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Quantitative determination of amino acids in earthworm meal (*Eisenia andrei*) by a Surveyor HPLC system in conjunction with pre-column 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatization.

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RESUMEN

Objetivo. Evaluar la integración del sistema de derivatización AccQ•Tag en conjunción con el sistema de cromatografía CLAR Finnigan Surveyor Plus en la determinación de la composición de aminoácidos (aa) de las proteínas de harina de lombriz posterior a la hidrólisis.

Material y Método. Las lombrices de tierra (*Eisenia andrei*) fueron criadas en condiciones de laboratorio, reducidas a harina e hidrolizadas con HCl 6 M a 110ºC por 24 horas en un sistema cerrado. El producto de la hidrólisis se neutralizó y los aa se derivatizaron con 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). Los aminoácidos derivatizados se separaron por cromatografía liquida de alta resolución (CLAR) en FR y se detectaron por fluorescencia.

Resultados y Conclusiones. La integración propuesta conformada por el diseño modular del sistema CLAR Surveyor Plus (versatilidad y flexibilidad) y las principales características del sistema de derivación AccQ•Tag (estabilidad y reproducibilidad) resultó óptima. Los parámetros analíticos de validación fueron estudiados antes y después de la derivatización con AQC originando datos dentro de los intervalos aceptables, incluyendo un límite de cuantificación en el orden de pmol por inyección. Los aminoacidos más abundantes (m/m) en la harina de lombriz fueron: Glu, Asp, Arg, Leu y Lys (4 % - 10 %), mientras que el contenido más bajo correspondió a Met (< 1,5 %), pero comparable a la harina de pescado. La propuesta de análisis se puede utilizar con seguridad en el control de calidad de la harina de lombriz de tierra con el fin de garantizar el contenido apropiado de aa para crear una dieta óptima para peces.

PALABRAS CLAVE: AQC, Carbamato de 6-aminoquinolil-N-hidroxisuccinimidilo, Cromatografía liquida, Harina, Lombriz de tierra .

ABSTRACT

Aim: The objective of this study was to evaluate the integration of the AccQ•Tag derivatization system with the Finnigan Surveyor Plus HPLC system to determine the amino acids (aa) composition of earthworm meal protein post-hydrolysis.

Materials and Methods: In lab cultivated earthworms (*Eisenia andrei*) were reduced to flour which was then hydrolyzed with 6M HCl at 110 °C for 24 hours in a closed system. The hydrolysis product was neutralized and their aa were derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). The derivatized-aa were separated by RP-HPLC and detected by fluorescence.

Results and Conclusion: The proposed integration makes optimal use of both the modular design of the Surveyor Plus HPLC for versatility and flexibility and the main features of the AccQ•Tag derivatization system in terms of stability and reproducibility. Analytical validation parameters were studied both before and after derivatization with AQC. The resulting data were within acceptable ranges for this type of analysis. Pre-column derivatization with AQC yielded appropriate sensitivities within the low pmol range per injection. Earthworm meal generated the following aa; the most abundant (w/w) being: Glu, Asp, Arg, Leu, and Lys (4 % - 10 %), whereas the lowest content corresponded to Met (< 1. 5%), which is comparable to fishmeal. The analytical proposal can be used with confidence in earthworm meal quality control to guarantee the appropriate aa content to create an optimum fish diet.

KEY WORDS: 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, AQC, earthworm meal, HPLC.

INTRODUCTION:

In fish farming, nutrition is critical because feed represents about 50% of production costs. Fish nutrition has advanced dramatically in recent years with the development of new balanced commercial diets that promote optimal fish growth and health [1]. Fishmeal as fish feed, however, is increasingly expensive for developing countries. Therefore, people that are working in fish farming employ alternative diets. One of the options is earthworm meal, a processed by-product of the vermiculture practice. The main characteristic of earthworm meal is high protein content, usually higher than 50% [2-4]. Proteins are formed by linkages of individual amino acids. For a variety of specific situations, determination of amino acid content is important [5]. Earthworm meal has been shown to have an amino acid profile very similar to that of fishmeal [6]. They are rich in high quality essential amino acids such as lysine and methionine with high digestibility due to low fiber content [4]. On the whole, earthworm meal will always be cheaper than fishmeal since earthworm production feedstuff generally constitutes free raw material [7]. Therefore, the determination of the amino acid profile of the earthworm flour in order to be used as a non-conventional ingredient in the formulation and preparation of diets for fish is very important.

The traditional method for the amino acid composition analysis of proteins is separation by ion-exchange chromatography and post-column derivatization with ninhydrin as the detection mode [8]. Given that conventional HPLC-equipment is more frequently available to most analytical laboratories, several HPLC methods have been developed. Several years ago, the Amino Acid Analysis Research Committee described amino acid composition analysis of proteins as "deceptively difficult" [9].

Hydrolysis is a difficult task. Amino acids are highly polar analytes and, therefore, not suitable for conventional RP-HPLC. Furthermore, not all amino acids have chromophores useful for UV/Vis detection. As a result, a derivatization step for HPLC analysis is often required. Several derivative agents have been proposed for both pre- and post-column derivatization of amino acids [10]. However, there is no ideal reagent for this purpose due to various disadvantages. They include extensive sample manipulation, considerable derivatization time, heating after derivatization, labile derivatives, and constant time from reaction to injection, among others [10]. In this regard, it is always practical to have a derivative agent with fewer disadvantages available. The AccQ•Tag derivatization system introduced by the Waters Corporation somehow meets the above requirements. The derivatization system can be considered as simple as *A, B, C*. The derivatization procedure takes approximately one minute and the amino acid derivatives, including secondary amino acids are quite stable.

As can be expected, each analytical laboratory has a HPLC system from a different manufacturer. With this in mind, the objective of the present work was to explore the possibility of using the robust Waters AccQ•Tag derivatization system in conjunction with the features of the Finnigan Surveyor Plus HPLC system for amino acid analysis composition of samples containing proteins. The applicability of the method was explored by determining the amino acid composition of hydrolysate earthworm meal.

MATERIALS AND METHODS:

Apparatus:

Analyses were performed using a Surveyor Plus liquid chromatography system (Thermo Scientific, San Jose, USA), equipped with a modular design conformed by autosampler, quaternary pump with vacuum degasser, and a full-featured time-programmable fluorescence detector connected to a computer with ChromQuest Software.

Solvents, Reagents and Materials:

Acetonitrile HPLC-grade and phosphoric acid 85 % were purchased from Fischer Scientific (New Jersey, USA). Triethylamine from Himedia (Mumbai, India). Sodium hydroxide, sodium acetate trihydrate and hydrochloric acid from Riedel-de Haên (Seelse, Germany). All samples were filtered using a Millipore syringe with 0.22 µm membrane filters (Milford, USA). Vials, 12 mm x 32 mm, with septa and screw cap 1.8 mL from Thermo Electron Corporation (San Jose, USA). Pyrex glass tubes, 2.2 cm x 17.5 cm, with black screw caps were used for hydrolysis. Amino acid hydrolysate standard mixture 2.5 mM was purchased from Pierce Chemical Co. (Rockford, IL, USA). Individual amino acids were purchased from Sigma-Aldrich (St Louis, USA). AQC reagent was acquired as an "AccQ•Fluor reagent kit" containing borate buffer, reagent powder (AQC), and reagent diluent (acetonitrile) was purchased from Millipore Corporation (Milford, MA, USA).

HPLC Mobile Phase:

A mobile phase AccQ•Tag (acetate–phosphate aqueous buffer) concentrate was purchased from Millipore Corporation (Milford, MA, USA). For chromatographic analysis, the AccQ•Tageluent concentrate was diluted by mixing 100 mL of the concentrate with 1000 mL of Milli-Q water. A home-made eluent concentrate was prepared as

published elsewhere [11].

Samples:

Earthworms, identified as *Eisenia andrei*, were cultivated in laboratory conditions at the Department of Food Science at the University of Los Andes, Mérida, Venezuela. Earthworm flour was obtained from washed earthworms submerged in an air insufflated water container at 19 °C for 18 h, until their digestive systems were emptied. They were then submerged in boiling water for 1 min. Finally, they were dried in an oven with air circulation at 40 °C for 24 h. Flour was obtained by grinding dried earthworms in a classical Oster food grinder and sieved through a 1 mm pore size mesh [12]. For each sample, 3 g of fat from earthworm meal samples was extracted by triplicate with petroleum ether at 110 °C for 40 min by using an automated soxhlet extractor SER 140 Series VELP Scientifica (USA). The fat content of earthworm meal is 9 % - 10 % (w/w) depending on humidity. The protein content of non-defatted earthworm meal is 58 % ± 1 % (w/w).

Sample Hydrolysis:

Two lots of defatted samples of earthworm flour were sieved through a 0.5 mm pore size mesh. Each sample was weighed into a hydrolysis tube, 7.0 mg for lot 1 and 7.7 mg for lot 2. Then, 4 mL of a constantly boiling 6 M HCl solution, containing 0.1% (w/v) phenol, was added and gently mixed together with 3 boiling glass beads to ensure uniform acid hydrolysis. Subsequently, the reaction glass tubes were purged with nitrogen for 1 min to remove oxygen. Sealing of the hydrolysis tubes with the screw caps was reinforced and secured by using 100 % PTFE Teflon tape. Finally, hydrolysis tubes were maintained at 107 °C - 108 °C, equivalent to 110 °C, for 24 h.

Preparation of Sample Solutions:

The hydrolyzed contentof each tube was quantitatively transferred to a 25 mL volumetric flask with the aid of up to 20 mL of diluted NaOH solution. A 0.5 mL aliquot of 2.5 mM L-Nleu was added before the flask was filled to the mark with just Milli-Q water. The final alkalinized hydrolyzed-sample (pH 9.1 ± 0.4) was filtered through a 0.22 µm filter. An aliquot of 0.5 mL of the filtered solution was stored at 4 °C for further analysis within the first 24 h. Each sample vial was flushed 10 seconds with nitrogen prior to closing it.

Preparation of Standard Solutions:

Each amino acid standard solution was prepared by mixing 0, 10, 20, 40, 60, 80 and 100 µL of the amino acid hydrolysate standard mixture of 2.5 mM concentration with 40 µL of internal standard (L-Nleu) stock solution 2.5 mM and up to 2 mL diluted with Milli-Q water. They were stored at -20 °C for up to one month following Waters AccQ•Tag Chemistry Package, Millipore Corporation (USA) instruction manual.

Derivatization Procedure:

Initially, the reaction sample vial of 1.8 mL containing 40 µL of either the final hydrolysed sample solution or the final standard solution was spiked with 120 µL of AccQ•Fluor borate buffer (pH 9.0), and then shaken in a vortex for 15 seconds. Finally, the resulted buffered sample solution was spiked with 40 µL of reconstituted AccQ•Fluor reagent (AQC) and then quickly shaken in a vortex for 30 seconds. Completion of the derivatization was accomplished by heating the vial in a preheating water bath at 55 °C for 10 min. Before performing the analyses, all sample and standard solutions were further diluted by addition of 600 µL of a blank solvent mixture of 1:3:1, by vol water/borate buffer/acetonitrile. This procedure represented an equivalent modification of 800 µL instead of 100 µL of the standard procedure in order to achieve greater volume for chromatographic work.

Procedure for Plotting Calibration Curve:

After preparation of the standard solutions, derivatization, and dilution to a convenient final volume, the resulting concentration of each amino acid oscillated between 625 and 6250 nM. For the standard addition assay, 20 µL sample solutions were spiked with aliquots of 200 µL of calibration standard, 2500 nM each amino acid, and diluted by the addition of 580 µL of blank solvent mixture of 1:3:1, by vol water/borate buffer/acetonitrile. Calibration curves were constructed by using Origin® 7.0 SR0 software, OriginLab Corporation (MA, USA).

Chromatographic Separation:

The chromatographic separation was carried out in a Nova-Pak™ C_{18} column (3.9 mm x 150 mm, 4 µm) fitted with a Nova-Pak™ C_{18} Sentry™ Guard column (3.9 mm x 20 mm, 4 µm), both of them from Waters Corporation (Milford, MA, USA). Mobile phase A consisted of a diluted homemade AccQ•Tag eluent. Before beginning the gradient, the column was equilibrated as published elsewhere [13]. Detection was accomplished by fluorescence (λ_{exc} 250 nm and λ_{em} 395 nm).

Quantitative Analysis:

Amino acid contents were estimated by linear regression calibration using the internal standard method. The response factor A_s/A_{is} was plotted versus amino acid content (nM); where A_s, amino acid area in standard/sample, A_{is}, internal standard area, and nM, nmol/L. Recovered concentrations

were calculated from the corresponding calibration graphs obtained by comparing the amino acid response with the increment response reached after the addition of the standard. The method was validated according to analytical method validation guidelines [14, 15].

RESULTS AND DISCUSSION:

Hydrolysis:

Protein hydrolysis is known to be the most critical part of analysis and is mainly responsible for analysis errors [16]. Some progress has been made in protein acid hydrolysis directed mainly towards automatic methods and shorter analysis time [17-20]. A review of specialized literature shows many authors leading to the same conclusion. Significant advances have been made in the protein hydrolysis procedure but there is no method likely to displace the established 6M HCl, 110 ºC, 24 h hydrolysis method used by the majority of analytical laboratories [21,22]. Under the above premise, a procedure equivalent to the old slow acid hydrolysis way was used in this work. A quantity of the sample weighing between 5 mg and 15 mg and containing between 0.5 and 4 mg of protein was weighed into a hydrolysis tube and subsequently heated with 1 mL of 6 M HCl for about 24 h at 110°C in an oxygen-free atmosphere [23]. After hydrolysis, the pH of the solution was adjusted with a NaOH solution as published elsewhere [24,25]. Precision and accuracy of the developed procedure for sample hydrolysis is discussed throughout the text.

Chromatographic Analysis of Amino Acids:

A few years ago, an amino acid analysis overview and strategic planning, discussing a range of methodologies and issues, was published [23,26]. Recently, standardized methods for determining amino acid food and feed contents were also reviewed [21]. Although many methods have been developed, RP-HPLC with pre-column derivatization using AQC for fluorescence detection still is being used in quantitative determination of amino acids from samples rich in proteins [5,23,27,28]. Consequently, this last approach was used in the present work.

Chromatographic Gradient Selection:

Application of the chromatographic conditions, previously established by the Waters AccQ•Tag amino acid analysis instruction manual using the FSP-HPLC system, failed to provide sufficient resolution for a standard mixture of AQC-derivatized amino acids. We found a suitable gradient program (Table 1-B) after conducting several modifications in 2% increments of the first third of the gradient program (Table 1-A) and following guidelines of former works [11,13,28]. This provided an acceptable resolution for the AQC-derivatized amino acids. Fig. 1 shows the obtained chromatographic separation using the optimized conditions.

Analytical Parameters:

System Suitability:

The Waters AccQ•Tag derivatization system for amino acid composition analysis of protein samples has been amply studied [11,13,29]. Typical pH variations usually affect the resolution of Asp and Glu [11]. The pH of the home-made eluent-A was adjusted to 5.17 at 22 ºC by comparison with the commercial eluent-A. Since it was prepared from the original information on ingredients (WAT052888, Waters Corporation) mobile phase composition did not changed. A solvent-strength error was avoided by using 100 % acetonitrile as the strong eluent instead of an aqueous mixture of 60 % (v/v). In contrast, the gradient table required optimization in order to obtain amino acid

Table 1. Summary of the assayed HPLC gradient conditions.

Table 1-A
Gradient generated for Waters 510 HPLC pumps used for the FSP-HPLC System[a]

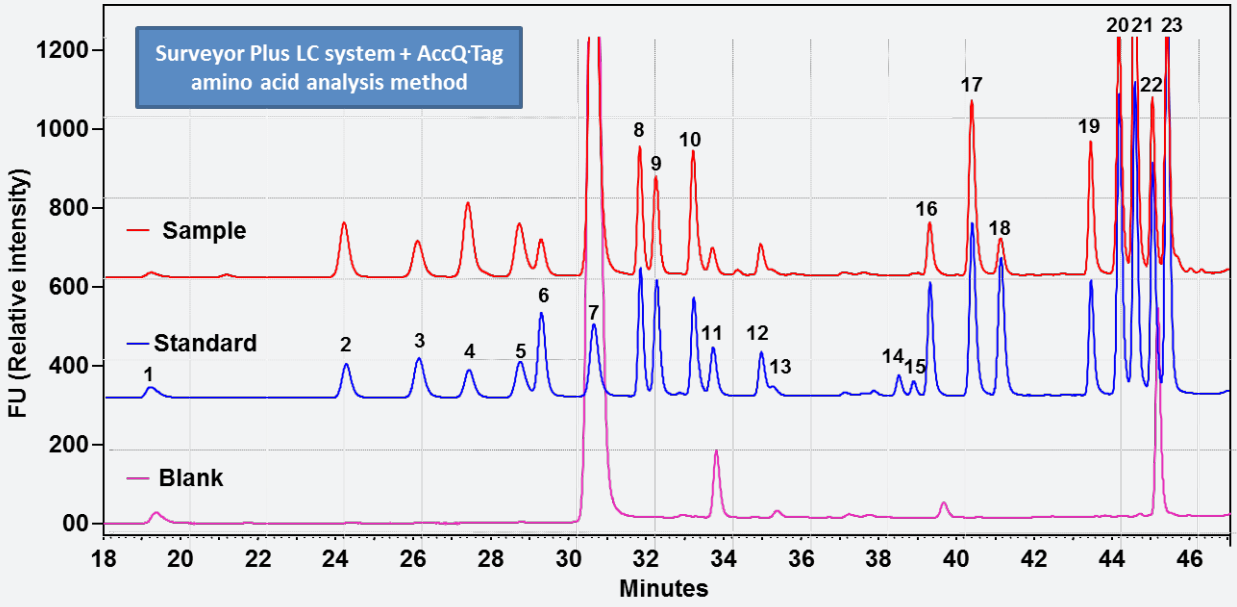
Time (min)	FM-A (Buffer)	FM-B (ACN)	FM-C (H_2O)	Colum Temp
0.05	100	0.0	0.0	37 ºC
0.5	99.0	1.0	0.0	
18.0	95.0	5.0	0.0	
19.0	91.0	9.0	0.0	
29.5	83.0	17.0	0.0	
40.0	70.0	30.0	0.0	
50.0	0.0	60.0	40.0	
60.0	0.0	60.0	40.0	
65.0	100	0.0	0.0	
75.0	100	0.0	0.0	

Table 1-B
Gradient generated for Waters 626 HPLC pumps modified to be used with the FSP-HPLC System[a]

Time (min)	FM-A (Buffer)	FM-B (ACN)	FM-C (H_2O)	Colum Temp
0.01	100	0.0	0.0	34 ºC
25.0	95.0	5.0	0.0	
29.0	91.0	9.0	0.0	
39.0	83.0	17.0	0.0	
47.0	70.0	30.0	0.0	
50.0	0.0	60.0	40.0	
53.0	0.0	60.0	40.0	
54.0	100	0.0	0.0	
64.0	100	0.0	0.0	
-	-	-	-	

[a] FSP, Finnigan Surveyor Plus. Linear gradient program was followed in all cases, except in those where the column was shut down in another mobile phase channel. Note that the steps at 50 min and later shut down the column in eluent A and do not impact the separation.

Figure 1. Overlay of three chromatograms measured with fluorescence-detection: AQC-derivatized blank, AQC-derivatized amino acid standard, and a representative AQC-derivatized hydrolyzed earthworm meal sample.



Identification: (1) AMQ, (2) Asp, (3) Ser, (4) Glu, (5) Gly, (6) His, (7) NH_3, (8) Arg, (9) Thr, (10) Ala, (11) Imp, (12) Pro, (13) Imp, (14) Unknown, (15) Cys, (16) Tyr, (17) Val, (18) Met, (19) Lys, (20) Ileu, (21) Leu, (22) Nleu, (23) Phe.

separation. This last parameter was complemented by small changes in column and sample tray temperatures.

System efficiency was demonstrated for all peaks. As it was discussed earlier, resolution was demonstrated to ensure that closely eluting amino acids were resolved from each other and from the internal standard (Fig. 1). The coefficient of variation for instrument precision, or injection repeatability, was insignificant since all of them resulted less than one percent (Table 2). Part of the intermediate precision was estimated by using the same hydrolysate mixture standard and by determining one standard solution on three different days. The variation coefficient for the relative area of the peaks (A_s/A_{is}) was less than two percent. The intra-assay precision, depending on reproducibility of derivatization replications, was acceptable. All resulted less than one percent, except for Hys which was 3.4 % (Table 3). The intra-assay precision depending on the reproducibility of hydrolysis replications also resulted acceptable, all of them less than 10 %, except for Lys and Phe, but both of them less than 15 % (Table 3). Analytical precision for feed samples is largely dependent on the sample matrix, the concentration of the analyte, the performance of the equipment, and the analysis technique. Therefore, the precision criterion for any assay method, for this type of sample, is considered to vary between 2 % and 20 % [5].

Peak symmetry was demonstrated for most of the peaks considering an asymmetry factor no greater than 1.2 at 10% peak height. For Asp, Ser, Tyr, Nleu, and Phe considering values as high as 1.5 were considered acceptable. The greater chromatographic peak shape distortion was found for Lys (1.60), but acceptable for quantitative purposes due to the very high signal response of this particular amino acid (Table 2).

Linearity and Range:

Linearity was established using five standard solutions whose concentrations spanned 25 % – 200 % of the 2500 nM target analyte concentrationfor the majority of the amino acids. The proposed method obeyed the typical equation $Y = a + b[X]$, with a y-intercept less than a few percent of the response obtained for each amino acid at the target level. "a" was zero within the 95 % confidence limits and the correlation coefficient r was greater than 0.999. Consequently, it was better than the acceptable criterion for any evidence of suitable fit of the data to the regression line (Table 4).

Limit of Detection and Limit of Quantitation Limits:

The *LOD* was determined using the standard deviation of the response where slope (b) of the calibration curve was calculated as $3a/b$, and a is the SD of the y-intercept. Under chromatographic conditions and the detection system, appropriate sensitivities within the low pmol range per injection (Table 4) were yielded. The dynamic interval spanned an overall concentration for the lower limit (*LOQ*) between 27 nM and 536 nM with an upper limit of 5000 nM, except for Pro (3750 nM).

Table 2. Determination of the precision of the method and chromatographic peak tailing factor.

Amino acid	Standard[a]		Sample[b]	Standard[c]
	Retention time (min) ± RSD (%)	Relative area (A_s/A_{is}) ± RSD (%)	Relative area (A_s/A_{is}) ± RSD (%)	Tailing factor
Asp	24.26 ± 0.28	0.181 ± 0.97	0.463 ± 0.45	1.40
Ser	26.15 ± 0.30	0.237 ± 0.19	0.287 ± 0.25	1.23
Glu	27.49 ± 0.48	0.156 ± 0.06	0.619 ± 0.22	1.00
Gly	28.79 ± 0.41	0.211 ± 0.09	0.470 ± 0.13	1.08
His	29.35 ± 0.47	0.393 ± 0.89	0.364± 0.59	1.00
Arg	31.73 ± 0.45	0.389 ± 0.03	0.555 ± 0.47	1.09
Thr	32.27 ± 0.26	0.638 ± 0.10	0.495 ±0.96	1.18
Ala	33.19 ± 0.09	0.368 ± 0.29	0.681± 0.22	1.18
Pro	34.97 ± 0.23	0.164 ± 0.18	-	1.20
Tyr	39.11 ± 0.56	0.379 ± 0.38	0.260 ± 0.20	1.45
Val	40.36 ± 0.13	0.637 ± 0.12	0.962 ± 0.26	1.10
Met	41.13 ± 0.25	0.801 ± 0.33	0.192 ± 0.37	1.20
Lys	43.49 ± 0.25	0.355 ± 0.47	0.557 ± 0.43	1.60
Ileu	44.19 ± 0.27	0.897 ± 0.07	1.124 ± 0.12	1.00
Leu	44.58 ± 0.24	0.932 ± 0.04	1.833 ± 0.05	1.00
Nleu	45.04 ±0.20	0.997 ± 0.22	-	1.45
Phe	45.40 ±0.17	1.237 ± 0.05	1.068 ± 0.09	1.30

[a]The data cover the specified range for the procedure: 625 nM – 5000 nM, five concentrations, each two replicates.
[b]Data belong to six determinations of the same sample at 100% of the test concentration. As, sample area; Ais, internal standard area.
[c]Calculated as the peak asymmetry factor at 10% peak height. RSD, relative standard deviation.

Table 3. Amino acid concentration of earthworm flour samples and intra-assay precision.

AA	Amino acid concentration in earthworm flour samples (g/100g)[a]				
	Intra-assay precision (Derivatization, Lot 1)[b]		Intra-assay precision (Hydrolysis, Lot 2)[c]		
	Mean ± SD	RSD (%)	Mean ± SD (H1)	Mean ± SD (H2)	RSD (%)
Asp	5.930 ± 0.025	0.42	6.103 ± 0.002	6.130±0.017	0.31
Ser	2.054 ± 0.004	0.20	2.360 ± 0.010	2.362±0.002	0.01
Glu	9.810 ± 0.022	0.20	10.66 ± 0.020	10.441±0.002	1.5
Gly	2.986 ± 0.004	0.13	3.140 ± 0.026	3.053±0.006	2.1
His	1.780 ± 0.061	3.40	2.000 ± 0.012	1.897±0.001	3.7
Arg	4.480 ± 0.021	0.47	4.940 ± 0.023	4.752±0.001	2.7
Thr	2.620 ± 0.021	0.80	2.808 ± 0.004	2.788±0.004	0.5
Ala	2.993 ± 0.007	0.23	3.140 ± 0.011	3.202±0.002	1.4
Pro	3.200 ± 0.035	1.10	-	-	-
Tyr	2.197 ± 0.004	0.18	2.411 ± 0.002	2.621 ± 0.004	5.9
Val	3.172 ± 0.008	0.25	3.343 ± 0.005	3.212 ± 0.001	2.8
Met	0.933 ± 0.003	0.32	1.068 ± 0.001	0.928 ± 0.002	9.9
Lys	4.150 ± 0.018	0.43	4.530 ± 0.050	3.900 ± 0.018	11.0
Ileu	2.939 ± 0.004	0.14	3.073 ± 0.002	2.853 ± 0.005	5.3
Leu	4.701 ± 0.003	0.06	5.021 ± 0.001	4.658 ± 0.001	5.3
Phe	2.560 ± 0.002	0.08	2.910 ± 0.014	2.41 ± 0.012	13.2

[a]Results obtained using defatted earthworm meal. [b]Derivatization of the same sample lot (S1), two independent derivatizations (D1 and D2), each analyzed by duplicate. [c]H1 and H2 represent two independent hydrolysis of the same sample lot (S2); data represent the average of 2 replicates. SD, standard deviation. Intra-assay precision calculated as relative standard deviation (RSD).

Selectivity:

Discrimination of analytes: a chromatogram showing 17 hydrolysate amino acids from the amino acid hydrolysate standard solution was registered and contrasted with the standard chromatogram given by the instruction manual of the Waters AccQ•Tag Chemistry Package (Fig. 1). The obtained chromatogram in the present study had a profile quite similar to the standard chromatogram with regard to both percentage signal and behavior for elution time order.

Evaluation of the separation of the calibration standard: Resolution of critical pairs such as Gly/His, Arg/Thr, Ala/Imp, Cys/Tyr, Ileu/Leu, Leu/Nleu, and Nleu/Phe (Fig. 1) were as follows: 91, 97, 92, 97, 95, 95, and 95, respectively. As a result, all of them exceeded 90 % resolution demonstrating the selectivity of the proposed method for the intended application. **Peak purity**: For all, except the pair Pro/Imp, which exhibited a resolution of

76 % peak purity was demonstrated. Additionally, the Phe peak in the samples showed an additional marginal peak in the tail of less than 5 % of peak height that did not exceed the stated resolution.

Accuracy:

Accuracy was assessed by analyzing the sample and comparing the measured value to the true value, together with confidence intervals. Additionally, accuracy confidence was considered acceptable when the established precision, linearity and selectivity were taken into account. Inaccuracy owing to the introduction of any error during the derivatization and so on was minimized by using Nleu as the internal standard. Pre-column derivatization with AQC occurs on specific compounds, eliminating in some way the matrix effect, very common for complex samples. The reaction of this reagent with amino acids is known not to be highly matrix sensitive. The presence of salts, detergents, lipids, and many other sample components do not interfere with reaction [5]. Consequently, both the derivatization procedure and detection mode contributed to the specificity of the method and consequently to its accuracy. The recovery values of amino acids ranged from 93 % and 107 %, within the acceptable mean recovery range of 80 % - 110 % as a function of the analyte concentration [15] (Table 5).

Application of the Method:

Analyses of real samples were performed in order to determine the amino acid contents of hydrolysed earthworm meal proteins. The most abundant amino acids were Glu and Asp at > 6 %, w/w; most of which were found within 2 % and 3 % (w/w). The amino acid with the lowest content was Met at 1.1 %, w/w. In order to understand the above results, it is important to keep in mind that after sample hydrolysis the method allows determination of some amino acids. This represents related amino acids, e.g. Gln and Asn which are deaminated, resulting in Glu and Asp, respectively. Consequently, Asp content could represent the content of Asn + Asp in the sample. The same principle is applicable to Glu, which could represent Glu+Gln in the sample. During acid hydrolysis, Trp and Cys are destroyed and Ser and Thr are also partially lost, while Met can undergo oxidation [30]. In the present study, partial degradation of Tyr, Thr, and Ser was prevented using phenol as a scavenger thus avoiding somewhat oxidation by using an atmosphere of nitrogen [24].

Connotation of the Amino Acids Content Found in the Earthworm Meal:

Returning to the importance of earthworm meal for feeding fishes, 10 amino acids cannot be synthesized by fish. Two of the latter, Lys and Met, are often the first limiting amino acids[1]. In the present work, the analyzed earthworm meal from *E. andrei* provided amino acid content in a representative amount and compared it to those established for fish [31] and earthworm meal from *Eisenia ssp* [32]. Glutamic acid was the amino acid found in greatest concentration. This latter finding is important because Glu, although nonessential, is an amino acid thought to imparts palatability to fishmeal [33]. At present, with respect to Asp, there is a little information regarding supplementation of aspartate or asparagine to fish diets [33]. Our results also revealed high Tyr content in earthworm meal from *E. andrei* compared to earthworm meal from *Eisenia ssp* [32,34]. This result is also highly significant because adding Tyr to diets for fish can reduce Phe requirements. Furthermore, Tyr is a common precursor of important hormones and neurotransmitters, which have important regulatory roles [35]. In spite of not reporting Pro content due to peak purity analytical problems, we found a relatively high content for this secondary amino acid of approximately 3g/100g defatted earthworm meal. Pro is traditionally thought to be an indispensable amino acid for fish and promotes feed intake[35]. Met content in earthworm meal from *E. andrei* resulted slightly lower than those reported in fishmeal [31]. This is understandable since Met can be partially lost during acid hydrolysis [5].

The availability of the above information is useful in order to determine if extra amounts of a particular amino acid must be added to any feed in tended to be used in a fish diet or as a source of non-conventional proteins in order to promote optimal growth and health of fish. Our research group has already carved a path in this regard [4,12,36,37].

CONCLUSIONS

If the conventional hydrolysis method is standardized, the AccQ•Tag derivatization system can be used in conjunction with the Finnigan Surveyor Plus HPLC System in the amino acid composition analysis of earthworm meal proteins with high precision and acceptable accuracy. Problems such as the stability of reagents and derivatives, a too long derivatization procedure, reproducibility of injections of an identical sample, and the reproducibility of hydrolysis replications, among others, are overcome. The main drawback of the proposed analytical application is the long analysis time, which could be improved using a modern shorter RP-HPLC column. In order to guarantee the appropriate amino acids content to create an optimum fish diet, the analytical proposal can be used with confidence in the quality control of earthworm meal.

Table 4. Linear regression correlation and derived analytical parameters.

Amino acid	Intercept (*a*)	Slope (*b*)	*r*	LOQ (nM)[a]	LOQ (pmol)[b]
Asp	0.001 8	0.000 074	0.9997	499	2.50
Ser	- 0.005 6	0.000 107	0.9999	179	0.90
Glu	- 0.008 4	0.000 067	0.9997	536	2.68
Gly	0.004 8	0.000 084	0.9999	262	1.31
His	0.009 4	0.000 153	0.9999	292	1.46
Arg	0.012 8	0.000 151	0.9998	344	1.72
Thr	0.008 9	0.000 158	0.9999	312	1.56
Ala	0.010 7	0.000 142	0.9999	178	0.89
Pro	- 0.001 2	0.000 066	0.9999	64	0.32
Tyr	0.007 8	0.000 148	0.9999	27	0.14
Val	- 0.006 1	0.000 257	0.9999	329	1.65
Met	0.019 6	0.000 197	0.9992	402	2.01
Lys	0.011 4	0.000 137	0.9997	543	2.71
Ileu	0.011 6	0.000 355	0.9999	82	0.41
Leu	0.026 2	0.000 360	0.9998	456	2.28
Phe	0.031 2	0.000 478	0.9999	157	0.79

[a]Limit of quantification was determined using the standard deviation of the response as slope (b) of the calibration curve (Y = a + b[X]), calculated as 10α/b, where α is the SD of the y-intercept, and [X] is the amino acid concentration. [b]Chromatographic injection volume, 5 µL.

Table 5. Accuracy of the AccQ•Tag amino acid analysis method in conjunction with the Surveyor Plus LC system.

	Chromatographic mean peak area (n = 2)[a]			Recovery
Amino acid	Present (sample)	Added (standard)	Found (total)	(%)
Asp	992 527	3 547 413	467 613 820	103 ± 5.5
Ser	6 947 519	4 639 480	1 181 873 898	102 ± 4.6
Glu	1 397 011	3 048 704	466 800 075	105 ± 3.2
Gly	1 017 881	4 130 336	509 673 483	99 ± 2.9
His	518 418	7 681 738	762 614 508	93 ± 14.9
Arg	1 250 832	7 613 021	850 929 888	96 ± 2.4
Thr	1 104 221	12 485 103	1 372 521 724	101 ± 4.5
Ala	1 505 282	7 193 940	896 019 866	103 ± 6.3
Tyr	618 211	7 410 122	859 031 631	107 ± 10.8
Val	2 056 158	12 465 648	1 423 136 988	98 ± 1.9
Met	4 201 303	15 668 334	1 907 485 152	96 ± 3.4
Lys	1 178 607	6 937 306	803 475 387	99 ± 5.5
Ileu	2 365 447	17 544 980	1 951 221 846	98.0 ± 0.3
Leu	3 934 532	18 235 791	2 217 032 300	100.0 ± 0.3
Phe	23 013 540	24 188 113	4 814 568 606	102 ± 7.9

[a]Analytical data processing for the average recovery calculation did not include the response factor A_s/A_{is} because of the concentration of the internal standard was changed in the analyte addition technique.

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