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Accelerated Hydrolysis Method To Estimate the Amino Acid Content of Wheat (*Triticum durum* Desf.) Flour Using Microwave Irradiation

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ABSTRACT: The technique of microwave-assisted acid hydrolysis was applied to wholegrain wheat (*Triticum durum* Desf. cv. Balcali 2000) flour in order to speed the preparation of samples for analysis. The resultant hydrolysates were chromatographed and quantified in an automated amino acid analyzer. The effect of different hydrolysis temperatures, times and sample weights was examined using flour dispersed in 6 N HCl. Within the range of values tested, the highest amino acid recoveries were generally obtained by setting the hydrolysis parameters to 150 °C, 3 h and 200 mg sample weight. These conditions struck an optimal balance between liberating amino acid residues from the wheat matrix and limiting their subsequent degradation or transformation. Compared to the traditional 24 h reflux method, the hydrolysates were prepared in dramatically less time, yet afforded comparable ninhydrin color yields. Under optimal hydrolysis conditions, the total amino acid recovery corresponded to at least 85.1% of the total protein content, indicating the efficient extraction of amino acids from the flour matrix. The findings suggest that this microwave-assisted method can be used to rapidly profile the amino acids of numerous wheat grain samples, and can be extended to the grain analysis of other cereal crops.

KEYWORDS: microwave hydrolysis, wheat, *Triticum durum* Desf., amino acid analysis, accelerated hydrolysis, analytical technique

INTRODUCTION

In human and animal systems, a balanced intake of the 20 amino acids from various protein sources plays a major role in sustaining metabolic functions such as modulation of gene expression,[1] upkeep of intestinal integrity,[2] protein synthesis,[3] and cellular signaling and regulation.[4] Cereals represent the greatest share of the total diet, with a 47% global fraction on average, and a fraction nearing 70% in many countries such as Bangladesh. Among the cereals, wheat contributes most to the nutrient intake of humans and livestock in many developing countries.[5,6]

An inadequate quantity and diversity of the dietary protein intake continues to predominate in many developing countries,[7,8] particularly in regions where the diet is heavily based on cereals.[5,9] Efforts led by the United States Department of Agriculture (USDA) World Wheat Collection screening program have indicated that the protein content can vary from 7 to 22% among the different modern wheat genotypes. While wheat does in fact contain all of the essential amino acids, the content of lysine, threonine, cysteine and methionine is low compared to foods of animal origin.[10] Many mutagenesis and conventional breeding attempts have been implemented to improve both the protein content and amino acid composition. A most noteworthy example relates to the selection performed at the International Maize and Wheat Improvement Center (CIMMYT), which resulted in opaque-2 genotypes containing remarkably high concentrations of lysine.[11−13] Still, the amino acid constitution of wheat continues to play a decisive role in preventing or defining the nature of malnutrition in developing countries, particularly in the context of child development.[14,15]

In most cases, the total amounts of nitrogen (N) and protein are quantified and serve as a standard measure of the nutritional quality of cereal grains. Very little attention has been paid to the amount and composition of the amino acid constituents, in spite of their direct impact on human nutrition and development, as described before. One major constraint responsible for the limited data on amino acid profiles in cereal grains is related to the lack of simple, quick and sensitive methods for the determination of amino acids. Among the sample manipulations employed, acid hydrolysis describes a critical step in enabling the successful analysis of amino acids in various biological samples.[16,17] Several factors are known to influence the hydrolysis process and, hence, the liberation of amino acids. In particular, parameters such as time, temperature, sample matrix, sample mass, and hydrolyzing agent are established considerations.[16,18,19] Even under optimal conditions, one of the ongoing practical challenges of the hydrolysis step has been related to improving the speed and efficiency of amino acid liberation. In keeping with the rate-accelerating traits and cost-efficiency of microwave irradiation, the use of microwave-assisted hydrolysis has grown increasingly popular, especially in the food industry and protein chemistry field.[16,18,20,21] Depending on the sample matrix, it has been possible to reduce the hydrolysis time from about 24 h under standard reflux conditions to only a few hours or a few minutes.[18,21,22]

In the routine amino acid analysis of insoluble composites such as cereal grain samples, conventional acid hydrolysis techniques still predominate. Of the available methods, the application of 6 N HCl at 110 °C for 24 h has proved most common.[17,23−25] Given the advantages reported for model proteins, the extension of microwave assistance to the hydrolysis

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of grain seemed to depict a logical progression, with clear benefits particularly anticipated in the analysis of large sample numbers.[21] Still, microwave irradiation has been rarely applied to cereal grain samples.[26] In fact, no systematic characterization of amino acid recoveries has been reported following microwave-assisted hydrolysis. In view of the dearth of available data, a range of values reflecting three hydrolysis parameters, namely, the temperature, time and sample weight, was examined to ascertain the optimum hydrolysis conditions. The parameter set corresponding to the hydrolysis temperature (130 °C, 150 °C 170 °C), time (1 h, 2 h, 3 h and 4 h), and sample weight (100 mg, 200 mg, 300 mg, 400 mg, and 500 mg) was arbitrarily chosen in light of prior work highlighting the microwave-assisted hydrolysis of noncereal as well as cereal protein samples.[18−21] A fourth parameter, namely, the acid type, was fixed as 6 N HCl in order to facilitate yield comparisons against the conventional reflux method.[16,17,28,30,31] Herein, the merit of microwave-assisted hydrolysis was demonstrated using wholegrain flour samples of a durum wheat cultivar (*Triticum durum* Desf. cv. Balcali 2000). The hydrolysis parameters were first optimized using a multiple-sample microwave instrument. Second, the individual as well as total recoverable amino acid yields were correlated against those obtained by the traditional 24 h acid reflux method. Lastly, the % recovery was determined by comparing the total recoverable amino acid values against the total protein content, the latter being derived from the total N assay.

MATERIALS AND METHODS

Materials. Durum wheat seeds (*Triticum durum* Desf. cv. Balcali 2000) were obtained from the Cukurova University Research Farm, Adana, Turkey. The standard reference material SRM 8436 Durum Wheat Flour was purchased from the National Institute of Standards and Technology, Gaithersburg, MD, USA. Ninhydrin solution, citrate-borate buffer 0.5 M, pH 8.6) and sodium citrate buffers (0.2 M, pH 2.2, 2.65, 3.35 and 4.25) were purchased from Biochrome Ltd., Cambridge, U.K. Sterile syringe-tip filters with polyethersulfone (PES) membrane (0.22 μm pore size) were purchased from Techno Plastic Products AG, Trasadingen, Switzerland. Standards were prepared by diluting the mixed Sigma AAS18 amino acid standard (i.e., L-alanine, ammonium chloride, L-arginine, L-aspartic acid, L-cystine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine and L-valine at 2.5 μmol/mL in 0.1 N HCl, except L-cystine at 1.25 μmol/mL) in sodium citrate loading buffer (0.2 M, pH 2.2). All other chemicals were obtained as high-grade materials from commercial suppliers and were used without further purification. Deionized water was employed throughout the study.

Sample Preparation. Seed material (20 g) was dried (40 °C, 2 h) and milled (700 rpm, 5 min) in a vibrating agate cup mill (Pulverisette 9, Fritsch GmbH, Idar-Oberstein, Germany) to afford finely pulverized wholegrain flour. The standard reference material SRM 8436 Durum Wheat Flour was used as received.

Microwave-Assisted Hydrolysis and Prequantification Protocol. A closed-vessel microwave system (MarsExpress, CEM Co., Matthews, NC, USA) was employed in all hydrolysis experiments. The system was able to simultaneously process up to 40 samples in individual 55 mL Teflon vessels capped with fixed-pressure release values. Three variables, i.e., sample weight, hydrolysis period and hydrolysis temperature, were optimized using the ninhydrin color yields as a measure of amino acid recovery. In a commonly applied run, wholegrain wheat flour samples (i.e., 100, 200, 300, 400, or 500 mg [±1 mg]) were delivered to the vessels, and hydrolysis solution (5 mL, 6 N HCl) was added. To limit oxidation of the amino acids during hydrolysis, the suspensions were purged with N_2 gas (1 min) and thereafter the vessels were immediately capped. The samples were hydrolyzed under temperature control and autogenous pressure. In efforts to ascertain the optimal hydrolysis conditions, the microwave system was programmed to ramp to a fixed temperature (i.e., 130, 150, or 170 °C [±5 °C]) within 30 min, and to maintain that temperature for a given time (i.e., 1, 2, 3, or 4 h) by using a maximum of 1200 W output power at 2.45 GHz.

Like any acid-mediated technique, microwave-assisted hydrolysis will only report on the population of acid-stable amino acids.[28−34] Hence, Trp is destroyed, the side chains of Gln and Asn are hydrolyzed, and the amino acids Cys and Met are partially degraded and oxidized. The lattermost two can be quantified by applying a revision to the method herein.[35] For practical reasons, only the free amino acids, and not incompletely hydrolyzed peptides, have been detected and quantified as a function of time. Hence, it is not possible using the method herein to extract detailed kinetic information potentially relating to mechanisms, induction periods, rate constants or reaction order. If desired, these finer details can be elucidated as per the method of Wattanapat et al.[36]

Upon completion of the hydrolysis period, the vessels were cooled to room temperature in a water bath. Each hydrolysate was topped to 10 mL with 6 N HCl, and 1 mL of the resultant mixture was treated with 0.55 mL of 32% (w/v) NaOH to give a final pH value of 2.2 [±0.2]. Five milliliters of 200 mM sodium citrate loading buffer was added to each sample, bringing the total volume to 6.55 mL. Lastly, the samples were forced through syringe-tip polyethersulfone filters (pore size 0.22 μm) into 2 mL glass vials. The vials were placed in batches into a refrigerated (+4 °C) autosampler rack of an amino acid analyzer, and the filtrates were analyzed sequentially as soon as possible. Since up to forty samples could be hydrolyzed simultaneously, the bottleneck of this quantification was no longer the hydrolysis process, but rather the amino acid analysis itself, with a single sample run typically requiring about 1 h. When analyzing the sample batches using one amino acid analyzer, samples in the queue faced delays up to five days. Still, these delays had no significant effect on the amino acid analysis results of hydrolysates and standards kept at +4 °C (data not shown).

Amino Acid Resolution and Quantification. The hydrolysates and standards were analyzed using an automated amino acid analyzer (Biochrom 32 Oxidised Hydrolysate System, Biochrom Ltd., Cambridge, U.K.) configured with postcolumn ninhydrin-derivatization. A calibration standard was prepared by diluting the Sigma AAS18 mixed standard in sodium citrate loading buffer, yielding a final concentration of 5 nmol of each amino acid per 20 μL of volume. A fixed injection volume of 20 μL was applied to the sample runs as well as to the standard. The ninhydrin derivative of Pro was detected at 440 nm whereas the remaining amino acid derivatives were detected at 570 nm. The ninhydrin color yields were equated to the amino acid recovery, and these values were interpreted as an apparent measure of the absolute amino acid concentration (i.e., g of recoverable amino acid per 100 g of wholegrain wheat flour).

Validation. SRM 8436 Durum Wheat Flour was hydrolyzed using the optimum microwave conditions, and the hydrolysates were subjected to amino acid analysis. The outcome was compared against the 24 h reflux method in a round robin assessment. Two independent laboratories (i.e., Biochrom Ltd. and Ansynth Service B.V. laboratories) were selected to implement the 24 h reflux method.

Protein Concentration of Flour. The total protein concentration of flour samples was calculated by multiplying the total N concentration of the samples by a protein conversion factor (i.e., Jones factor) of 5.83.[27] The total N concentration was determined in triplicate using an automated N analyzer (TruSpec CN, LECO Corp., St. Joseph, MI, USA), and the mean value and standard deviation were determined and propagated thorough all relevant calculations (*vide infra*). All results were validated against the certified N value of SRM 8436 Durum Wheat Flour.

Table 1. Recoverable Concentration of Amino Acids (g/100 g of Whole Wheat Flour) as a Function of Sample Mass

amino acid	100 mg	200 mg	300 mg	400 mg	500 mg
Ala	0.40[a] ± 0.02[b] B[c]fg[d]	0.44 ± 0.02 Ah	0.41 ± 0.03 Bdef	0.41 ± 0.02 Bef	0.38 ± 0.01 Bef
Arg	0.45 ± 0.03 BCef	0.54 ± 0.03 Aef	0.46 ± 0.06 Bdef	0.42 ± 0.01 BCef	0.38 ± 0.02 Cef
Asp	0.55 ± 0.02 Ad	0.59 ± 0.02 Aef	0.55 ± 0.05 Acde	0.52 ± 0.04 ABd	0.46 ± 0.06 Bde
Glu	3.21 ± 0.19 ABa	3.70 ± 0.13 Aa	3.12 ± 0.53 Ba	3.22 ± 0.12 ABa	2.87 ± 0.14 Ba
Gly	0.46 ± 0.01 ABef	0.48 ± 0.02 Agh	0.39 ± 0.11 ABdef	0.43 ± 0.03 ABe	0.38 ± 0.04 Bef
His	0.34 ± 0.01 Agh	0.32 ± 0.01 ABi	0.30 ± 0.02 Bf	0.13 ± 0.04 Ci	0.16 ± 0.02 Cj
Ile	0.18 ± 0.02 Ci	0.36 ± 0.05 Ai	0.27 ± 0.05 Bf	0.28 ± 0.02 Bh	0.26 ± 0.05 Bghi
Leu	0.66 ± 0.04 Ac	0.72 ± 0.02 Ac	0.70 ± 0.15 Abc	0.77 ± 0.03 Ac	0.70 ± 0.04 Ac
Lys	0.32 ± 0.02 ABh	0.35 ± 0.02 Ai	0.26 ± 0.07 BCf	0.27 ± 0.01 BCh	0.24 ± 0.01 Cij
Phe	0.48 ± 0.02 Ae	0.47 ± 0.03 Agh	0.35 ± 0.05 Bef	0.36 ± 0.01 Bfg	0.34 ± 0.01 Bfgh
Pro	0.95 ± 0.06 Bb	1.16 ± 0.06 Ab	0.90 ± 0.21 Bb	0.95 ± 0.03 Bb	0.87 ± 0.05 Bb
Ser	0.61 ± 0.03 ABcd	0.65 ± 0.01 Ad	0.61 ± 0.05 Acd	0.58 ± 0.05 ABd	0.49 ± 0.11 Bd
Thr	0.28 ± 0.02 Bh	0.36 ± 0.02 Ai	0.31 ± 0.05 ABef	0.30 ± 0.01 Bgh	0.25 ± 0.03 Bhi
Tyr	0.58 ± 0.01 Ad	0.33 ± 0.01 Bi	0.33 ± 0.01 Bef	0.28 ± 0.04 Bh	0.21 ± 0.05 Cij
Val	0.34 ± 0.01 Bgh	0.52 ± 0.06 Afg	0.39 ± 0.11 Bdef	0.36 ± 0.04 Befg	0.35 ± 0.07 Bfg
total[e]	9.80 ± 0.21 B	11.00 ± 0.18 A	9.34 ± 0.63 B	9.27 ± 0.16 B	8.34 ± 0.23 C
recovery[f] (%)	75.8 ± 1.7	85.1 ± 1.4	72.2 ± 4.9	71.6 ± 1.3	64.5 ± 1.8

[a] Mean value of three independent analyses. [b] Standard deviation of three independent analyses. [c] Different uppercase letters indicate statistically significant yield differences ($P < 0.05$) for a given amino acid type over the range of sample masses tested, with "A" signifying the highest recoverable yield. [d] Different lowercase letters indicate yield differences ($P < 0.05$) among the different amino acid types at a given sample mass, with "a" signifying the highest yield. [e] Total values are the sum of all amino acids analyzed excluding Trp, Cys and Met. [f] Recovery values were calculated as the ratio of total recoverable amino acids to total protein (i.e., % N × 5.83). The total protein was determined as 12.93% (± 0.03).

Numerical and Statistical Analysis. All amino acid determinations as well as total N assays (above) were performed in triplicate and their standard deviations calculated. The average (mean) amino acid values were reported (Tables 1, 2 and 3) along with their standard deviations. Uncertainties in precision were propagated through all subsequent summation and division operations as per standard error treatment methods.[37] The statistical analysis of analytical data, as denoted by the upper- and lowercase letter notation (Tables 1, 2 and 3), was performed using JMP software (SAS Institute, Cary, NC).

RESULTS

The recoverable concentration of amino acids (g/100 g of whole wheat flour) as a function of sample weight has been illustrated in Table 1. Among the range of sample weights evaluated (i.e., 100−500 mg), the 200 mg sample weight generally afforded the highest amino acid recoveries. One noteworthy exception was Tyr, with a maximum recovery clearly noted at 100 mg. Other exceptions were His, with a maximum recovery slightly favoring 100 mg, and Leu, with a maximum recovery independent of the sample weight. Incrementally increasing the sample weight beyond 200 mg to 500 mg was generally accompanied by lowered recoveries, particularly in the case of His, Thr, Phe and Ser. Tyrosine yields consistently dropped beyond 100 mg. In decreasing order, the most abundant amino acids noted in the 200 mg sample were Glu (Gln contributions inclusive), Pro and Leu. The lowest absolute concentrations corresponded to Lys, Tyr and His.

As presented in Table 2, the recoverable concentration of total amino acids was highest after 3 h of hydrolysis, the shorter or longer hydrolysis periods (i.e., 1, 2, or 4 h) yielding lower amino acid recoveries. With Pro as the only exception, the recoverable concentration of individual amino acid types was also greatest after 3 h of hydrolysis. Proline, the second most abundant amino acid in the wheat flour, was unusual in that it exhibited an exceptional insensitivity to hydrolysis time over the range of 2 to 4 h. The most abundant wheat constituent, Glu, together with two lesser constituents, Ala and Gly, was substantially influenced by the hydrolysis period. The recoveries of these amino acids were markedly lower at the 1 h, 2 h and 4 h time points. A hydrolysis period of 1 h resulted in an unsatisfactory recovery of many amino acids. For instance, compared to the optimum hydrolysis period of 3 h, the recoveries of Phe, Ser, Tyr and Thr had dropped by a factor of 3−3.5.

Table 3 illustrates the recoverable concentration of amino acids as a function of hydrolysis temperature. The total amino acid recovery as well as the recoveries of most of the different component amino acids was highest at 150 °C within the temperature range of 130−170 °C. Increasing the hydrolysis temperature from 150 to 170 °C improved the liberation of Val, Ile and most notably Leu. In contrast, the recovery of Asp was severely reduced at 170 °C. Among nearly all the amino acids, a reduction from 150 to 130 °C was accompanied by a statistically insignificant or significant drop in recovery. The sole exception was Tyr, which afforded a marginally improved yield at 130 °C. The recoveries of Arg, Glu, Gly, His, Pro and Thr were noted to have decreased by a statistically insignificant amount at 130 °C as well as 170 °C.

As described in Figure 1, the established wheat standard, i.e., SRM 8436 Durum Wheat Flour, was digested using either the optimized microwave-assisted method or the traditional 24 h reflux method. The amino acid analysis results indicated a strong correlation between the two methods (Figure 1, left and center). A strong correlation was also noted among the two 24 h reflux trials, which had been conducted at independent institutions (Figure 1, right).

Overall, the % recovery of total amino acids was subject to variability (see Tables 1, 2 and 3, bottom row). Still, it was calculated as 85.1% when quantified under optimal hydrolysis conditions. Percent recovery values were derived from the percentage ratio of total recoverable amino acids to total protein,

Table 2. Recoverable Concentration of Amino Acids (g/100 g of Whole Wheat Flour) as a Function of Hydrolysis Period

amino acid	1 h	2 h	3 h	4 h
Ala	0.20[a] ± 0.02[b] D[c]de[d]	0.28 ± 0.01 Bgh	0.44 ± 0.02 Ah	0.26 ± 0.00 Cg
Arg	0.20 ± 0.01 Cdef	0.34 ± 0.02 Bef	0.54 ± 0.03 Aef	0.32 ± 0.01 Bef
Asp	0.21 ± 0.03 Cde	0.37 ± 0.01 Bde	0.59 ± 0.02 Aef	0.34 ± 0.00 Be
Glu	1.85 ± 0.09 Da	2.67 ± 0.08 Ba	3.70 ± 0.13 Aa	2.43 ± 0.02 Ca
Gly	0.18 ± 0.02 Ddefg	0.31 ± 0.01 Bfg	0.48 ± 0.02 Agh	0.27 ± 0.00 Cg
His	0.13 ± 0.02 Cfgh	0.21 ± 0.00 Bi	0.32 ± 0.01 Ai	0.20 ± 0.00 Bhi
Ile	0.17 ± 0.01 Befgh	0.19 ± 0.02 Bi	0.36 ± 0.05 Ai	0.21 ± 0.02 Bh
Leu	0.34 ± 0.03 *Cc*	0.53 ± 0.02 Bc	0.72 ± 0.02 Ac	0.51 ± 0.01 Bc
Lys	0.14 ± 0.01 Cefgh	0.21 ± 0.01 Bi	0.35 ± 0.02 Ai	0.20 ± 0.00 Bh
Phe	0.13 ± 0.02 Cfgh	0.26 ± 0.04 Bh	0.47 ± 0.03 Agh	0.30 ± 0.04 Bf
Pro	0.94 ± 0.10 Bb	1.16 ± 0.01 Ab	1.16 ± 0.06 Ab	1.06 ± 0.03 ABb
Ser	0.19 ± 0.06 Cdef	0.41 ± 0.01 Bd	0.65 ± 0.01 Ad	0.37 ± 0.01 Bd
Thr	0.12 ± 0.01 Cgh	0.22 ± 0.01 Bi	0.36 ± 0.02 Ai	0.21 ± 0.00 Bh
Tyr	0.10 ± 0.01 Ch	0.19 ± 0.01 Bi	0.33 ± 0.01 Ai	0.17 ± 0.00 Bi
Val	0.25 ± 0.02 Bd	0.29 ± 0.03 Bgh	0.52 ± 0.06 Afg	0.30 ± 0.02 Bf
total[e]	5.14 ± 0.16 C	7.66 ± 0.10 B	11.00 ± 0.18 A	7.16 ± 0.06 B
recovery[f] (%)	39.8 ± 1.2	59.3 ± 0.8	85.1 ± 1.4	55.4 ± 0.5

[a] Mean value of three independent analyses. [b] Standard deviation of three independent analyses. [c] Different upper case letters indicate statistically significant yield differences ($P < 0.05$) for a given amino acid type over the range of hydrolysis times tested, with "A" signifying the highest recoverable yield. [d] Different lower case letters indicate yield differences ($P < 0.05$) among the different amino acid types at a given hydrolysis time, with "a" signifying the highest yield. [e] Total values are the sum of all amino acids analyzed excluding Trp, Cys and Met. [f] Recovery values were calculated as the ratio of total recoverable amino acids to total protein (i.e., % N × 5.83). The total protein was determined as 12.93 % (± 0.03).

the latter being extrapolated from the total N concentration of wheat using an established protein conversion factor (Jones factor) of 5.83.[27] For obvious reasons, acid-labile amino acids and incompletely hydrolyzed peptides contributed neither to the total ninhydrin color yield nor to the resultant total recoverable amino acid concentration.[28]±

DISCUSSION

The underlying goal of this study was to address the urgent need to expedite and to simplify the profiling of amino acids in wheat grain samples. To achieve this end, the rate-accelerating property of microwave irradiation was applied to the hydrolysis step. By way of this approach, hydrolysis times could be reduced from the routine 24 h incubation to just 3 h without sacrificing the analytical quality. The accelerated hydrolysis process reflected a weighted interplay of physicochemical events[38−43] leading to amino acid liberation as well as to transformation or destruction.[28,30−34,44] Acid permeation into the dry wheat matrix defined a precondition to sustain effective hydrolysis and product release.[41,42] With permeation realized, a predominantly heterogeneous-phase hydrolytic process was initiated. The process featured, to a first approximation, the essential physicochemical elements of solid−liquid interfacial chemistry, particularly reaction constraints related to hydrolysis.[40,42,43,45] Over time, the mode of hydrolysis shifted from a heterogeneous to a homogeneous regime, much like the scenario presented for rice husk.[43] Initially, large peptide solutes predominated in the acidic medium, followed by smaller peptides and eventually free amino acids.[28] Some of the freed amino acids and larger fragments likely reacted with matrix-derived glucose according to the Maillard mechanism, detracting from the overall yield.[44,46] Imine formation was also plausible by reaction of free amino groups and hydroxymethylfurfural, a hydrolysis product of glucose.[45] Other reactions of amino acids and matrix elements have been forecast or established; among the myriad of reactions potentially operative during acid hydrolysis, some amino acids have apparently assumed a catalytic role.[36]

As shown in Table 1, the best overall amino acid yields, with exception to Tyr, corresponded to the hydrolysis trials using 200 mg of sample. The outcome was noteworthy in the sense that proceeding from 200 mg (or 100 mg in the case of Tyr) to higher sample weights had in fact lowered the recoverable yields. An explanation was conveniently rationalized by referring to mechanistic studies of proteins subjected to conventional acid hydrolysis conditions.[28] In particular, the recoverable amino acid yields were perceived to have reflected the net interplay of two classes of competing chemical pathways; formation pathways, which liberated amino acids from the protein, and removal pathways, which removed amino acids via subsequent transformation or destruction. The formation pathways were perceived to reflect a weight-averaged contribution of pseudo first-order and possibly second-order as well as pseudo second-order kinetics in the rate-limiting steps. The pseudo first-order contributions accounted for the interplay between dilute protein, and concentrated HCl or water, in forming a rate-determining bimolecular transition state; the second-order contributions accounted for the interplay between dilute protein, and dilute in situ derived catalysts, in forming a rate-determining bimolecular transition state; last, the pseudo second-order contributions additionally incorporated the participation of concentrated HCl or water in a rate-determining ternary transition state.[45,36] In contrast to formation, the removal pathways were perceived to follow second, pseudo second or even greater order kinetics in the rate-determining steps. Once again, the reaction order accounted for the interplay between dilute amino acids, and one or more other reaction participants, in forming a rate-determining bimolecular or possibly ternary transition state.[45,38] Some non-amino acid participants included dilute in situ derived catalysts, dilute reactive wheat matrix byproducts, concentrated

Table 3. Recoverable Concentration of Amino Acids (g/100 g of Whole Wheat Flour) as a Function of Hydrolysis Temperature

amino acid	130 °C	150 °C	170 °C
Ala	0.42[a] ± 0.01[b] B[c]fg[d]	0.44 ± 0.02 Ah	0.43 ± 0.01 ABfg
Arg	0.44 ± 0.01 Af	0.54 ± 0.03 Aef	0.53 ± 0.06 A de
Asp	0.53 ± 0.04 Ade	0.59 ± 0.02 Ae	0.31 ± 0.04 Bhi
Glu	3.34 ± 0.07 Aa	3.70 ± 0.13 Aa	3.47 ± 0.19 Aa
Gly	0.45 ± 0.03 Aef	0.48 ± 0.02 Agh	0.45 ± 0.04 Af
His	0.29 ± 0.02 Ahij	0.32 ± 0.01 Ai	0.31 ± 0.04 Ahi
Ile	0.27 ± 0.06 Bj	0.36 ± 0.05 ABi	0.45 ± 0.03 Af
Leu	0.64 ± 0.04 Cc	0.72 ± 0.02 Bc	0.80 ± 0.02 Ac
Lys	0.29 ± 0.00 Bij	0.35 ± 0.02 Ai	0.34 ± 0.03 ABhi
Phe	0.35 ± 0.06 Bjhi	0.47 ± 0.03 Agh	0.37 ± 0.02 Bgh
Pro	1.03 ± 0.03 Ab	1.16 ± 0.06 Ab	1.15 ± 0.10 Ab
Ser	0.59 ± 0.05 Acd	0.65 ± 0.01 Ad	0.47 ± 0.08 Bef
Thr	0.30 ± 0.02 Ahij	0.36 ± 0.02 Ai	0.31 ± 0.04 Ahi
Tyr	0.37 ± 0.09 Afgh	0.33 ± 0.01 Ai	0.28 ± 0.03 Ai
Val	0.34 ± 0.10 Bghij	0.52 ± 0.06 Afg	0.59 ± 0.03 Ad
total[e]	9.65 ± 0.19 C	11.00 ± 0.18 A	10.27 ± 0.26 B
recovery[f] (%)	74.6 ± 1.5	85.1 ± 1.4	79.4 ± 2.1

[a] Mean value of three independent analyses. [b] Standard deviation of three independent analyses. [c] Different upper case letters indicate statistically significant yield differences ($P < 0.05$) for a given amino acid type over the range of hydrolysis temperatures tested, with "A" signifying the highest recoverable yield. [d] Different lower case letters indicate yield differences ($P < 0.05$) among the different amino acid types at a given hydrolysis temperature, with "a" signifying the highest yield. [e] Total values are the sum of all amino acids analyzed excluding Trp, Cys and Met. [f] Recovery values were calculated as the ratio of total recoverable amino acids to total protein (i.e., % N × 5.83). The total protein was determined as 12.93 % (± 0.03).

HCl and water. In keeping with the above kinetic schemes, the net amino acid accumulation and, hence, recoverable yield were expected to drop in response to any change of reaction parameter, which might cause the rate of amino acid removal to become disproportionately increased relative to the rate of amino acid formation.[38] Proceeding to higher sample weights exemplified such a scenario. For instance, if second-order removal kinetics and pseudo first-order formation kinetics applied on average during hydrolysis, a 2-fold rise of sample weight would be expected to quadruple the rate of amino acid removal, yet only double the rate of amino acid formation.[38]

What distinguished Tyr from the other amino acids in Table 1 was the pronounced extent of its removal. This distinction was consistent with the oxidative halogenation of phenol or the phenolic ring of model compounds by highly reactive electrophiles such as chlorine, chlorinium or bromonium species.[47,33,45] As marginal amounts of halide-transforming oxidants and transition metal ion catalysts were likely retained by the wheat matrix, this mode of removal was a probable contributor. Histidine afforded a similar recovery versus sample weight profile, albeit not as distinguished as Tyr. Even Phe, with a relatively unreactive ring, suffered losses that exceeded the average trend in proceeding to higher sample weights. Also noteworthy in Table 1 were the greater-than-average losses of Ser and Thr in proceeding from 200 mg to 500 mg. Rationalizing this observation were reactions distinct to Ser and Thr. Serine has been shown to decompose into pyruvic acid upon acid hydrolysis,[32] and protonated Thr is known to readily lose water.[48] Heating aqueous Ser and Thr at 120 °C and 4 h has produced pyrazine derivatives.[49] Also, Ser and Thr hydroxyl groups may have been transformed in the presence of certain metal ion catalysts and traces of residual or matrix-trapped oxidant.[45] It followed to again reason that the above removal scenarios were facilitated by the higher sample weights, as any reactive participants derived from the matrix would be available at higher concentrations. Even thermolysis alone has exerted a profound effect on Ser, causing decarboxylation at temperatures between 200 and 300 °C.[50] Leucine, Val and Ile did not yield a self-consistent weight versus recovery profile, despite the relatively similar character of the side-chain groups. The underlying reason is unclear.

As reported in Table 2, all amino acid recoveries, as well as the total amino acid recovery, were highest following 3 h of hydrolysis. Hydrolysis for 1 h proved overly brief, yielding largely incomplete hydrolysates. Two-hour hydrolysates appeared to still be substantially composed of dipeptides and larger peptides, despite their homogeneous appearance. This premise was consistent with the postcolumn ninhydrin detection scheme and the near-doubled amino acid recoveries noted at the 3 h time point. Beyond the 3 h time point, hydrolysis weighed more heavily on the removal pathways, detracting from the net accumulation of amino acids. The ongoing competition between formation and removal pathways was not assessed in detail, as the rates of amino acid liberation and removal had clearly varied in time and under the influence of many factors. Still, the strikingly constant Pro recoveries observed from 2 to 4 h of hydrolysis contrasted against the significant losses observed by four hours time in the case of the primary amino group amino acids. Since Pro, the only imino acid, is incapable of imine formation,[45] it followed to suppose that a significant fraction of the amino acid removal pathways had been mediated by imine formation with in situ generated aldehydes. The greater-than-average loss of Glu at 4 h of hydrolysis was possibly related to one or more of its three functional groups. In view that Glu and the structurally similar Asp differed in their time versus recovery profiles, this drop in recovery did not likely reflect a general event. Supporting this premise were established reactivity studies. Aspartic acid, for instance, was shown to liberate ammonia when treated at 180 °C and pH 8 for 2 h. Glutamic acid, with the same functionalities, tested nearly inert under the same conditions.[51] The greater-than-average loss of Gly and Ala at 4 h of hydrolysis was thought to be related to thermal decarboxylation.[50] This possibility was later discouraged, given the similar recoveries of both Gly and Ala following 3 h hydrolysis at either 150 or 170 °C (Table 3).

As shown in Table 3, a hydrolysis temperature of 150 °C, with some exceptions, appeared to have struck a balance between liberating amino acids from the wheat matrix and limiting their premature destruction or transformation. The improved recovery of hydrophobic amino acids at 170 °C was anticipated, as Val, Leu and Ile amide bonds are notorious for their hydrolytic stability.[28,45] Clearly, a higher hydrolysis temperature had accelerated the formation pathways compared to the removal pathways. In contrast, the recoverable yield of Asp proved substantially lower at 170 °C compared to 150 °C. While the specific removal pathways were not elucidated, thermal treatment of Asp at 180 °C and pH 8 for 2 h is known to lead to deamination.[51] Also, a pH-independent deamination of Asp to fumaric acid has been observed at 135 °C and pH values

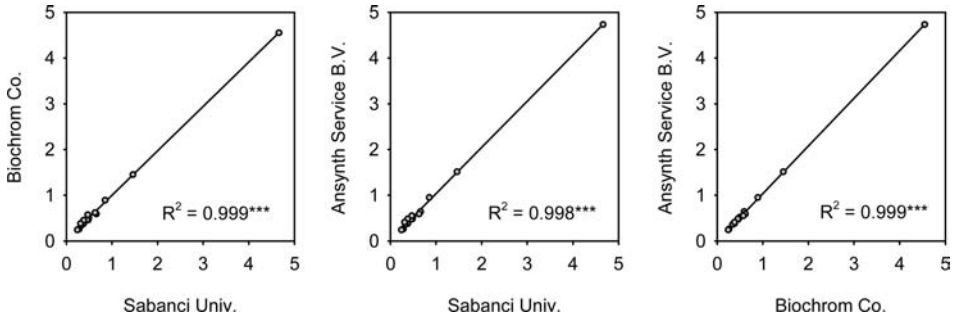

Figure 1. Correlated amino acid analysis results following the round robin testing of SRM 8436 Durum Wheat Flour (National Institute of Standards and Technology, Gaithersburg, MD, USA). Identical samples were hydrolyzed using either the optimized 3 h microwave-assisted method (i.e., Sabanci University) or the traditional 24 h reflux method (i.e., Biochrom Co. and Ansynth Service B.V. laboratories).

below 1.[52] The established β-decarboxylation of Asp to Ala[53] did not likely apply herein, as the Ala recoveries appeared to be unaffected by temperature (Table 3). The high recovery of Tyr at 130 °C compared to 150 °C and especially 170 °C was consistent with ring halogenation. While this mode of removal was not validated by direct measurement, the halogenation of phenol is known to proceed easily and to be prompted by heating.[33,45]

In comparing Tables 1, 2 and 3, the recoverable amino acid yields appeared most significantly influenced by the choice of hydrolysis time. The second most influential parameter was the sample weight. The hydrolysis temperature did not appear to impact the amino acid recoveries to the same extent as did the above two parameters. As the recoverable yields proved high and reproducible, a final matter was to examine how samples prepared using microwave assistance might fare against those prepared using the established reflux method.

As presented in Figure 1, the amino acid recoveries derived from SRM 8436 Durum Wheat Flour were found to strongly correlate between one laboratory utilizing microwave assistance and two laboratories utilizing the established reflux method (R^2 = 0.999, 0.998 and 0.999, left to right). A question remained as to whether or not the overabundance of Glu and Pro in the data set had inadvertently skewed the overall correlation. This concern was dispelled by removing Glu and Pro from each data set. The less abundant amino acids still correlated strongly among themselves, yielding R^2 = 0.93, 0.93, and 0.98. These findings clearly attested to the credibility and comparability of the microwave and traditional 24 h reflux methods. The total recovery of 85.1%, as defined by the ratio of total recoverable amino acids to total protein content, lay within the range of previous work.[27] A discrepancy on the order of 14.9% was conceivable, as the accuracy of both quantification methods was challenged by the formation of wheat-matrix interferents during sample preparation. As such, the total N assay had likely reflected the combined contributions of proteinaceous and nonproteinaceous N sources, consistent with the compositional heterogeneity of wheat grain. In contrast, the total ninhydrin color yield reflected only that fraction of amino acids which had survived acid hydrolysis, avoided reaction with numerous wheat matrix byproducts, and successfully interacted with ninhydrin. Also, it is important to point out that this deviation of 14.9% was ascertained using the arbitrary value of 5.83 as a protein conversion factor.[27] Some have put to question the veracity of this factor, implying that it is too high.[14,54] Thus, if a value of 5.36 had been applied as stated by Moose,[14] a more impressive total recovery of 92.6% would have been realized. This value is similar to the recovery of 93% obtained by Fujihara et al.[54] using a protein conversion factor of 5.47. Therefore, the 14.9% deviation between the total recoverable amino acids and total protein likely defined the limit of experimental variability in determining the upper % recovery.

In conclusion, this study clearly established the merit of microwave irradiation as a simple hydrolysis approach to expedite the amino acid analysis of wheat grain without sacrificing analytical quality. The extension of a microwave-facilitated technique to the preparation of wheat grain was especially noteworthy in that no rapid hydrolysis method had been previously implemented to address the inconveniences inherent to such analytes. In addition to offering time-savings, the wheat samples were easily processed in parallel within a restricted space, adding a new dimension to the convenience of the method. Appropriately conducted, it follows to reason that this method may offer a rapid means to compare the constitution of different genotypes of wheat, and to hasten the assessment of commercially important cereal crops and their end products.

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