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Spectrophotometric Method for Determination of Tryptophan in Protein Hydrolysates

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Summary

A novel spectrophotometric method for determination of tryptophan content in protein hydrolysates has been developed. The reagent used is diphenylamine sulphonate which is oxidised to diphenylbenzidine sulphonic acid after reacting with sodium nitrite in the sulphuric acid medium. The unstable oxidation product reacts quickly with sodium nitrite to produce a diazotized intermediate. When the diazotized intermediate is coupled with tryptophan, a pink colour product is developed, which is stable for at least 1 h at the ambient temperature. This coloured product has the absorption maximum at 522 nm and the molar absorptivity is $0.89 \cdot 10^4$ L/(mol·cm). Beer's law is obeyed in the range of 0.30–12 μg/mL. The method is applied for the analysis of tryptophan content in grass carp protein hydrolysates. Moreover, it is compared with the reversed-phase high-performance liquid chromatography analysis. There is no significant ($p<0.05$) difference between the two results. The method is simple, rapid and accurate compared to the previous methods.

Key words: protein hydrolysates, tryptophan, spectrophotometry, diphenylamine

Introduction

Tryptophan (Trp) is an essential amino acid for humans and is considered exceptional in its diversity of biological functions (*1*). It is a vital constituent of proteins and indispensable in human nutrition for establishing and maintaining a positive nitrogen balance (*2*). In particular, Trp is the precursor of the neurotransmitter serotonin and plays an important role in brain function and related regulatory mechanisms (*3*). In addition, Trp is an important and frequently used starting material in the chemical synthesis of a range of pharmaceuticals (*4*). It has been used in the treatment of depression, schizophrenia, and hypertension (*5*). Besides, some of its derivatives are potent drugs (*6*). Trp is widely used in food industry. It is sometimes added to dietary and feed products as a food fortifier in order to maintain the amino acid balance of the food and correct possible dietary deficiencies. Trp can also be used to study structure and dynamics of the proteins because of its indole moiety (*7*). Its nutritional and biochemical importance emphasises the need for reliable analytical methods for the determination of Trp in food and feed proteins.

The analytical methods used for the determination of Trp content in biological media, in food or in pure form include titrimetry (*8*), capillary electrophoresis (*4, 9*), voltammetry (*2,10–11*), chemiluminescence (CL) (*3,12–14*), amperometry (*15,16*), polarography (*17*), high-performance liquid chromatography (HPLC) (*18–22*), fluorescence spectroscopy (*23*) and spectrophotometric method (*24,25*). Among these methods, titrimetry is the simplest, but it is insensitive and only the amino acids of samples with more than millimolar concentration (mmoL/L) can be accurately analyzed (*8*). Although capillary electrophoresis has generated considerable attention because of its high resolution (*9*), the operation is very complicated and it is essential to adopt good working practices in or-

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der to maintain consistent migration times and resolution (*4*). Voltammetric methods are relatively inexpensive and sensitive. The detection limit is about 1.7 μmoL/L (*2*). However, the extracting qualitative and quantitative information from electrochemical data may result in a difficult task, which is the main disadvantage of the voltammetric methods. CL methods also have lower detection limits in the femtomole (10^{-15}) range (*13,14*), and protein and ascorbic acid in the samples may have negative interference in Trp determination (*12*). So far as the amperometry method is concerned, Trp should first be separated from common amino acids by anion-exchange chromatography and that pre-treatment makes the procedure tedious and complicated (*15,16*). HPLC method is widely used for the determination of Trp. However, the analysis of the Trp remains problematic due to its lability to acid hydrolysis, often a prerequisite for HPLC analysis. So it is common to employ the sophisticated, time-consuming alkaline hydrolysis during the assay of Trp by HPLC (*18–22*). Although Trp is a fluorescing chromophore, there is another fluorescing chromophore, tyrosine, in protein hydrolysates (*23*). The spectrophotometric analysis is generally adopted because it does not need expensive laboratory apparatus and it is relatively easy to operate compared with other methods. Unfortunately, the previous spectrophotometric method already reported (*24,25*) also suffers from some disadvantages such as time-consuming heat pretreatment. Sometimes, complicated extraction of samples to avoid the interference of other chemicals is required.

Thus, there is a need for the development of more direct and rapid techniques that are capable of determining Trp (usually in conjunction with other dissolved amino acids) in protein hydrolysates. In the present study, a simple spectrophotometric method is proposed, which involves an azo-coupling reaction of diphenylamine sulphonate with Trp in sulphuric acid system to produce a pink colour product with 522 nm of absorbance maximum. Compared with the previous methods, this method is shown to be sensitive and much easier to operate. It can be employed for the analysis of Trp content in protein hydrolysates because it is free of interference from other amino acids.

Materials and Methods

Raw materials

Standards of Trp and diphenylamine sulphonic acid sodium (DSAS) were purchased from Sigma (Beijing, China). Hydrochloric acid, sodium nitrite, sulphamic acid, sulphuric acid and other chemicals used in this study were of analytical grade. Deionized water was used to prepare all solutions. Phenylisothiocyanate (PITC), triethylamine (TEA) and acetonitrile were of chromatographic purity. Sodium acetate, sodium hydroxide and ethanol were of analytical grade. Alcalase 2.4 L ($6.9 \cdot 10^4$ U/g), Flavourzyme 500 MG ($1.6 \cdot 10^4$ U/g) and PTN 6.0S ($3.5 \cdot 10^4$ U/g) were provided by courtesy of Novo Nordisk Co. (Beijing, China).

Spectrophotometric method

Sample preparation

Trp solution (0.1 g/L) was prepared in deionized water with a few drops of 1 mol/L sodium hydroxide, and the Trp solution was suitably diluted before being used as a standard. A concentration of 2 g/L of DSAS solution was prepared with deionized water. Solutions of 1 mol/L of sodium nitrite and 25 g/L of sulphamic acid were prepared in deionized water, stored in refrigerator avoiding direct light and used within one week. A solution of 2 mol/L of sulphuric acid was also prepared.

Standard procedure

A volume of 3 mL of DSAS (2 g/L) solution was transferred into a set of 25-mL calibrated flasks, and then the flasks were cooled in the ice bath to maintain a constant temperature of about 5 °C. A volume of 6 mL of sulphuric acid was added into each flask and mixed gently. After 5 min, 2.5 mL of sodium nitrite were added and the mixture was placed in the ice bath for 5 min. A volume of 3 mL of sulphamic acid solution was added and cooled with occasional shaking for further 5 min. The Trp solutions were then added to the flasks, the volume was made up to 25 mL with sulphuric acid, mixed thoroughly, and incubated for 15 min. The absorbance of the resulting pink-coloured solution was scanned in the range of 450 to 600 nm by a spectrophotometer (Unico UV-2102 PC) with 1.0 cm matched cells. Absorption maximum at 522 nm was recorded.

RP-HPLC method

Sample preparation

For tryptophan determination by RP-HPLC using Waters system (Milford MA01757, USA) with a Pico-Tag® column (3.9×150 mm), the protein hydrolysate samples were first hydrolyzed using 4.3 mol/L of sodium hydroxide for 24 h at 110 °C (*26*). Precolumn derivatization of amino acids was performed by means of PITC.

Derivatization procedure

TEA solution including TEA-water-ethanol (1:2:2) was added to the samples and evaporated to dryness under vacuum. The PITC solution including PITC-ethanol-TEA-water (1:7:1:1) was added to the dried samples and reacted at ambient temperature for 20 min (*27*). The elution was carried out using sodium acetate buffer, pH=6.4 (eluent A) and 60 % (by volume) aqueous acetonitrile (eluent B).

Chromatographic conditions

Chromatographic analysis was performed by the gradient elution under the following conditions: 0–10 min, linear gradient 0–46 % B; 10–12 min, linear gradient 46–100 % B; 12–15 min, 100 % B; flow-rate 1 mL/min; column temperature 38 °C; detection wavelength 254 nm.

Statistical tests

Analysis of variance (ANOVA) for recovery, Dunnett's *t*-tests for recovery and Paired-samples *t*-test for the comparison of RP-HPLC method and the proposed method were all performed using SPSS 10.0 statistical

software. The analysis of the response values obtained by response surface methodology model was conducted using software Design Expert 6.0 (Stat-Ease Inc., USA).

Results and Discussion

Reaction mechanism

Under low temperature and high acidity conditions, DSAS was oxidised to diphenylbenzidine sulphonic acid with sodium nitrite in the sulphuric acid medium. The unstable oxidation product reacted quickly with sodium nitrite to produce a diazotized intermediate. Sulphamic acid solution was added to eliminate the remaining nitrite. Finally, the Trp solution was transferred into the mixture mentioned above and the indole moiety of Trp reacted with the diazotized intermediate to produce the pink-coloured products. The presumed reaction mechanism is illustrated in Fig. 1.

Optimization of reagent volume

It was found that the stable and coloured product was produced in the range of 2–4 mL of DSAS solution (Fig. 2). Added sulphuric acid solution, sodium nitrite solution and sulphamic acid were in the range of 2–8, 1–3 and 1–4 mL, respectively (data not shown). Response surface methodology was employed to optimize the reaction parameters and 30 experiments were done according to the design by Design Expert 6.0 software. The 3D surface plot of volume of DSAS and sulphuric acid solutions against absorbance is shown in Fig. 3 with the volumes of sodium nitrite and sulphamic acid fixed at 2.5 and 3.0 mL, respectively. The others are not shown because they demonstrated equivalent information as Fig. 3. The optimization results showed that the product had the strongest colour intensity under the system of 3.09 mL of DSAS solution, 6.07 mL of sulphuric acid solution, 2.58 mL of sodium nitrite solution and 3.19 mL of sulphamic acid solution. For practical use, 3 mL of DSAS, 6 mL of sulphuric acid, 2.5 mL of sodium nitrite and 3 mL of sulphamic acid solutions were finally chosen for the following experiment.

Optimization of reaction conditions

The azo-coupling reaction should be performed at 0–5 °C due to the susceptibility of the diazotized reagent to decomposition. The reaction time for azo-coupling was chosen to be 5 min. It was found that the reagents reacted incompletely and resulted in the unstable product if the diazotization time was less than 5 min. For the same reason, 15 min for colour-formation were recommended. The temperature for colour-formation was controlled at 30 °C in the water bath. It was difficult to detect the colour formation at below 25 °C because of its relatively light hue. On the other hand, the product was unstable and easy to break down at above 40 °C. It was important not to shake the solutions during colour formation because vigorous shaking makes the detection inaccurate. The pink colour product was stable for at least 1 h under the conditions stated above.

Standard curve

The total volume of DSAS, sulphuric acid, sodium nitrite and sulphamic acid solutions was 14.5 mL, while the total volume of the flask was 25 mL, therefore, the volume of Trp solution could not be much more than 10.5 mL. A concentration of 1 mg/mL of Trp solution was suitably diluted in order to vary the Trp mass in seven flasks from 7.50 to 300 μg during preparation of

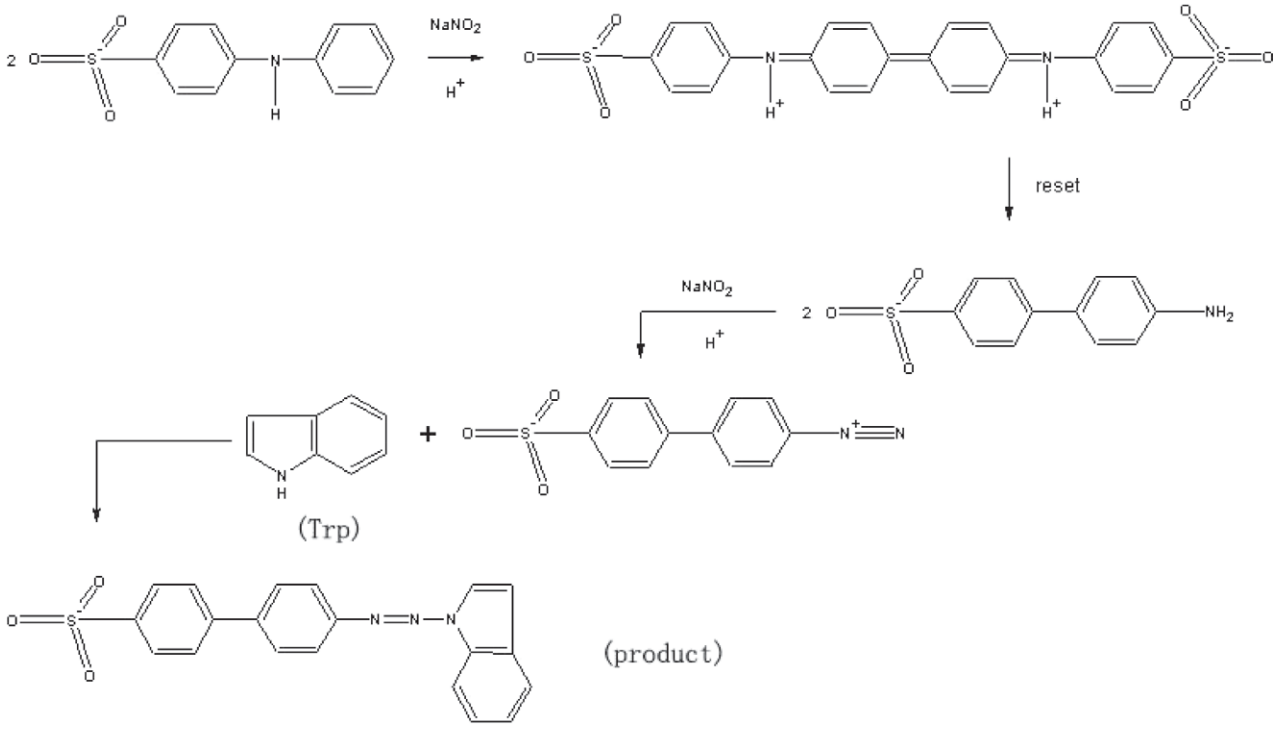

Fig. 1. Reaction mechanism for the production of azo-coupling

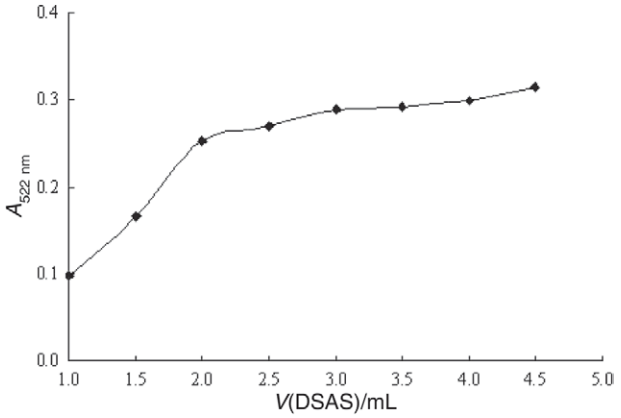

Fig. 2. Plot of volume of DSAS solution against absorbance. The values are means of triplicate. Sulphuric acid solution, 5 mL; sodium nitrite solution, 2 mL; sulphamic acid solution, 2.5 mL

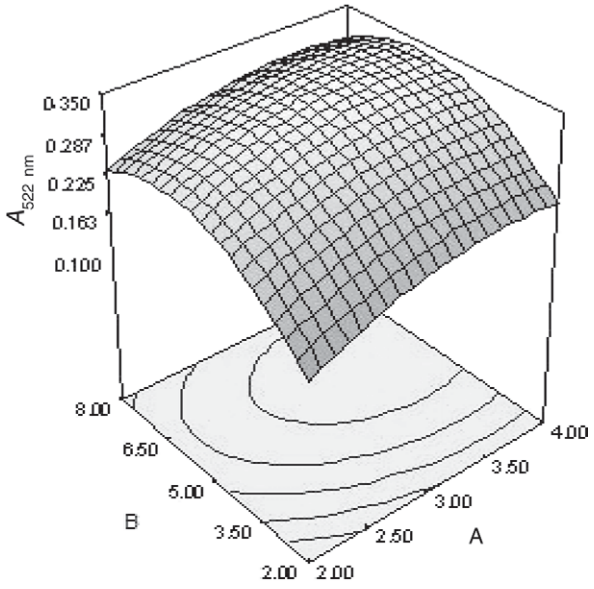

Fig. 3. 3D surface plot of volumes of DSAS and sulphuric acid against absorbance. A, DSAS volume (mL); B, sulphuric acid volume (mL)

the standard curve, while the volume of Trp solution for each flask was the same as the others. Volume of Trp solution was fixed at 2.5, 5.0 and 10.0 mL for the further test. The linearity and sensitivity of the method with different sample volume were statistically examined and the results are given in Fig. 4 and Table 1. The regression coefficient (R^2) was 0.9983, 0.9993 and 0.9992 for sample volume of 2.5, 5.0 and 10.0 mL, respectively. The slope was 0.0017, 0.0024 and 0.0023 in the same order. Therefore, the sample volume of 5.0 mL was strongly

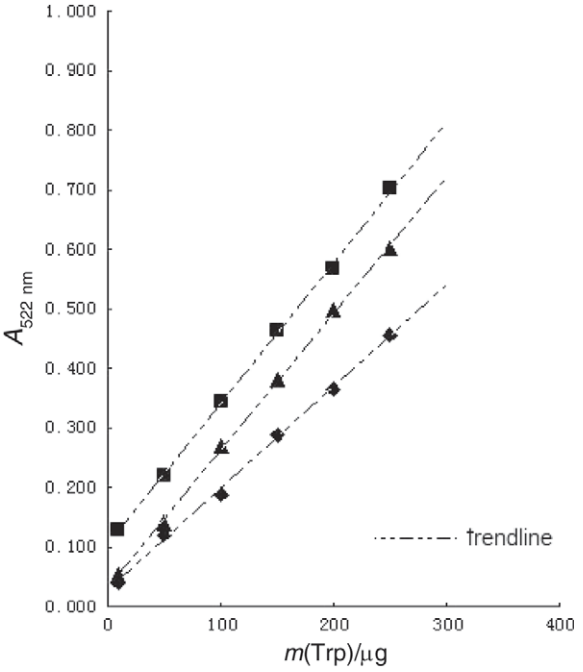

Fig. 4. The effect of the sample volume on the linearity of the absorbance. -■- 5.0 mL, -▲- 10.0 mL, -◆- 2.5 mL. The preparation was done according to the standard procedure of the proposed spectrophotometry method

recommended because of the best linearity (R^2=0.9993) and sensitivity (slope of 0.0024 and y-intercept of 0.1048). The resulting regression equation was as follows:

$$y=0.0024x+0.1048 \qquad /1/$$

where y means the content of Trp and x means the absorbance value.

The absorbance values at 522 nm for a series of solutions with varying contents of the tested samples were used to investigate the accordance with Beer's law. Beer's law was obeyed in the Trp concentration range of 0.3–12 μg/mL.

Interference effect

In the diazotization process, some of the other amines such as aniline, morpholine, piperidine, pyrrole, and some phenols had a positive reaction. However, in protein hydrolysates, these amines are not present, and hence there will be no interference from them in this method. The effect of various amino acids present in the protein hydrolysates on the Trp content was studied. A mass of 100 μg of the amino acids was added along with 100 μg of Trp to the diazotized DSAS (Table 2). Tables 3 and 4 show the statistical analysis of the interfer-

Table 1. Statistical analysis of the estimated linear regression model for different volumes

V/mL	Regression coefficient (R^2)	Standard error	y-intercept	Slope	Linear equation
2.5	0.9983	0.0073	0.0270	0.0017	y=0.0017x+0.0270
5.0	0.9993	0.0062	0.1048	0.0024	y=0.0024x+0.1048
10.0	0.9992	0.0066	0.0320	0.0023	y=0.0023x+0.0320

Table 2. The effect of the addition of other amino acids on the determination of Trp

Group	Substances	*m*(amino acid)/μg	Recovery*/ %	*m*(amino acid)/μg	Recovery*/ %
1	Trp	100	99.30±0.36	100	99.30±0.36
2	Tyrosine+Trp	100+100	98.66±0.87	200+100	98.67±0.65
3	Phenylalanine+Trp	100+100	99.00±0.18	200+100	99.27±0.51
4	Leucine+Trp	100+100	99.28±0.45	200+100	99.55±0.79
5	Glycine+Trp	100+100	99.91±0.17	200+100	99.53±0.10
6	Alanine+Trp	100+100	99.79±0.36	200+100	99.85±0.28
7	Glutamic acid+Trp	100+100	99.10±0.60	200+100	99.14±0.53

*Means of triplicate

Table 3. One-way ANOVA for the recovery

	Sum of squares	df	Mean square	F	p*
m(amino acids)=100 μg					
Between groups	3.426	6	0.571	2.443	0.079
Within groups	3.272	14	0.234		
Total	6.697	20			
m(amino acids)=200 μg					
Between groups	2.514	6	0.419	1.180	0.371
Within groups	4.969	14	0.355		
Total	7.484	20			

*at 95 % confidence

Table 4. Dunnett's *t*-tests of recovery for the difference between the Trp group and the interference group

	Group I	Group J	Mean difference (I-J)	S.E.[b]	p[c]
m(amino acids)=100 μg					
Dunnett's (2-sided) *t*-test[a]	2.00	1.00	–0.6367	0.3947	0.429
	3.00	1.00	–0.3033	0.3947	0.929
	4.00	1.00	–0.0167	0.3947	1.000
	5.00	1.00	0.6067	0.3947	0.474
	6.00	1.00	0.4867	0.3947	0.670
	7.00	1.00	–0.2000	0.3947	0.989
m(amino acids)=200 μg					
Dunnett's (2-sided) *t*-test[a]	2.00	1.00	–0.6333	0.4865	0.624
	3.00	1.00	–0.0300	0.4865	1.000
	4.00	1.00	0.2467	0.4865	0.989
	5.00	1.00	0.2333	0.4865	0.992
	6.00	1.00	0.5500	0.4865	0.737
	7.00	1.00	–0.1567	0.4865	0.999

[a]Dunnett's *t*-tests treat one group as a control, and compare all other groups against it
[b]Standard error
[c]at 95 % confidence

ence on the determination of Trp content. It is significant to note that various amino acids did not interfere because of p>0.05 (p=0.079). This was further confirmed by using Dunnett's *t*-tests. Multiple comparisons showed that difference between group I (I=2, 3, 4, 5, 6, 7) and group J (J=1) was not significant because p was 0.429, 0.929, 1.000, 0.474, 0.670 and 0.989 for group (2-1), group (3-1), group (4-1), group (5-1), group (6-1) and group (7-1), respectively, and all these p values were larger than 0.05 (at 95 % confidence). The analysis of the interference of 200 μg of the added amino acids on the determination of Trp content was also studied and the conclusion was the same (Tables 2, 3 and 4). This illustrated the advantage of the above mentioned method.

Application and comparison

The applicability of the proposed method was examined for grass carp protein hydrolysates. The hydrolysis was carried out with Alcalase 2.4 L (ratio of enzyme to substrate (E/S), 1000 U/g) and Flavourzyme 500 MG (E/S, 250 U/g), for 6 h under 55 °C using heated magnetic stirrer and pH=6.8, then PTN 6.0S (E/S, 750 U/g) was added to the system for another 6 h. The enzyme was inactivated for 10 min at 100 °C. The resulting hydrolysates were cooled to about 25 °C and centrifuged at 4800 rpm for 30 min. The lipoprotein layer, the supernatant and the residues were collected. Trp content was determined by the proposed spectrophotometric method (Table 5) and RP-HPLC method (Fig. 5 and Table 5), respectively. The figures for lipoprotein and resi-

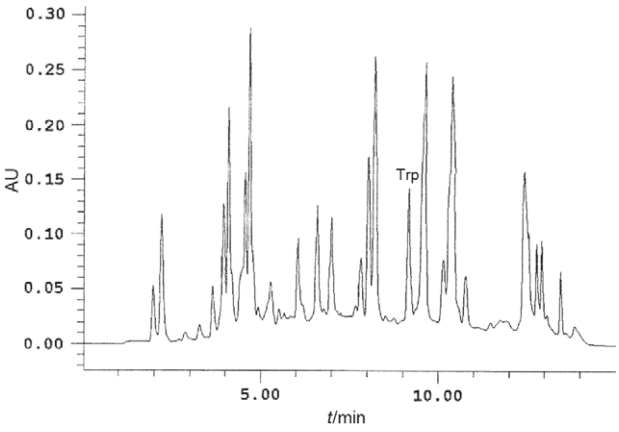

Fig. 5. Determination of tryptophan in supernatant fluid. The supernatant fluid was prepared by centrifuging the grass carp protein hydrolysates. The assay of Trp content in supernatant fluid was done according to the RP-HPLC method

Table 5. Determination of Trp in grass carp protein hydrolysates

Sample	m(Trp)[a]/(100 mg/g)	
	RP-HPLC method	Proposed method
Lipoprotein	177.2±0.2[A]	175±1[A]
Supernatant fluid	187.8±0.3[B]	184±1[B]
Residues	396.8±0.3[C]	390±3[C]

[a]Expressed as means±S.D. of triplicates. Values followed by the same letter were defined as pair, and pair A, pair B and pair C were used for further paired-samples *t*-test by SPSS 10.0

dues by RP-HPLC are not given here because they illustrate equivalent information as Fig. 5.

Paired-samples *t*-test was used to analyze the difference between the two methods (Table 6). The p (2-tailed) values for pair A, pair B and pair C were 0.055, 0.058 and 0.077, respectively. No significant ($p<0.05$) difference between these two results was noticed.

Table 6. Paired-samples *t*-test for the assay of Trp by RP-HPLC method and proposed method

Pair*	Paired differences		*t*	p/2-tailed
	Standard deviation	Standard error of the mean		
A	1.0812	0.6242	4.069	0.055
B	1.4799	0.8544	3.987	0.058
C	3.5052	2.0237	3.390	0.077

*Pair A for lipoprotein, pair B for supernatant fluid, pair C for residues

Conclusion

The proposed method for the determination of Trp is simple, rapid and sensitive. The method does not require heating treatment or extraction. The statistical parameters and the recovery study data clearly indicate the reproducibility and accuracy of the method. The method could be considered for the determination of Trp content.

Acknowledgements

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