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Tryptophan determination in milk-based ingredients and dried sport supplements by liquid chromatography with fluorescence detection

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Abstract

Sport supplements formulas are driven to a sector of consumers which a restrictive diet. Tryptophan is essential amino acid and its bioavailability should be assessed from these commercial products. A robust and fast HPLC procedure is applied to determine total tryptophan content from dairy-ingredients, mostly whey-based ingredients, and commercial dried-sport supplements, mostly whey-enriched sport supplements. The nutritional quality of the protein used in the formulation could be extrapolated to the expected tryptophan content by using a regression curve built from milk-based ingredients. Samples that not conform the correlation are suspected from inadequate industrial practices, such as over-processing, and/or the use of heat-damage ingredients.

Keywords: Tryptophan; HPLC analysis; Sport supplements; Whey protein

1. Introduction

Tryptophan is essential amino acid for brain functions and neuronal regulatory mechanisms. Because serotonin is synthesised from the dietary L-tryptophan (Trp), brain serotonin concentrations are directly affected from an unbalanced dietary intake of Trp (Markus, Olivier, & de Haan, 2002). Processing or cooking practices might reduce its bioavailability from the dietary intake, mainly by oxidative degradation or cross-linking among proteins (Moreaux & Birlouez-Aragon, 1997; Nielsen, Klein, & Hurrell, 1985b). Then accurate tryptophan database is mandatory from a legislative, economical, medical and processing control point of view. Literature cited many efforts to apply a general method for tryptophan analysis in foodstuffs (i.e., Molnár-Perl, 1999; Nielsen & Hurrell, 1985a). Tryptophan is degraded during classical aminogram analysis of proteins by acid hydrolysis. Several approaches have been proposed for direct tryptophan determination or after derivatisation; such as second-derivative spectroscopy, *p*-dimethylaminobenzaldehyde, *p*-phenylenediamine, *O*-phthaldehyde, and ninhydrin. Acidic (HCl, HCl with additives), alkaline (NaOH, LiOH) and enzymatic (pronase, pepsin, enzyme cocktail) hydrolysis of the protein has been carried out, followed by chromatographic (ion exchange or reversed-phase), colorimetric or fluorimetric determination of tryptophan. (i.e., Aitken & Learmonth, 1996; Fabian, Pinter-Szakacs, & Molnar-Perl, 1990; Fletouris, Botsoglou, Papageorgiou,

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& Mantis, 1993; Hugli & Moore, 1972; Ng, Pascaud, & Pascaud, 1987; Slump, Flissebaalje, & Haaksman, 1991). Thus, depending on their structures, milk proteins exhibits a characteristic fluorescence emission spectrum defined by its maximum emission wavelength and the tryptophan quantum yield (Friedman & Cuq, 1988; Lakowicz, 1983). Recently, Ravindran and Bryden (2005) have proposed a chromatographic method based on ion-exchange separation and post-column *O*-phthaldehyde derivatization.

The high nutritive value of the whey proteins is mainly due to their high content of essential amino acids, such as tryptophan (147 mg Trp/g total nitrogen), as compared the wheat, beef, soy bean or eggs (FAO, 1973). In this sense, whey-based sport supplements are gaining popularity among consumers. Nowadays, health-care concepts are linked to well-being and social acceptance, apart from competition skills, such as body-builders. Intakes of proteins, mostly from a dairy source, represent a high proportion of the total diet for this type of consumers. Then, it is necessary to ensure the high quality of the protein consumed as well as their efficient biotransformation. Quality of whey proteins used in the formulation is highly related to the technology applied for its production (Holt et al., 1999). Whey used for sport supplements could be grouped according to the protein content and technologies used. Whey protein concentrates (WPC) usually contains less than 25% of protein and whey protein isolates (WPI) usually contains more than 70% protein. The addition of whey proteins from whey powders into food products has two goals, one nutritive (as dietary supplements) and the other technological (solubility, foam formation, gel formation, emulsion, water binding, viscosity, etc.) (Morr & Ha, 1993). Concentrated whey powders that contain more than 80% proteins are used in a wide range of food applications (infant formula, health foods, and drinks) as nutritional and functional ingredients (Fox, 1989).

The aims of this investigation were: (a) to develop a robust and cost-effective method for tryptophan analysis in dairy-based formulations; (b) to evaluate the levels of tryptophan in widely distributed sport-supplements as well as representative whey-based commercial proteins used as ingredients.

2. Materials and methods

2.1. Reagents

L-Tryptophan (Trp), bovine serum albumin (BSA) and 5-methyl-triptophan (M-Trp) were purchased from Sigma (Sigma Chemicals Co., St. Louis, MO, USA). Chloridric acid, sodium hydroxide, and acetonitrile of HPLC grade were from Merck (Darmstadt, Germany). All other chemicals used were of analytical purity.

2.2. Samples

Representative samples of milk-based powder (ingredients $n = 18$, and formulas $n = 16$) were obtained directly from manufacturers (Tables 1 and 2). Most samples are directly marketed as supplied except for P12–P16 which were produced at industrial scale but not commercially distributed. Ingredients were distributed in WPC (whey protein concentrate $n = 4$), WPI (whey protein isolates $n = 11$) and calcium-caseinates ($n = 3$) according to technical information supplied by manufacturers. Most of the final products ($n = 12$) have a protein content higher than 60% (expressed in dried weight). Products containing low proportions of non-dairy proteins, such as soy-protein and egg-albumin were also included in order to evaluate potential bias in during the analysis. Two homogeneous portions were packed in plastic containers sealed under vacuum, light protected to minimize possible tryptophan oxidation and stored at 4 °C until analysed.

2.3. Alkaline hydrolysis procedure

Samples (9–10 mg, 0.1 mg precision) were dissolved in 3 ml of 4 N sodium hydroxide and 150 μl 5-methyl-tryptophan (16 mg/100 ml) as internal standard was added. Hydrolysis tubes were sealed under nitrogen. Samples hydrolysed in an oven at 110 °C for 18 h. The hydrolysates were aerated and cooled at 4 °C. Then carefully acidified to a pH 6.5 with HCl, diluted to 25 ml with sodium borate buffer (0.1 M, pH 9.0) and allowed to stand for 15 min. Samples were centrifuged at

Table 1
Ingredients description and technical information supplied by manufacturers

ID	Protein source	Protein (%)[a]	Carbohydrates (%)[a]
A	WPC	35.0	n.d.
B	WPI	93.0	0.5
C	WPI	90.0	1.0
D	Hydrolysed-casein	86.0	<3.0
F	WPI (high α-la)	85.4	<3.0
G	WPI (high α-la)	80.0	<3.0
H	WPC	14.0	70.0
I	WPC	12.0	73.0
J	WPC	16.0	72.0
K	WPC, casein, soybean	n.d.	n.d.
M	Hydrolyzed-WPI	84.0	n.d.
N	WPI	76.5	4.0
O	Ca-caseinate	89.6	<3.0
Q	Ca-caseinate	88.0	<3.0
R	Ca-caseinate	90.0	<3.0
S	WPI	n.d.	n.d.
T	WPI (high α-la)	90.0	3.0
U	WPI	90.0	4.0

n.d., not declared. *Protein source*. WPC, whey protein concentrate; WPI, whey protein isolate; α-la, α-lactalbumin.
[a] Data expressed as g/100 g of product.

Table 2
Formula description and technical information supplied by manufacturers

ID	Type	Protein source	Protein (%)[a]	Carbohydrates (%)[a]
P1	Commercial	Ca-caseinate, WPC	50.0	41.6
P2	Commercial	WPC, WPI, hydrolysed-WPI	81.0	10.0
P3	Commercial	WPC, hydrolysed-WPC	21.8	69.0
P4	Commercial	WPI	76.0	11.0
P5	Commercial	Ca-caseinate, WPC	59.0	33.2
P6	Commercial	Ca-caseinate, WPI	88.0	4.0
P7	Commercial	Ca-caseinate, WPC	90.9	3.8
P8	Commercial	Ca-caseinate, WPC	18.0	78.0
P9	Commercial	WPC, WPI, hydrolysed egg albumin	73.6	19.0
P10	Commercial	Ca-caseinate,WPC hydrolysed egg albumin	92.4	6.1
P11	Commercial	WPI, hydrolysed egg albumin	88.0	2.9
P12	No-commercial	Ca-caseinate, WPC	n.d.	n.d.
P13	No-commercial	Ca-caseinate, WPI	n.d.	n.d.
P14	No-commercial	Ca-caseinate, WPI	n.d.	n.d.
P15	No-commercial	WPI	n.d.	n.d.
P16	No-commercial	n.d.	n.d.	n.d.

n.d., not declared. *Protein source*. WPC, whey protein concentrate; WPI, whey protein isolate.
[a] Data expressed as g/100 g of product.

10,000*g* for 4 min and supernatant filtered through 0.2 μm nylon filter membrane into HPLC vials. Trp losses during hydrolysis were corrected by internal standard calculation, where the tryptophan contents were calculated by dividing the area of the peak by the area of the internal standard and multiplying this value by the weight of the internal standard and the response factor of tryptophan. Samples were hydrolysed in quadruplicate.

2.4. Instruments

A Kontron Instruments (Milan, Italy) chromatographic system was used for tryptophan and methyl-tryptophan analysis, with a pump (MD-420), UV–vis detector (MD-432), spectrofluorimeter (SMF-25), refractive index (Erma, Inc., Tokyo, Japan) and a DT-450/MT-2 v.3.90 computing integrator connected to a PC.

2.5. Determination of tryptophan by reversed-phase liquid chromatography

A degassed mobile phase was prepared with acetonitrile:water (25:75; v/v) containing 0.1% of trifluoroacetic acid and the flow rate was set at 1.0 ml/min. An Excell ODS-2 S5 analytical column (25 × 0.40 cm, 5 μm, Tecknokroma, Barcelona, Spain) was used. Column was maintained at 32 °C during analysis. Samples (20 μl) were injected into the column. Tryptophan and methyl-tryptophan were detected at 280 and 340 nm for excitation and emission wavelengths, respectively. Retention times were 3.8 and 5.8 min for tryptophan and methyl-tryptophan, respectively. Duplicate analysis of each hydrolysate was analysed.

2.6. Total protein content

Samples (0.800–1.000 g) were heated to 1050 °C following AOAC 992.15 (AOAC, 1995) in a LECO model FP-2000 (Leco Instruments, Madrid, Spain) protein/nitrogen analyser calibrated with EDTA (Dumas method). The nitrogen-to-protein conversion factor considered was 6.38%. Results were expressed as grams of protein/100 g of products.

2.7. Statistical analysis of data

Statistical analysis of data were performed by multiple analysis of variance following a simple, balanced one-way model. Student's *t*-test was used to compare means and the level of significance was set at 95%. Statgraphic v.2.0 software (Statistical Graphics Corp., Rockville, MD, USA) was used.

3. Results and discussion

The repeatability of the Trp quantification was investigated by performing 12 determinations at two different levels of the standards (2.35 and 9.40 μmol/l) spiked with M-Trp (160 mg/l). Residual standard deviations was found to be 1.3% (2.35 μmol/l, $n = 12$) and 1.68% (9.40 μmol/l, $n = 12$). Detection limit (DL, 0.46 μmol/l) and quantification limit (QL, 1.37 μmol/l) for Trp were determined by the standard deviation approach in the estimated limit of quantification. Quantification limit was confirmed experimentally with 2.5-fold diluted sample P3 (1.62 μmol/l, RSD. 3.12%, $n = 3$).

In order to validate the alkaline hydrolysis procedure for Trp analysis to whey-based sport supplements and

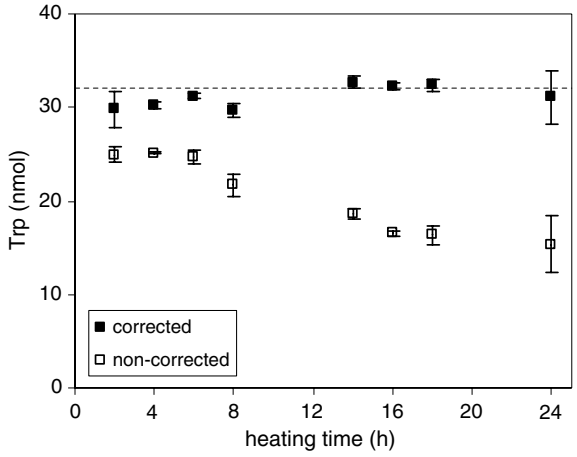

Fig. 1. Stability of Trp during alkaline hydrolysis (empty symbol) and Trp corrected with the recovery of M-Trp (full symbol). Error bars denote standard deviation from two independent analyses. Theoretical value is drawn as a dotted line.

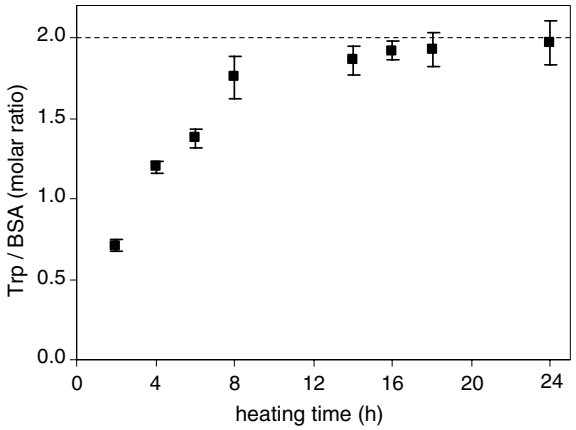

Fig. 2. Determination of Trp in pure bovine serum albumin (2 Trp residues per molecule) during alkaline hydrolysis. Trp values are corrected with the recovery of M-Trp. Error bars denote standard deviation from two independent analyses. Theoretical value is drawn as a dotted line.

ingredients, several investigations were carried out. It was investigated the stability of free L-tryptophan in 4 M NaOH during hydrolysis up to 24 h. Free Trp is most susceptible to degradation during alkaline hydrolysis. Fig. 1 shows Trp is rapidly degraded during alkaline hydrolysis from 19.2% (2 h) to 58.2% (24 h), but results remains constant if Trp area value is corrected by the M-Trp one. This evaluation agrees with reported by Nielsen and Hurrell (1985a).

Accuracy of the analytical procedure was evaluated with bovine serum albumin which literature reports that contains two residues of Trp per molecule (Hugli & Moore, 1972). Fig. 2 shows the recovery of Trp residues from the protein at different heating times in reproducibility conditions. Hydrolysis times from 16 to 24 h were acceptable, and 18 h (1.93 ± 0.102 Trp/BSA molar ratio) were selected for further analysis. The 96.5% of the expected Trp was released and efficiently measured from BSA. Heating times higher than 18 h provides higher uncertainty on the reported Trp value and lower precision for the M-Trp recoveries, but higher Trp recoveries. Fig. 3 shows Trp and M-Trp were resolved at baseline by applying the procedure to different type of samples (ingredients and commercial products). There was not detected interference from other compounds which might be present in the sample. This observation was confirmed by spiking recovery studies performed on a commercial sample with two levels of tryptophan Trp concentration (data not shown). The recovery of tryptophan in sample P15 was 93.4% and 96.6% at two levels of Trp concentration.

A homogeneity study of the test sample was also performed by taking portions from different part of the container. Residual standard deviation was found to be 2.05% for eight independent analysis of sample P2 (0.487 ± 0.010 g Trp/100 g protein). It was calculated

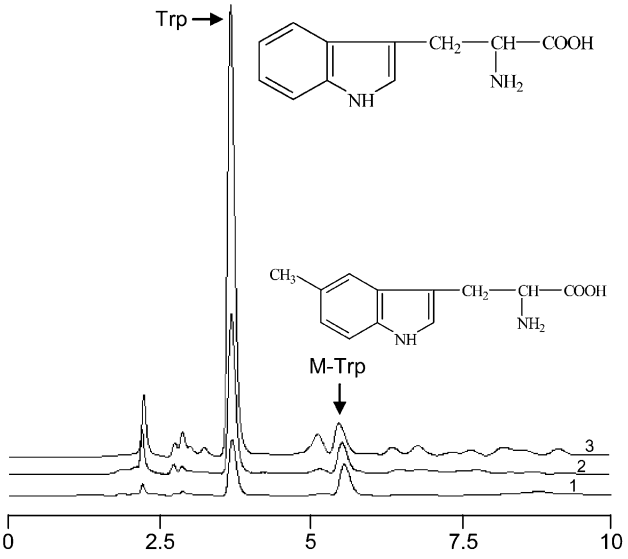

Fig. 3. Classical chromatogram profile of Trp analysis form alkaline digestion: (1) sample A1 (WPC-ingredient); (2) sample P5 (commercial sport formula); (3) sample F1 (WPI-ingredient). 1.21, 0.90 and 1.89 g Trp/100 g protein for samples A1, P5, and F1, respectively.

an M-Trp average recovery of $94.7 \pm 5.53\%$ ($n = 8$). Table 3 reported the study of precision on sample P-15. Sample was analysed on three non-consecutive days to determine the precision of the method. It was evaluated repeatability and intermediate precision or reproducibility. Results show a good intermediate precision of 9.30% for Trp and an average M-Trp recovery of 99.1%.

The relationship between tryptophan content and protein content of ingredients and whey-based sport supplements was studied (Fig. 4). It is noteworthy to keep in mind that companies did not supply the full

Table 3
Precision study (repeatability and intermediate precision) of tryptophan determination (g/100 g product) in a whey protein sport supplement containing 81.3 g of protein per 100 g of product

	Sample	IS recovery (%)	Average Trp content (g/100 g product)	Minimum (g/100 g product)	Maximum (g/100 g product)	RSD (%)	*n*
Day-1	P-15	101.8 ± 2.13	0.910 ± 0.0025	0.873	0.954	2.73	8
Day-2	P-15	98.8 ± 1.56	1.093 ± 0.0158				2
Day-3	P-15	96.6 ± 1.77	0.979 ± 0.1027				2
Intra-day	P-15	99.1 ± 2.61	0.994 ± 0.0924	0.910	1.093	9.30	3

IS, internal standard;, *n*, number of independent analysis. All samples analysed by duplicate.

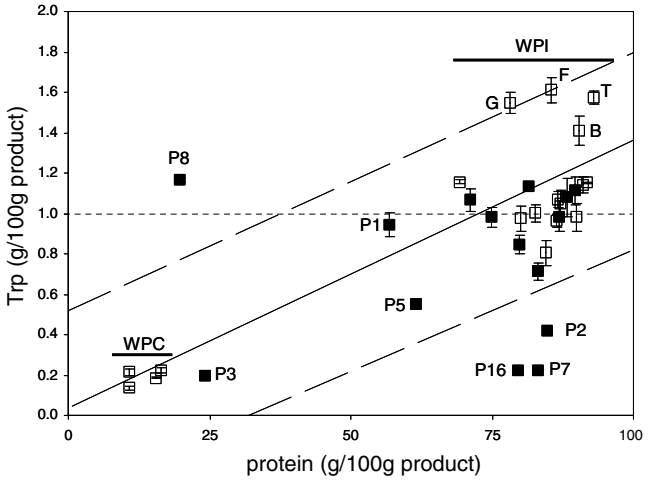

Fig. 4. Relationship between Trp content (g/100 g of product) and protein content (g/100 g product) for ingredients (WPC and WPI), and commercial samples. Dotted lines indicate limits of confidence at 95%. Solid line indicates correlation for whey-based ingredients. Ingredients (empty symbol), commercial formulas (solid symbols). Error bars denote standard deviation from two independent analyses.

composition of the unprocessed formulas. Then, it was not able to evaluate the final Trp contain directly form the ingredients in order to correlated with the Trp content of the processed formula. A regression analysis was carried out within the laboratory characterised ingredients (mostly whey proteins). Tested ingredients are widely applied for body building formulas at commercial scale. Ingredients were grouped in WPC (total protein content lower than 25%) and WPI (total protein content higher than 70%). It was observed a significant correlation ($p < 0.01$) between Trp (g/100 g of product) and protein content (g/100 g of product). Obviously, a high correlation is expected for pure mixture of raw protein but the regression agreement could be reduced according to the extent of the heat load applied.

As previously mentioned, WPC and WPI production have different processing conditions where more severe heat load is applied to WPC. Whey proteins are thermolabile being α-la > β-lg > BSA classified in order of thermostability (Fox, 1989). For instance, whey proteins dried by roller procedure instead of spray will be affected with higher denaturation rates (Morales, Romero, & Jiménez-Pérez, 2000). On the other hand, WPC contains higher lactose proportions (6–8%) compared with WPI (<1%) (Holt et al., 1999; Morr & Foegeding, 1990). Lactose could reacts thought the Maillard reaction reducing the availability of amino acids. Finally, Trp residues could be more susceptible to oxidation during different stages of processing (Moreaux & Birlouez-Aragon, 1997; Simat & Steinhart, 1998).

The experimental relationship between Trp and total protein content for commercial whey-based ingredients is useful to study the processing conditions applied to sport supplements. Most of samples are included between the limit of confidence for the regression analysis obtained from commercial whey-based ingredients (Fig. 4). There are four samples that tryptophan content expected is not related to the protein content calculated. Samples P2, P7 and P16 shown a low ratio Trp:protein content. It can be hypothesised that either a tryptophan residue oxidation or/and the addition of non-Trp-rich proteins (such as caseins) could reduce the ratio Trp:protein. Indeed, sample P2 contain a fraction of hydrolysed casein and P7 contain casein apart from whey proteins. Concerning to sample P16, it is suspected that non-protein nitrogen was added in the formulation (private communication). On the opposite, sample P8 had an unusual high ratio of Trp:protein content. There is not a clear explanation for this behaviour. This effect can be due to the enrichment with Trp-rich proteins like α-la (4 mol of Trp/mol of protein) as compared with β-lg (2 mol of Trp/mol of protein). Or even due to an unusual high proportion of lysozyme (6 mol Trp/mol molecule) or α-chymotrypsin (8 mol Trp/mol molecule). But, this hypothesis has to be further confirmed by chromatographic or immunological analysis of the protein fraction.

The declarated Trp content (g/100 g protein) by manufactures was compared with the calculated Trp content in this study. Differences among ingredients were lower than 15% except for ingredient U (calculated Tp was a 37.0% lower than declarated Trp). Concerning to commercial samples, differences were lower than 25% except for samples P7 and P2, being 79.2% and 75.5%, respectively. Once again, a over-processing is the most plausible explanation for the lack of linearity of these samples as compared with the ingredients. There were not observed significant differences among the declarated content of protein (g protein/100 g of product) and the

measured one, except for sample P7 with a 8.6% of protein lower than measured. Differences among declarated protein content and measured protein content was lower than 2% and 4.5% for ingredients and samples, respectively.

In conclusion, a rapid and robust methodology is described for analysis of tryptophan in whey-based sport supplements. The procedure is not subject to interference by other molecules in whey-based sport supplements and can by applied routinely. The nutritional quality of the protein used in the formulation could be extrapolated by the expected tryptophan content by using a regression curve built from milk-based commercial ingredients. Then, the possible over-processing practices could be detected from the lack of relationship between the Trp content and total protein content of the commercial samples.

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