± 0.09c	0.45 ± 0.06c	0.53 ± 0.08c	0.71 ± 0.03b	0.78 ± 0.07a
Resilience	0.05 ± 0.01a	0.05 ± 0.01a	0.04 ± 0.02a	0.05 ± 0.01a	0.05 ± 0.01a
Springiness	0.83 ± 0.07b	0.76 ± 0.03b	0.79 ± 0.05b	0.84 ± 0.04b	0.94 ± 0.02a
Loaf volume[d] (cm^3)	759.0 ± 24.8c	637.2 ± 36.9d	764.1 ± 12.7c	791.0 ± 21.0b	805.0 ± 42.1a

[a]Means ± SD, n = 12. Values in the same row with the same letter are not significantly different ($P < 0.05$).
[b]Addition of proline was based on 22.62 μmol of proline per gram of wheat flour.
[c]Addition of glutamine was based on 43.14 μmol of glutamine per gram of wheat flour.
[d]Loaf volume of 100 g bread was determined using AACC Method 10-10.

Significant increase in resistance to extension was observed while proline and glutamine were both added. Because resistance to extension is the degree of force required for the dough to change its shape as it resists deformation (Cauvain 1998), when only a modest force is applied, the dough that is more sticky in nature reverts faster and easier to its original shape. Similarly, a slight decrease in loaf volume was observed when Sisson 36 was treated with proline alone, whereas treatment with glutamine appeared to have no change on the volume. The loaf volume was significantly increased when both proline and glutamine were added. These observations could possibly be attributed to a synergistic effect on the stickiness of dough when both proline and glutamine were added.

As seen in Table 2, the dough and bread properties of Sisson 36 treated with both proline and glutamine were still inferior to those of Karl 92, despite the significant increases from untreated Sisson 36 or Sisson treated with one of the amino acids. It is generally agreed that soft winter wheat (in this case, Sisson 36) gives less desirable breadmaking performance than that of hard winter wheat (Karl 92), which is currently used in the industry for making many breads (Atwell 2001). The dough and bread properties that showed synergistic increases that were unseen when either proline or glutamine was added individually suggested that both amino acids are needed to achieve the desirable texture attributes. For instance, dough extensibility, which is defined by the distance that the center of the dough piece can be stretched, is closely linked to dough elasticity, which is dependent on the development of molecular network, as it is defined by the amount of stress that can be applied before the dough breaks (Cauvain 1998; Stauffer 1998).

Increasing glutamine that readily forms hydrogen bonds with electron donors such as other amides and water molecules should increase intermolecular reactions in the dough matrix. In fact, individual hydrogen bonds are relatively weak, but the presence of many of them lends overall strength to the interchain reactions. Aside from interchain reactions, hydrogen bonds serve to stabilize the β-turn spirals in the central portions of glutenin molecules, influencing solubility and viscosity properties (Ewart 1972; Mita and Matsumoto 1984; Stauffer 1998). The high proline content in glutenin provides gluten with the ability to form extended conformations, as proline residues cause bends and folds in the polypeptide chains, requiring force to unfold them. Once the internal molecular adhesion is overcome, a great capability for extension becomes possible (Ewart 1968). Proline residues favor the formation of β-sheets composed of β-turn spirals (connected by hydrogen bonding), which can be slightly extended and act as springs (Tatham and others 1985; Stauffer 1998). Hence, increases of proline would result in a more rigid, more intact dough matrix with increased tolerance to environmental stress.

Dependency of functional properties on proline and glutamine

The ability of proline and glutamine to modify the functional properties of dough and bread appears to be interdependent. The known ability of glutamine to provide more intermolecular and intramolecular H bonding during the mixing of dough (Belton 1999) could be leveraged when the conformational bends created by proline offers more points of contact for H bonding to occur (Nelson and Cox 2000; Tseng and Lai 2002). To further investigate the dependency of functional properties on proline and glutamine, as well as the differences between soft wheat and hard wheat, a 2nd approach was taken in which Karl 92, a hard red winter wheat, was used. The addition of proline and glutamine were fixed at 5% by weight of wheat flour. Initial tests were conducted in which 1% of proline or glutamine was added to Karl 92, but no significant effects were noted at this level. Table 3 summarizes the changes of dough and bread functional properties before and after the addition of 5% (w/w) proline and glutamine.

Based on these data, glutamine and proline exhibit opposite effects on the stickiness properties of the dough. Proline increased stickiness and dough cohesion/strength as well as the work of adhesion required. Glutamine, on the other hand, reduced dough stickiness, cohesion, and work of adhesion, which was opposite from what was seen when it was added to soft wheat (Sisson 36). The implications of these effects are crucial in the early stage of mixing because the quality and extent of dough and gluten development are determined at this stage in the breadmaking process (Chen and Hoseney 1995b; Stauffer 1998). Also different from soft wheat samples, while proline and glutamine addition both significantly increased dough extensibility to equivalent levels ($P < 0.05$), only glutamine was shown to affect resistance to extension and deformation by reducing such resistance, which could consequently reducing the elastic character of the dough.

On the other hand, bread firmness and springiness were not affected by any of the amino acid treatments. Measurements of chewiness, gumminess, cohesiveness, and resilience were designed to predict bread textural behavior when forces are exerted during the chewing process. Results showed that both proline and glutamine

Table 3—Comparison of dough and bread properties before and after respective addition of proline (Pro) or glutamine (Gln) at 5% by weight of flour to Karl 92 flours[a]

Textural properties	Karl 92	Karl 92 + Pro	Karl 92 + Gln
Dough properties			
Stickiness (g)	8.4 ± 1.0b	12.4 ± 1.7a	6.9 ± 1.2c
Work of adhesion (g-s)	0.50 ± 0.11b	0.80 ± 0.19a	0.30 ± 0.14c
Cohesion/strength (mm)	0.7 ± 0.1b	0.9 ± 0.2a	0.7 ± 0.2b
Resistance to extension (g)	50.0 ± 2.1a	50.1 ± 1.6a	46.2 ± 1.8b
Extensibility (mm)	14.5 ± 1.5b	17.1 ± 0.8a	18.0 ± 1.4a
Bread properties			
Firmness (g)	5.4 ± 0.2a	5.2 ± 0.2a	5.3 ± 0.1a
Chewiness (g)	2.4 ± 0.3a	2.3 ± 0.6a	1.2 ± 0.2b
Gumminess (g)	4.1 ± 0.4b	4.8 ± 0.9a	5.0 ± 0.7a
Cohesiveness	0.78 ± 0.07b	0.92 ± 0.17a	1.06 ± 0.14a
Resilience	0.05 ± 0.01b	0.06 ± 0.01a	0.07 ± 0.01a
Springiness	0.94 ± 0.02a	0.96 ± 0.01a	0.95 ± 0.03a
Loaf volume (cm^3)	805.0 ± 42.1c	885.0 ± 38.3a	831.2 ± 24.9b

[a]Means ± SD, n = 12. Values in the same row with the same letter are not significantly different (P < 0.05).

Table 4—Changes in proline and glutamine contents (μmol per gram of flour) before and after the breadmaking process (AACC Method 10-10B)[a]

Content	Before	After	% Difference
Karl 92			
Proline	152.0 ± 4.2a	66.1 ± 6.8b	–57%
Glutamine	223.2 ± 6.4a	59.6 ± 4.8b	–74%
Karl 92 + Proline			
Proline	589.1 ± 4.2a	530.1 ± 5.2b	–10%
Glutamine	223.7 ± 6.4a	205.9 ± 1.6b	–8%
Karl 92 + Glutamine			
Proline	152.4 ± 4.2a	147.8 ± 2.4a	N/D[b]
Glutamine	567.2 ± 6.4a	417.0 ± 9.8b	–26%

[a]Mean ± SD, n = 3. Values in the same row with the same letter are not significantly different (P < 0.05).
[b]N/D = not differentiable.

Table 5—Changes in gluten (%, dry basis), glutenin, and gliadin fractions (% per weight of gluten) of flour dough before and after mixing with optimum water absorption[a]

	Type of flour		
Components	Karl 92	Karl 92 + 5% Gln	Karl 92 + 5% Pro
Gluten			
Before mixing	13.6 ± 3.7a (A)	13.2 ± 3.1a (B)	13.0 ± 2.8a (A)
After mixing	14.2 ± 2.7b (A)	17.3 ± 1.8a (A)	11.1 ± 2.2c (B)
Glutenin			
Before mixing	61.1 ± 5.4a (A)	61.4 ± 3.2a (B)	60.2 ± 3.9a (B)
After mixing	59.3 ± 4.2c (A)	69.1 ± 2.5a (A)	64.1 ± 3.0b (A)
Gliadin			
Before mixing	38.8 ± 1.6a (A)	38.5 ± 3.7a (A)	39.7 ± 5.1a (A)
After mixing	40.6 ± 5.2a (A)	30.8 ± 2.6c (B)	35.8 ± 2.9b (B)

[a]Mean ± SD, n = 3. Values in the same row with the same lower-case letter or in the same column under the same component with the same letter in parentheses are not significantly different (P < 0.05).

affected sensory texture properties of bread in terms of gumminess, cohesiveness, and resilience. The loaf volume of Karl 92 bread was increased with the addition of 5% (w/w) proline, more significant than the addition of glutamine (5%, w/w), indicating the ability of proline to contribute more than glutamine to the network of bread structure. These observations were different from when adding glutamine and proline to Sisson 36 flours (Table 2), where proline caused a reduction in loaf volume and glutamine did not seem to have any effect when added alone. Therefore, it is obvious that the superior dough properties of Karl 92 flours to Sisson 36 are also dependent on other factors in addition to the proline and glutamine contents.

Effect of the breadmaking process on the retention of proline and glutamine

To understand the effect of the breadmaking process on the retention of proline and glutamine, Karl 92 flour was used to take advantage of its superior breadmaking properties. Table 4 summarizes the changes in proline and glutamine, the gluten content, as well as the glutenin and gliaden fractions, before and after baking using flours with or without adding proline or glutamine. The initial glutamine content of wheat flour was 223.2 μmol/g; initial proline 152.0 μmol/g. Addition of glutamine and proline to the wheat flour increased initial glutamine and proline to 567.2 μmol/g and 589.1 μmol/g, respectively. In proteins, the number of each amino acid species and the unique sequence of its incorporation through peptide bond formation determine the conformation of a protein molecule by defining the possibilities for interaction of any residue with other residues within or outside the chain (Kasarda and others 1971). Untreated flour resulted in the greatest losses in proline and glutamine concentrations after undergoing the breadmaking process. Treatment with proline increased proline concentration after baking and resulted in decreased glutamine losses compared with untreated flour. Treatment with proline or glutamine reduced glutamine and proline losses after the breadmaking process compared with untreated flour. Addition of either proline or glutamine appears to have a protective effect on the retention of the other amino acid.

In addition, the changes of gluten and its subunits, glutenin and gliadin, before and after dough mixing were measured (Table 5). The gluten content remained almost unchanged after mixing in the control Karl 92. Addition of 5% glutamine increased the overall gluten percentage per dry weight of the flour, in agreement with the enhanced dough properties reported in Table 3. In contrast, addition of 5% proline reduced the total percentage of gluten in the dough, which is in line with the reduced values of the majority of dough and bread properties (Table 3). Moreover, addition of proline and glutamine affected the composition of gluten. The glutenin subunit was increased in dough with amino acid addition, whereas the gliadin subunit was decreased after mixing (P < 0.05).

Glutenin is a very large molecule that consists of protein subunits connected by disulfide linkages (Atwell 2001). Increasing glutamine increases amide side chains of the gluten proteins that are responsible for the cohesiveness of the dough structure because of their great H-bonding capability. It could therefore be inferred that this capacity for H-bonding in turn strengthens the gluten network and forms a more stable dough matrix that would be more tolerant to environmental stress brought about by heating, pH changes, or ingredient addition and mixing. The increase of the glutenin subunit after mixing when proline was added to Karl 92 could be attributed to the fact that proline is known to stabilize β-sheets, which are held together by hydrogen bonds (Stauffer 1998), and proline's cyclic ring structure theoretically constrains the conformational flexibility of proline (Stepanian and others 2001).

The decrease in gliadin, which is composed of individual protein chains and imparts the viscous nature to gluten (Atwell 2001), after mixing when glutamine was added supports the previous observation that the viscous properties (for example, stickiness and resistance to extension) of dough was reduced when 5% glutamine was added to Karl 92 flour (Table 3). Conversely, the viscous properties of

Karl 92 dough added with 5% proline were increased (Table 3), in spite of the reduction in gliadin percentage (Table 5), indicating that the viscous properties of dough are more dependent on the overall gluten content than on glutenin or gliadin. The increase of loaf volume (Table 3) appeared to correlate well with the concentration shift in glutenin and gliadin (Table 5), regardless of the slight decrease in overall gluten content when 5% proline was added to Karl 92 flour. This is most likely due to the ability of the increased glutenin to form disulfide bonds during baking, yielding a product with better gas-holding capacity and, consequently, higher loaf volume.

Conclusions

The addition of proline and glutamine to winter wheat flour was proven to affect the functional properties of wheat dough as well as the bread. Addition of proline or glutamine individually did not provide significant improvements in soft wheat dough and bread properties. Combination of glutamine and proline could synergistically enhance the viscous properties of dough and bread attributes such as loaf volume in soft wheat. Addition of glutamine to hard wheat increased the glutenin subunit while enhancing the development of gluten during mixing. Conversely, despite the increase in glutenin subunit, proline hindered gluten formation. The findings in this study provide a foundation for characterizing the properties of dough and bread based on the contribution of key constituents and will be of interest to researchers and product developers for the development of specialty bread products. Additionally, should the degree of dependence of breadmaking properties on these 2 amino acids be elucidated, a better control of dough quality would be within reach, enabling selection criteria to be set for wheat breeders.

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