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Glutamine in Commercial Liquid Nutritional Products

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Total glutamine concentrations in commercial nutritional products have been determined by enzymatic hydrolysis followed by HPLC quantification of free glutamine and free pyroglutamic acid. Hydrolysis was accomplished by a published three-enzyme (Pronase, leucine aminopeptidase, prolidase), 20-h/37 °C digestion. Glutamine was determined as its FMOC derivative by reverse phase HPLC-fluorescence, and pyroglutamic acid was determined directly by organic acid HPLC-UV. Approximately 4.11% of the released glutamine is converted to pyroglutamic acid during the 20-h digestion. Experimental ratios of enzyme hydrolysis glutamine to acid hydrolysis glutamic acid + glutamine + pyroglutamic acid (GLX) indicate that the method recovers >90% of the protein-bound glutamine. The nutritional products with casein dominant intact protein systems typically deliver >9 g of glutamine/100 g of protein, or ∼40 g of glutamine/100 g of GLX.

KEYWORDS: Glutamine; pyroglutamic acid; enzyme hydrolysis; nutritional products; HPLC

INTRODUCTION

In their study of the sequences of 1021 unrelated proteins, McCaldron and Argos reported an average glutamine frequency of 4.0 residues per 100 amino acids (*1*, *2*). Glutamine occurs at more than twice that frequency in each of the major bovine casein proteins (α_{S1}-, α_{S2}-, β-, and κ-casein), as well as in both of the major soybean proteins (glycinin and β-conglycinin) (*3*−*13*). Accordingly, nutritional products based on caseinate, nonfat dry milk, condensed skim milk, milk protein isolate, and/or soy protein isolate are expected to deliver significant quantities of glutamine. The increased awareness of glutamine's metabolic significance—as a nitrogen transport vehicle, as a cellular redox state maintenance agent, as a metabolic intermediary, and as an energy source—in recent years has placed greater value on a quantitative knowledge of dietary glutamine, particularly in nutritional products (*14*). Unfortunately, the quantification of glutamine in proteins and in protein-containing nutritional products is complicated by the instability of glutamine's amide side chain to the conventional acid hydrolysis procedure (6 M HCl, 110 °C, 22−24 h), which is commonly used for the determination of most protein-bound amino acids. However, researchers have reported success with at least four alternate approaches for quantifying the total glutamine concentrations in foods, including (a) sequence-based estimates, (b) determination of amide nitrogen, (c) selective side-chain modification, and (d) glutamine release by enzymatic hydrolysis. Glutamine estimates can be made with knowledge of the protein system (total protein content and individual protein distribution) and amino acid sequence data. The glutamine levels in bovine milk (∼3.4 g/L) and in numerous enteral formulas have been estimated in this manner (*3*, *15*). A mild acid hydrolysis (typically 3 h at 100 °C in 2 M HCl) can be used to quantitatively release amide nitrogen (from the glutamine and asparagine side chains) as free ammonia (*16*−*18*). Subsequent determination of the released ammonia enables an estimate of [glutamine + asparagine]. In fact, such determinations of amide nitrogen can be used to provide experimental support to sequence-based estimates, bearing in mind the inability of the methodology to quantify glutamine or asparagine individually, but only as the sum of the two. In recent years, an approach that uses the reagent [bis(trifluoroacetoxy)iodo]benzene (BTI) to selectively modify protein-bound glutamine to the acid hydrolysis stable 2,4-diaminobutyric acid (DABA, which can be determined as its dansyl derivative by HPLC-fluorescence) has been developed, refined, and applied to the quantification of glutamine in enteral products (*19*−*23*). The application of the BTI method to enteral products requires extensive sample preparation (solvent extraction, centrifugation, dialysis, or ion exchange chromatography) "to remove interfering fat and carbohydrates" prior to the BTI treatment (*23*), and the method reportedly fails to accommodate free glutamine and N-terminal glutamine (*24*). Finally, various degrees of success in determining protein-bound glutamine via enzymatic hydrolysis have been described over the past 30 years (*25*−*28*), and Tsao and Otter recently reported the ability of an optimized enzyme digestion, followed by reverse phase HPLC-UV of phenylisothiocyanate (PITC) derivatized amino acids, to accurately quantify glutamine in purified milk proteins (*24*, *29*). In the present study, the optimized enzyme digestion has been applied to glutamine determination in liquid nutritional products with total protein concentrations ranging from 30 to 67 g/L. The released glutamine was quantified as its 9-fluorenylmethoxycarbonyl (FMOC) derivative by reverse phase HPLC-fluorescence, and

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the pyroglutamic acid (PGA) which formed during the digestion (upon glutamine deamination/cyclization) was quantified by direct HPLC-UV. The sum of the glutamine and the PGA determined in this manner exceeds 90% of the total glutamine concentration expected [based on published protein composition data (*3*)] to reside in the nutritional products.

MATERIALS AND METHODS

Standards, Enzymes, Proteins, Peptides, and Nutritional Products. Standard reference materials of L-glutamine, L-glutamic acid, and L-pyroglutamic acid were obtained from Fluka Chemical (Milwaukee, WI). Each was fully characterized in-house by a series of physical, spectrometric, and chromatographic analyses. On the basis of the analyses, purities of 99.2% (L-glutamine), 100.0% (L-glutamic acid), and 99.0% (L-pyroglutamic acid) were assigned. The proteolytic enzymes Pronase E (EC 3.4.24.31; protease from *Streptomyces griseus*, type XIV, ~4 units/mg of solid, containing calcium acetate), leucine aminopeptidase, microsomal (EC 3.4.11.2; from porcine kidney, type VI-S, lyophilized powder, 15−25 units/mg of protein, containing primarily phosphate buffer salts), and prolidase (EC 3.4.13.9; from porcine kidney, lyophilized powder, salt-free, 100−300 units/mg of protein) and the purified bovine milk proteins β-casein, β-lactoglobulin, and BSA as well as citric acid, HEPES, sodium azide, and boric acid were obtained from Sigma-Aldrich (St. Louis, MO). Each of the five N-terminal PGA peptides—pGlu-Gly-Arg-Phe amide, locustapyrokinin, physalaemin, pGlu-Asn-Gly, and pGlu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn (serum thymic factor)—was also obtained from Sigma-Aldrich. Unexpired batches of 12 commercial liquid nutritional products—AlitraQ, Ensure, Glucerna, Glucerna Shake, Jevity, Optimental, Osmolite, Osmolite HN, Osmolite HN Plus, PediaSure, Perative, and Pulmocare—were obtained from Ross Products Division, Abbott Laboratories, Columbus, OH.

Equipment. Determinations of FMOC-L-glutamine and FMOC-L-glutamic acid were performed on an Agilent model 1100 HPLC system (Agilent Technologies, Wilmington, DE) with a model G1321A fluorescence detector (FLD, Agilent Technologies). The FLD system was equipped with a 4.6 × 250 mm i.d., 5 μm, 120 Å, YMC-Pack ODS-AQ reverse phase column (Waters, Milford, MA). The column was maintained at 40 °C during analyses by a model G1316A thermostated column compartment (Agilent Technologies). Determinations of free pyroglutamic acid were performed on an Agilent model 1100 HPLC system with a model G1315A diode array detector (DAD, Agilent Technologies). The DAD system was equipped with a 7.8 × 300 mm i.d. Aminex HPX-87H organic acid analysis column (Bio-Rad Laboratories, Hercules, CA). This column was also maintained at 40 °C during analyses.

Chromatographic Conditions. On the FLD system, the flow rate was 0.6 mL/min using vacuum-degassed mobile phases [A, 30% (v/v) acetonitrile/70% (v/v) 0.05 M citric acid, pH 3.56 with NaOH; B, 80% (v/v) acetonitrile/20% (v/v) 0.05 M citric acid, pH 3.56]. The elution program (step gradient) was as follows: 0.0−45.0 min, 100% A; 45.1−55.0 min, 100% B; 55.1−65.0 min, 100% A. The excitation wavelength was set at 262 nm, the emission wavelength was set at 310 nm, and the gain was set at 10. The injection volume was 5 μL. On the DAD system, the flow rate was 0.4 mL/min using vacuum-degassed 5 mN sulfuric acid as the mobile phase. The primary detection wavelength was 214 nm, with reference at 590 nm. A second detection wavelength, 229 nm with reference at 590 nm, was used to verify the purity of the PGA peak. The injection volume was 10 μL, and the run time was 50 min per injection.

Enzyme Hydrolysis. The release of protein-bound glutamine was accomplished by the enzymatic digestion described by Tsao and Otter (*24*), which was a modification of the procedure reported by Henle et al. (*29*). A quantity of (as fed) nutritional product containing ~15 mg of protein was diluted to 10 mL with 0.10 M HEPES, pH 7.5, containing sodium azide at 0.1% (w/v). To 3.00 mL of the buffered sample suspension were added 150 μL of Pronase E (prepared at 9.2 units/mL in buffer), 60 μL of leucine aminopeptidase M (prepared at 24 units/mL in Milli-Q water), and 30 μL of prolidase (prepared at 180 units/mL in Milli-Q water). The mixture (digest volume = 3240 μL) was aliquoted in equal portions (~1620 *l*L each) into two glass HPLC autosampler vials (12 × 32 mm; 1.8 mL volume), which were then crimp-sealed and incubated at 37 °C for 20 h. An enzyme blank preparation (enzymes added to 3.00 mL of buffer only) was included in the analysis to quantify the glutamine and pyroglutamic acid contributions from enzyme autolysis.

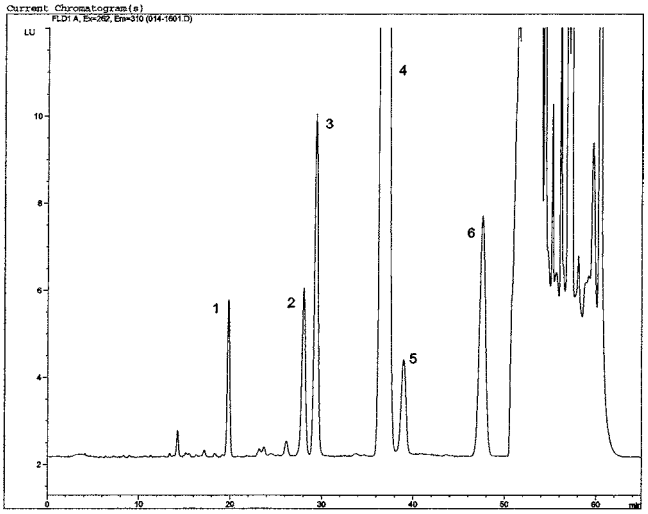

Figure 1. HPLC-FLD chromatogram of enzyme-digested Glucerna, after FMOC derivatization. Peak identities: (1) FMOC-arginine; (2) FMOC-asparagine; (3) FMOC-glutamine; (4) FMOC-HEPES; (5) FMOC-aspartic acid; (6) FMOC-glutamic acid. The detection wavelengths were 262 nm (excitation) and 310 nm (emission).

Determination of Glutamine. Upon completion of the 20-h digestion, 1.00 mL of digest was diluted to 50 mL with Milli-Q water. This diluted digest was prepared for HPLC analysis as follows: (a) 100 μL was pipetted into a 10 mL volumetric flask; (b) 500 μL of 0.40 M sodium borate, pH 8.5, was added to the flask; (c) 500 μL of 9-fluorenylmethyl chloroformate (FMOC-Cl; Fluka Chemical, Milwaukee, WI) prepared at 1.5 mg/mL in acetonitrile was added to the flask; (d) the flask was swirled gently to mix the reagents; (e) the mixture was allowed to stand at room temperature for 5 min (only) and (f) then brought to volume with FLD system mobile phase A. Also derivatized (in separate 10 mL flasks) at the same time, and by the same procedure, were (a) 100 μL of a standard blank, (b) 100 μL of a 50−55 μM solution of L-glutamine reference standard in Milli-Q water, and (c) 100 μL of a 24−28 μM solution of L-glutamine reference standard in Milli-Q water. The derivatized standard and sample solutions were tested for FMOC-L-glutamine by the FLD system described above (**Figure 1**). Glutamine in the sample solutions was calculated by linear regression from a three-point external standard curve of FMOC-L-glutamine peak area versus L-glutamine concentration.

Determination of Pyroglutamic Acid (PGA). Upon completion of the 20-h digestion, the undiluted digest was tested for free PGA by the DAD system described above (**Figure 2**). The analysis was calibrated with five PGA reference standard solutions, prepared at approximately 0, 25, 50, 75, and 100 μM in Milli-Q water. Free PGA in the digest was calculated by linear regression from a five-point external standard curve of PGA peak area (at 214 nm) versus PGA concentration. The PGA peak area at a second wavelength, 229 nm, was also determined, to verify the purity of the digest PGA peak (i.e., by comparing the 214 nm/229 nm ratio of the digest PGA peak to those of the PGA peaks in the standard solutions).

Determination of GLX. A quantity of (as fed) nutritional product containing ~5 mg of protein (the quantity ranged from 0.0800 to 0.1800 g of as fed nutritional product) was weighed into a tared 5 mL glass ampule (Wheaton, Millville, NJ), and 5.00 mL of 6 M hydrochloric acid was added to the ampule. The ampule was nitrogen-blanketed, flame-sealed, and placed in a 110 °C oven for 22 h. After cooling to room temperature, the ampule was opened, and the contents were quantitatively transferred into a 100 mL volumetric flask, using 0.4 M sodium borate, pH 8.5, to assist in the transfer. The acid was neutralized

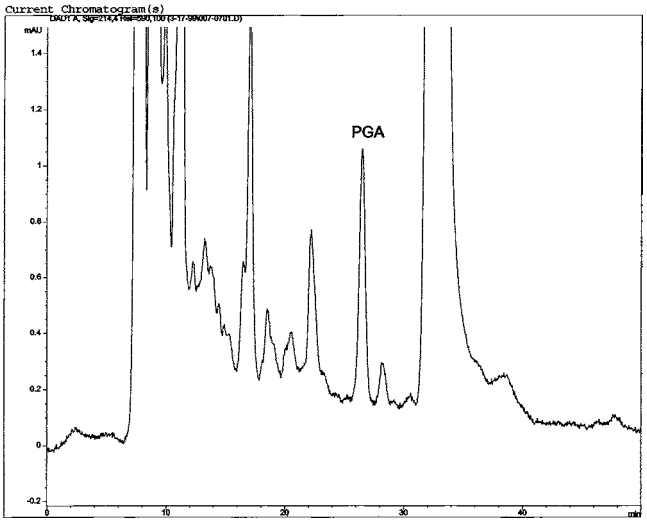

Figure 2. HPLC-UV chromatogram showing direct determination of free pyroglutamic acid (PGA) in enzyme-digested Glucerna. The detection wavelength was 214 nm.

by adding 3.2 mL of 10 N NaOH, and the mixture was diluted to volume with 0.4 M sodium borate, pH 8.5. The diluted sample was then prepared for HPLC analysis as follows: (a) 100 μL was pipetted into a 10 mL volumetric flask; (b) 500 μL of 0.40 M sodium borate, pH 8.5, was added to the flask; (c) 500 μL of FMOC-Cl prepared at 1.5 mg/mL in acetonitrile was added to the flask; (d) the flask was swirled gently to mix the reagents; (e) the mixture was allowed to stand at room temperature for 5 min (only); and (f) the flask was brought to volume with FLD system mobile phase A. Also derivatized (in separate 10 mL flasks) at the same time, and by the same procedure, were (a) 100 μL of a standard blank (Milli-Q water), (b) 100 μL of a 150−160 μM solution of L-glutamic acid reference standard in Milli-Q water, and (c) 100 μL of a 75−80 μM solution of L-glutamic acid reference standard in Milli-Q water. The derivatized standard and sample solutions were tested for FMOC-L-glutamic acid by the FLD system described above. GLX in the sample solutions was calculated by linear regression from a three-point external standard curve of FMOC-L-glutamic acid peak area versus L-glutamic acid concentration.

Glutamine Determination by Small-scale Enzyme Digestion. The release of protein-bound glutamine can be accomplished by a scaled-down version of the enzyme hydrolysis described above. In this small-scale enzyme digestion, a quantity of (as fed) nutritional product containing ~30 mg of protein is weighed into a 10 mL volumetric flask; a 1.00 mL aliquot of a Pronase E solution prepared at 8.5 units/mL in 0.10 M HEPES, pH 7.5, containing sodium azide at 0.1% (w/v), is added to the flask, and the mixture is diluted to 10 mL with 0.10 M HEPES, pH 7.5, containing sodium azide at 0.1% (w/v). A 100 μL aliquot taken from the 10 mL mixture is pipetted into a 200 μL limited volume glass insert (obtained from SUN SRI, Wilmington, NC), which is positioned in a glass HPLC autosampler vial, and into the same insert are added 50 μL of a leucine aminopeptidase M solution prepared at 1.78 units/mL in 0.10 M HEPES, pH 7.5, containing sodium azide at 0.1% (w/v), and 50 μL of a prolidase solution prepared at 6.67 units/mL in 0.10 M HEPES, pH 7.5, containing sodium azide at 0.1% (w/v). The autosampler vial is sealed and incubated at 37 °C for 20 h. The vial is cooled to room temperature, the seal is removed, and a 100 μL aliquot is diluted to 5 mL with Milli-Q water. The glutamine concentration is then determined as specified above under Determination of Glutamine, beginning with "this diluted digest was prepared for HPLC analysis as follows...". An enzyme blank preparation was included in the analysis to quantify the glutamine contribution from the enzyme autolysis.

RESULTS AND DISCUSSION

Method Validation. Every sample analysis was calibrated by duplicate injections of external standards the concentration range of which encompassed all sample analyte concentrations. The linearity of each system has been characterized as the coefficient of determination (r^2) average and the middle standard residual average, calculated for 10 consecutive standard curves. For the determination of FMOC-L-glutamine, the averages were $r^2 = 0.99998 \pm 0.00003$ and residual = 0.2%, over the range 0−0.55 μM; for FMOC-L-glutamic acid, the averages were 0.99998 ± 0.00005 and 0.3%, over the range 0−1.6 μM; and for PGA, the averages were 0.99990 ± 0.00008 and 0.2%, over the range 0−100 μM. The precision of each method was assessed as the average relative standard deviation (RSD) accompanying analyte determination in 12 nutritional products on three different days (12 products × 3 days = 36 determinations). For the determination of glutamine, this overall RSD was 0.9% (range = 0.1−3.2%); for GLX determination, the overall RSD was 0.7% (range = 0.2−1.4%); and for PGA determination, the overall RSD was 2.1% (range = 0.5−3.9%). Method accuracy was evaluated as spike recovery at the 100% spiking level. In the case of glutamine, four enzyme digests of Ensure (after the 20 h/37 °C incubation, but prior to FMOC derivatization) were spiked with L-glutamine at ~1.0 mM on each of three days, and a spike recovery average of $99.0 \pm 1.2\%$ ($n = 12$) was obtained. For GLX, two samples of Ensure (prior to acid hydrolysis) were spiked with L-glutamic acid at ~5 mM on each of three days. The spiked (and unspiked) samples were acid hydrolyzed and tested for GLX, and a spike recovery average of $100.6 \pm 1.5\%$ ($n = 6$) was obtained. For PGA, on each of three days, enzyme digests of two different products (Osmolite and/or Osmolite HN Plus and/or Pulmocare) were spiked with L-pyroglutamic acid at ~38 μM immediately prior to HPLC analysis. The spiked (and unspiked) samples were tested for PGA, and a spike recovery average of $96.2 \pm 0.8\%$ ($n = 6$) was obtained. Assessment of FLD system selectivity was based on its demonstrated capacity to separate FMOC-L-glutamine and FMOC-L-glutamic acid from the FMOC derivatives of all other common amino acids (Ala, Arg, Asn, Asp, Cys, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, and Tyr) and on the demonstrated absence of chromatographic interference in a reagent blank. DAD system selectivity was evaluated by comparing the 214 nm/229 nm peak area ratio for each digest PGA peak to the corresponding ratio calculated for the PGA calibration standards. The limit of analyte quantitation (LOQ) was determined experimentally via HPLC analysis of a low-level standard solution whose analyte response was ~10 times the signal-to-noise ratio. For glutamine and for glutamic acid the LOQ was 2 nM, well below the sample analyte range of ~100−1000 nM. For PGA, the LOQ was 2 μM, whereas the digest PGA ranged from ~20−100 μM. It should be noted that the glutamine contribution from the enzyme autolysis consistently comprised ~2.5% of the free glutamine in the nutritional product digest. The PGA contribution from the enzymes failed to exceed the LOQ of the DAD system and was thereby regarded as negligible.

The effect of enzyme activity variation on glutamine release and on PGA conversion (during the 20 h/37 °C digestion) was assessed by determining these analytes in a nutritional product (Glucerna Shake), using three enzyme activity levels: 0.80*X*, 1.00*X*, and 1.20*X*, where *X* is the enzyme activity specified above; that is, *X* = (150 μL of Pronase E prepared at 9.2 units/mL + 60 μL of leucine aminopeptidase M prepared at 24 units/mL + 30 μL of prolidase prepared at 180 units/mL). At 0.80*X* enzyme activity, glutamine release and PGA conversion were $98.2 \pm 1.1\%$ ($n = 5$) and $101.5 \pm 4.6\%$ ($n = 5$), respectively, versus the 1.00*X* enzyme activity control. At 1.20*X* enzyme

Table 1. Nutritional Product Protein Systems

nutritional product	protein system
AlitraQ	29.8% SPH,[a] 26.3% free L-glutamine, 9.4% WPC,[b] 6.3% free L-arginine, 6.0% lactalbumin hydrolysate, and 10 additional free amino acids (which total 22.2%)
Ensure	65% caseinate, 25% SPI,[c] and 10% WPC
Glucerna	100% caseinate
Glucerna Shake	80% caseinate, 20% SPI
Jevity	80% caseinate, 20% SPI
Optimental	67% SPH, 28% hydrolyzed caseinate, 5% free L-arginine
Osmolite	84.1% caseinate, 15.9% SPI
Osmolite HN	84.1% caseinate, 15.9% SPI
Osmolite HN Plus	100% caseinate
PediaSure	82% caseinate, 18% WPC
Perative	65% hydrolyzed caseinate, 25% lactalbumin hydrolysate, 10% free L-arginine
Pulmocare	100% caseinate

[a] Hydrolyzed soy protein. [b] Whey protein concentrate. [c] Soy protein isolate.

Table 2. PGA Formation during the 20-h, 37 °C, pH 7.5 Enzyme Digestion

test material	free PGA, mol % of (free glutamine + free PGA)	free PGA, mol % of (total glutamine + total PGA)
purified proteins (see **Table 4**)	4.11 ± 0.11 (n = 15)	N/A
nutritional products	4.23 ± 0.13 (n = 27)	N/A
free L-glutamine	4.68 ± 0.04 (n = 3)	N/A
PGA-Gly-Arg-Phe-NH_2	N/A	<0.1
PGA-Asn-Gly	N/A	<0.2
PGA-ADPDKFYGLM[a]	N/A	<0.6
PGA-AKSQGGSN[b]	N/A	<2.6
PGA-DSGDEWPQQPFVPRL[c]	N/A	<3.2

[a] Also known as Physalaemin [2507-24-6]. [b] Also known as Serum Thymic Factor [63958-90-7]; this peptide has one glutamine (Q) residue. [c] Also known as Locustapyrokinin [132293-87-9]; this peptide has two glutamine (QQ) residues.

activity, glutamine release and PGA conversion were 100.6 ± 0.6% (n = 5) and 106.6 ± 8.2% (n = 5), respectively, versus the 1.00X enzyme activity control. On the basis of this assessment, small deviations from the method-specified enzyme additions (i.e., from an enzyme activity = X) are not expected to significantly affect the glutamine result or the PGA conversion.

Nutritional Product Protein Systems. Twelve commercial nutritional products with various protein systems (**Table 1**) were selected for analysis. A single batch of each product was tested in triplicate for glutamine and for PGA (after enzyme hydrolysis) and for GLX (after acid hydrolysis).

PGA Formation during Enzyme Digestion. A significant and measurable fraction of the glutamine released by enzyme hydrolysis deaminated to free PGA over the course of the 20 h/37 °C digestion. This free PGA formation has been quantified in the digests of several relevant test materials, including purified proteins, nutritional products, free glutamine, and five N-terminal PGA peptides (**Table 2**). The average rate of glutamine conversion to PGA in a total of 15 digests of three purified proteins (β-casein, β-lactoglobulin, and BSA) is 4.11% and is notably uniform (overall RSD = 2.7%; n = 15). The average rate is only slightly higher in the commercial nutritional products (AlitraQ was excluded from this set because it is fortified with free L-glutamine; Optimental and Perative were excluded due to matrix interference with the PGA peak) and is slightly higher

Table 3. Glutamine and GLX Determined in 12 Nutritional Products

nutritional product	GLN,[a] g/L actual	GLN,[b] g/L adjusted	GLN,[b] g/1500 kcal	GLX, g/L	protein,[c] g/L
AlitraQ (as fed)[d]	15.9	15.8	23.6	19.6	52.5
Ensure	3.41	3.40	4.81	8.18	38.0
Glucerna	3.87	3.85	5.78	9.55	41.8
Glucerna Shake	3.90	3.89	6.27	9.24	41.8
Jevity	4.12	4.09	5.78	10.1	44.3
Optimental	3.60	3.60	5.40	11.2	51.3
Osmolite	3.46	3.43	4.86	8.65	37.1
Osmolite HN	4.09	4.09	5.79	10.3	44.3
Osmolite HN Plus	5.24	5.23	6.53	13.1	55.5
PediaSure	2.67	2.67	4.00	6.85	30.0
Perative	4.76	4.76	5.49	13.4	66.6
Pulmocare	5.85	5.84	5.84	14.8	62.6

[a] Concentrations include the actual free PGA concentration, which was determined by direct HPLC-UV in the enzymatically hydrolyzed product, at the conclusion of the 20-h/37 °C digestion. [b] Concentrations include a 4.11% positive adjustment to compensate for the average glutamine deamination (to free PGA), which occurred during the enzyme digestion of purified milk proteins (n = 15). [c] Per product label. [d] AlitraQ is fortified with free L-glutamine.

Table 4. Glutamine Recoveries from Caseinate-Based Nutritionals and from Purified Proteins

nutritional product or purified protein	GLN/GLX, exptl	GLN/GLX, expected	glutamine recovery, %
Glucerna	0.407	0.442 (3)	92.0
Osmolite HN Plus	0.403	0.442 (3)	91.1
Pulmocare	0.398	0.442 (3)	90.1
av (n = 3)	0.403 ± 0.005	0.442	91.1 ± 1.0
β-casein	0.515	0.513 (3)	100.4
β-lactoglobulin	0.356	0.360 (3)	98.9
BSA	0.236	0.253 (4)	93.3
av (n = 3)			97.5 ± 3.7

yet (4.68%) when free L-glutamine is subjected to the same conditions. The enzymatic hydrolysis of the five purified N-terminal PGA peptides yielded significant free PGA only when the peptide contained bound glutamine. Inasmuch as the peptides that did not contain glutamine failed to release significant free PGA, the experimentation showed little indication that the enzyme hydrolysis released N-terminal PGA.

Glutamine in Commercial Nutritional Products. The average glutamine (n = 3) and average GLX (n = 3) concentrations determined in the commercial nutritional products are reported in **Table 3**. It should be noted that each glutamine concentration includes a 4.1−4.4% component, which has been included to compensate for the glutamine deamination that occurred over the course of the enzyme digestion. Total glutamine in the range of 4.00−6.53 g/1500 kcal was found in 11 of the 12 nutritionals. In the case of AlitraQ, free L-glutamine fortification elevates its total glutamine to 23.6 g/1500 kcal. To assess the extent of glutamine release accomplished by the enzyme digestion (and hence the accuracy of the determination), the molar ratio of glutamine (by enzyme hydrolysis) to GLX (by acid hydrolysis) was calculated, and this experimental GLN/GLX ratio was compared to the corresponding GLN/GLX ratios calculated from published protein distribution and sequence data (*3*). Such comparisons are shown in **Table 4** for the three nutritional products having protein systems that are exclusively caseinate, as well as for the same three purified milk proteins mentioned previously. The expected GLN/GLX ratio for caseinate (0.442) was calculated from published accounts of the

Table 5. Calculated GLN/GLX Ratios of Casein Proteins (Concentrations as Micromoles per Gram of Protein)

casein protein	% w/w (3)	GLN (3)	GLU (3)	GLX	GLN/GLX
α_{S1}-casein	40.3	593	1058	1651	0.359
α_{S2}-casein	10.5	634	951	1585	0.400
β-casein	33.2	834	792	1626	0.513
κ-casein	11.9	736	683	1419	0.519
γ-casein	4.1	980	588	1568	0.625
whole casein	100.0	710	895	1605	0.442

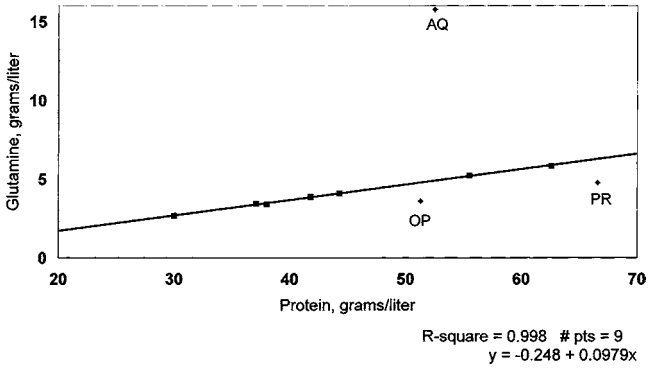

Figure 3. Glutamine variation with protein in 12 nutritional products. AlitraQ (AQ) is fortified with free L-glutamine. Optimental (OP) and Perative (PR) contain free L-arginine and hydrolyzed protein ingredients.

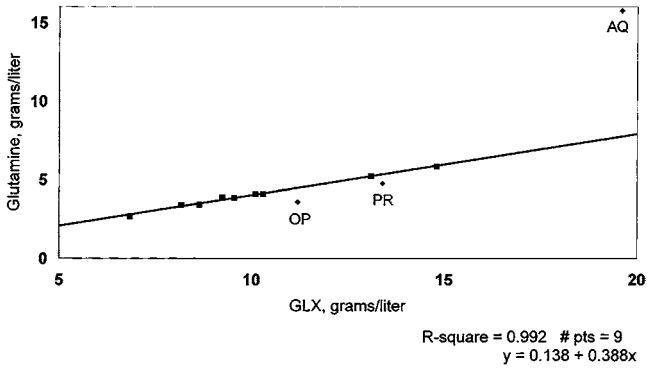

Figure 4. Glutamine variation with GLX in 12 nutritional products. AlitraQ (AQ) is fortified with free L-glutamine. Optimental (OP) and Perative (PR) contain hydrolyzed protein ingredients.

distribution of individual casein proteins in the casein fraction of bovine milk and of the glutamine (GLN) and glutamic acid (GLU) concentrations in each of these individual casein proteins (*3*), as shown in **Table 5**. By this assessment, the glutamine release from the three caseinate-based nutritional products averaged 91.1% of the expected glutamine total, and the glutamine release from the purified proteins averaged 97.5% of the expected total. These comparisons indicate that the enzymatic hydrolysis is capable of releasing >90% of the total glutamine bound by bovine milk proteins. Having established the quantitative capability of the methodology used in this study, it is interesting to examine the variation of glutamine with total protein (**Figure 3**) and with GLX (**Figure 4**). In the nine nutritional products having protein systems consisting of intact protein, with no free amino acid fortification, the ratio of glutamine to total protein is quite uniform (9.20 ± 0.17% [w/w]; $n = 9$), as is the ratio of glutamine to GLX (40.3 ± 1.0% [w/w]; $n = 9$). Both ratios are much higher in AlitraQ, by virtue of its free L-glutamine fortification. The ratios are significantly lower in Optimental and Perative, due to (a) the use of hydrolyzed proteins in both products [the hydrolysis of intact proteins often yields one or more peptides with a glutamine at the N terminus; these N-terminal glutamine residues are vulnerable to PGA conversion, particularly when heated, thereby reducing the glutamine/protein and glutamine/GLX ratios (*30*)], (b) free L-arginine fortification of both products, and (c) a substantial presence of whey protein in the Perative protein system (the GLN/GLX ratio of WPC is significantly lower than those of caseinate and SPI).

It should be noted that the cost of determining glutamine in each nutritional product, using the ("full-scale") procedure specified above, and at current reagent prices, is approximately $20 (U.S.), based on five determinations per HPLC analysis and excluding the costs of labor and instrumentation. Because the proteolytic enzymes consume the majority of the total cost (leucine aminopeptidase M > prolidase ≫ Pronase E), a small-scale enzyme digestion was developed, as described above. The use of this small-scale enzyme digestion would enable a 7-fold decrease in the enzyme cost component of the glutamine determination (at current prices). Although all data reported in this document were determined via the full-scale procedure, a comparison of glutamine determined by the small-scale procedure and by the full-scale procedure revealed no significant difference: glutamine in the Glucerna Shake by small-scale enzyme digestion = 99.4 ± 0.4% ($n = 3$) versus glutamine in the Glucerna Shake by full-scale enzyme digestion.

In conclusion, all indications are that the optimized enzyme digestion of Tsao and Otter provides a reliable and relatively simple means of quantifying total glutamine in nutritional products. The enzymatic digestion generally releases >90% of the protein-bound glutamine from casein dominant protein systems. Direct determinations of free PGA in the digests of purified milk proteins and nutritional products showed that 4.1−4.4% of the released glutamine can be expected to deaminate over the course of the 20 h, 37 °C, pH 7.5 incubation. The PGA results of extensive experimentation—15 digests of purified proteins (RSD = 2.7%) and 27 digests of nutritional products (RSD = 3.1%)—demonstrated the uniformity of the glutamine deamination. In fact, the deamination rate under these controlled conditions was sufficiently uniform to permit accurate glutamine quantification without an experimental determination of PGA, as seen from a comparison of **Table 3** "actual GLN" (which includes compensation for experimentally determined PGA) to "adjusted GLN" (which includes a 4.11% PGA compensation, based on the average for the 15 purified protein digests). The minimal variation in "actual GLN" and "adjusted GLN" illustrates that method accuracy was not significantly compromised by substituting a verifiable deamination estimate (4.11% in this case) for an experimentally determined free PGA increase. Finally, a survey of glutamine, protein, and GLX in nutritional products containing intact protein systems showed that total glutamine increases in an approximately linear fashion with total protein, and with GLX.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; FMOC, 9-fluorenylmethoxycarbonyl; UV, ultraviolet; GLX, glutamic acid + glutamine + pyroglutamic acid; BTI, [bis(trifluoroacetoxy)iodo]benzene; DABA, 2,4-diaminobutyric acid; PITC, phenylisothiocyanate; PGA, pyroglutamic acid; BSA, bovine serum albumin; FLD, fluorescence detector; DAD, diode array detector; HEPES, (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]); LOQ, limit of quantitation; SPH, hydrolyzed soy protein; WPC, whey protein concentrate; SPI, soy protein isolate; GLN, glutamine; GLU, glutamic acid.

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