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Reversed-phase liquid chromatographic analysis of *o*-phthaldialdehyde-derivatized free amino-acids in two types of goldfish muscles

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Abstract: *o*-Phthaldialdehyde-derivatized free amino-acids from muscle tissues of goldfish (*Carassius auratus* L.) were determined using reversed-phase liquid chromatography (3-μm Ultrasphere ODS) with gradient elution and UV detection of the analyte. An off-line, pre-column derivatization procedure was applied. A full analysis of the derivatives was completed within 40 min. The method showed good selectivity, sensitivity and reproducibility. The developed procedure was employed to quantify free amino-acids in white and red muscle of non-fasted, normoxic goldfish. The differences in the amounts of the individual amino-acids in white and red muscle are given. The rôle of two of the quantitatively most important free amino-acids in white and red muscle of goldfish, histidine and taurine is briefly discussed.

Keywords: *Reversed-phase liquid chromatography; pre-column* o-*phthaldialdehyde derivatization; amino-acids; muscle tissue.*

Introduction

During investigations on biochemical adaptations of teleost fish to lowered oxygen levels, the results of laboratory experiments indicated a possible involvement of amino-acids; this involvement was apparent from ammonia secretion during anaerobic energy production. In an attempt to study this phenomenon, the quantitative analysis of free amino-acids in goldfish tissues was investigated. Because of the necessity of accurate and sensitive measurements of these metabolites and because of the need that the method should be suitable for routine analysis in small laboratories, a rapid and simple liquid chromatographic (LC) procedure was applied.

LC analysis of amino-acids can be accomplished by either ion-exchange or reversed-phase (RP) strategies [1]. To achieve the required sensitivity it is necessary to derivatize the amino-acids with a suitable absorbance-enhancing or fluorescence-introducing reagent [2, 3].

In general, there are two approaches to execute the derivatization: derivatization after

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the chromatographic separation (e.g. ion-exchange) of the amino-acids (post-column derivatization); and derivatization before chromatographic separation (e.g. RP) of the derivatives (pre-column derivatization) [2, 3].

Post-column techniques using ninhydrin [2] or fluorescence-introducing reagents [2–8] have a long historical background and, because of the numerous protocols that are available, are therefore, accurate and reliable. However, post-column techniques require specialized equipment such as reaction coils [3] and additional high-pressure pumps for pulseless addition of liquids. Another disadvantage is, in some cases, the rather long analysis time, although this time can be shortened by using stationary phases with smaller particle sizes.

Pre-column derivatization is a more recent development for the derivatization of amino-acids. The quantitative determination of the derivatives of free amino-acids by RPLC is faster and more sensitive [9, 10] in comparison with that of the ion-exchange methods and, moreover, no complicated LC equipment is necessary.

Pre-column formation of dansyl [11, 12], dabsyl [13, 14], phenylthiohydantoin [15, 16], phenylthiocarbamyl [17, 18] and fluorenylmethylchloroformate [19, 20] derivatives have been widely described in the literature as suitable procedures to achieve the required sensitivity. However, all these methods have their disadvantages [3] and the major disadvantage is their lack of specificity for amino-acid analysis in biological matrices.

This objection is not valid for *o*-phthaldialdehyde (OPA) as a pre-column derivatization reagent. OPA in the presence of a suitable thiol like 2-mercaptoethanol (2-ME) reacts rapidly with primary amino-acids to form strongly absorbing thio-substituted isoindoles even in physiological fluids and other complex biological matrices [21]. The derivatization is quantitative in 1 min, it can be performed at room temperature, and each of the amino-acids shows one single product. The basic procedures for amino-acid derivative analysis by RPLC using OPA derivatization have been reviewed recently [1].

With a 3-μm stationary phase, the selectivity of the developed procedure was sufficient for the analysis of free amino-acids in muscle tissues of goldfish using a straightforward LC system. In order to obtain a stable baseline during the gradient elution, the column was thermostatted.

Experimental

Chromatography

The equipment comprised two high-pressure pumps (Model 6000 A and Model M-45) which were programmed by a Model 660 solvent programmer. A Model U6K injection system (fixed 100-μl loop) was used (all from Waters Assoc., Milford, MA, USA). Detection was performed with a LC-UV (Pye Unicam, Cambridge, UK) variable wavelength detector at 365 nm (0.005 a.u.f.s.). Peak area measurements were executed with a Model 603 integrating system (Packard, Delft, The Netherlands). A stainless steel column (75 × 4.6 mm, i.d.) packed with 3-μm Ultrasphere ODS (Beckman, San Ramon, CA, USA) was used.

Gradient elution system: eluent A was 0.03 M sodium acetate (adjusted to pH 4.0 with concentrated acetic acid)–acetonitrile (85:15, v/v), to which mixture, tetrahydrofuran (1% v/v) was added; eluent B was 0.03 M sodium acetate (adjusted to pH 7.4 with 1 M acetic acid)–acetonitrile (50:50, v/v). Both eluents were filtered through a 0.45-μm Millipore filter and de-aerated ultrasonically. The gradient profile was: $t = 0$, %B = 0; $t = 20$ min; %B = 50; according to a concave curve as given by curve number 7 of the

solvent programmer. Chromatography was performed at 30 ± 1°C and a flow rate of 0.6 ml min^{-1} was maintained.

Materials

All reagents and solvents were of analytical reagent grade and were used as received. The sodium salt of tetraborate decahydrate and 2-aminobutyric acid (2-ABA) were obtained from Janssen Chimica (Beerse, Belgium); OPA, perchloric acid, sodium hydroxide pellets and triethanolamine were purchased from Merck (Darmstadt, FRG); 2-ME was obtained from BDH (Poole, UK); methanol, ethanol, potassium carbonate, acetic acid, HPLC-grade sodium acetate trihydrate and tetrahydrofuran were received from Baker (Deventer, The Netherlands); and acetonitrile was obtained from Rathburn (Walkerburn, Scotland). The amino-acid standard H of Piece (Oud-Beijerland, The Netherlands) and asparagine (Asn), glutamine (Gln), and tryptophan (Trp) of Sigma (St. Louis, MO, USA) were used.

Throughout the study deionized water (Milli-Q water purification system, Millipore, Bedford, MA, USA) was used.

Solutions

Amino-acid standard H was mixed with Asn, Gln, Trp, and 2-ABA, dissolved in water. The final concentration of each of the amino-acids and the internal standard (2-ABA) was 1.25 mM. The resulting solution was used as the standard amino-acid solution.

The derivatization reagent solution was obtained by dissolving 7.6 mg of OPA in 300 μl of methanol; 2.2 ml of the 0.4 M sodium tetraborate buffer (pH 10.2 with 4 M sodium hydroxide) and 10 μl of 2-ME were then added. The mixture was filtered through a Millex-HV4 filter (Millipore) and was prepared freshly every day. OPA, 2-ME, and the derivatization reagent solution were stored in the dark at 4°C.

Sample pre-treatment

Extraction of free amino-acids from goldfish epaxial white muscle and lateral red muscle was carried out as follows. Non-starved, normoxic (pO_2 ~ 21 kPa) goldfish (~50 g), acclimatized to 20°C, were anaesthetized with 100 ppm tricaine methanosulphonate (Sigma, St. Louis, MO, USA) and decapitated. After the skin was stripped off, dorsal white muscle and lateral red muscle were excised and freeze-clamped, using liquid nitrogen-cooled aluminium clamps. The times between decapitation and freeze-clamping of dorsal white muscle and lateral red muscle were 20 and 60 s, respectively. Frozen tissue slices (2 mm thick) were powdered in a mortar under liquid nitrogen with 3.5 vol of perchloric acid (8%, v/v) in ethanol (40%, v/v). After transferring to a centrifuge tube, the powder was homogenized with a high-speed mixer. After centrifugation of the homogenate for 20 min at 30,000***g***, the pellet was re-extracted with 2.5 vol of perchloric acid (6%, v/v). The combined supernatants were neutralized to pH 6.0 with 3 M potassium carbonate in 0.5 M triethanolamine. Finally, the extracts were centrifuged for 20 min at 30,000***g***.

The extracts contained the equivalent of about 200 mg of tissue ml^{-1}. A known quantity of an internal standard was added to the extracts, and after derivatization, 100 μl was directly injected onto the chromatograph.

To check whether the transfer of the free amino-acids from the tissues to the extracts was quantitative, recovery experiments of the extraction method were carried out.

Derivatization

The derivatization procedure was as follows: 10 μl of the standard amino-acid solution, or muscle extract, were mixed with 20 μl of the tetraborate buffer and 20 μl of the derivatization reagent solution and mixed in a vortex mixer for 1 min. Immediately after derivatization, the sample was injected onto the chromatograph.

Results and Discussion

Chromatography and detection

The separation of the majority of the free, proteinaceous amino-acids as their OPA derivatives is shown in Fig. 1. Each of the peaks represents the injection of 5 nmol and as can be seen, the selectivity of the system is sufficient. The detection limits of this procedure (UV) are between 50–250 pmol, resulting in determination limits of the amino-acid derivatives in goldfish muscle extracts of better than 100 nmol g^{-1} (Fig. 2). Figure 3 shows the separation of derivatized, free amino-acids from the white muscle of goldfish.

Using absorbance detection, the response of the derivatives can be monitored at several wavelengths (e.g. 320, 340 and 365 nm). However, a wavelength of 365 nm gave the best compromise between sensitivity and selectivity, because at this relatively high wavelength the absorbance of the matrix was much decreased.

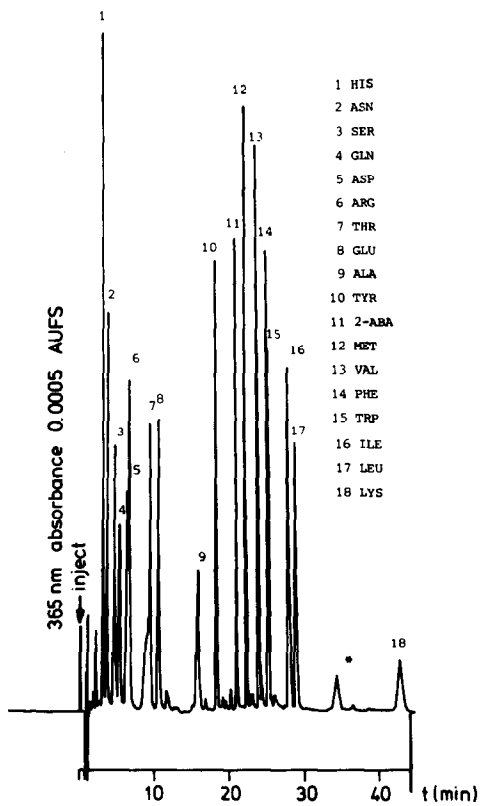

Figure 1
Gradient separation of 5 nmol of OPA amino-acid derivatives on a 3-μm Ultrasphere ODS column (75 × 4.6 mm, i.d.). *, Unknown component. Peak 7 represents two co-eluting amino-acids: Gly and Thr.

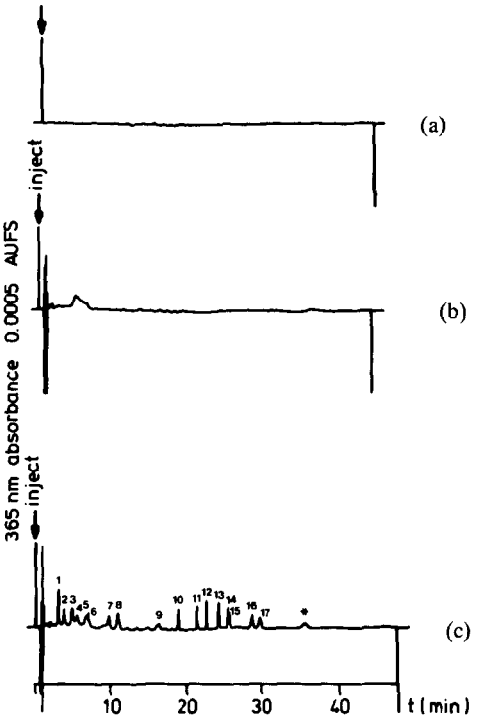

Figure 2
(a) Gradient-run without injection of amino-acid derivatives. (b) Blank — gradient-run after derivatization of water. (c) Gradient separation of 250 pmol of OPA amino-acid derivatives on a 3-μm Ultrasphere ODS column (75 × 4.6 mm, i.d.). Peak numbers as indicated in Fig. 1. *, Unknown component. Peak 7 represents two co-eluting amino acids: Gly and Thr.

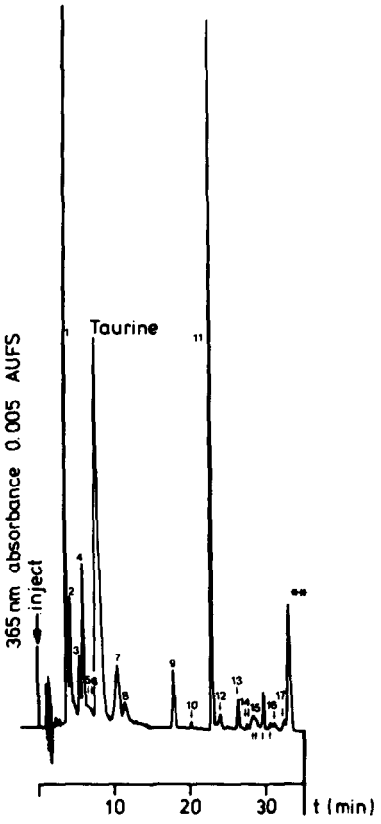

Figure 3
Gradient separation of OPA derivatized, free amino-acids from goldfish epaxial white muscle. Peak numbers as indicated in Fig. 1. **, Second internal standard; ε-amino-*n*-caproic acid. Unknown components are indicated by arrows facing up. Peak 7 represents two co-eluting amino-acids: Gly and Thr.

Thermostatting of the system was necessary in order to obtain a reproducible and stable baseline (Fig. 2a) and the results at 30°C were significantly better than those at 20°C. The total analysis time could be decreased by using a flow rate of 0.8 ml min^{-1} instead of 0.6 ml min^{-1} and, in spite of a somewhat higher backpressure in the system, about the same selectivity was achieved. Due to the limited stability of the amino-acid derivatives formed, a constant column equilibration time was necessary to obtain reproducible results.

The use of guard and/or scavenger columns was not necessary because the applied sample denaturation procedure (perchloric acid precipitation) was highly efficient; and over 200 samples could be analysed with the same analytical column without any significant loss in selectivity and reproducibility.

The only co-eluting derivatives in the system were the glycine (Gly) and threonine (Thr) derivatives and in spite of the addition of 1% of tetrahydrofuran, as repeatedly suggested by other investigators [10, 22], no baseline separation between these two derivatives could be achieved. The use of other concentrations of tetrahydrofuran to separate these products was not investigated. In the chromatograms (Figs 1–3) these two derivatives are indicated as peak 7 (Thr). Co-elution of OPA–Gly did not result in a considerable increase of the peak height of OPA–Thr because of the low absorbance of OPA–Gly (67.6% of the OPA–Thr response).

2-ABA was chosen as an internal standard because it forms a strongly absorbing OPA-adduct and it has a baseline resolution with all of the amino-acids in the system.

Derivatization

Some of the amino-acids give a poor yield of the light-absorbing OPA adduct, mainly due to the instability of the corresponding derivatives. The most unstable are the Gly- and lysine (Lys)-adducts [23, 24].

Many standard procedures to prepare OPA derivatives of amino-acids are based on short reaction times of approximately 1 min. In general, the isoindole adducts are unstable and susceptible to an enhanced degradation rate caused by the presence of excess OPA remaining in solution [26, 27]. In the present investigation, maximal sensitivity was achieved with a 15 s reaction time; sensitivity was not compromised when reaction times up to 4 min were used, as can be seen in Table 1.

The secondary amino-acids proline and cysteine cannot be detected using OPA as the derivatization reagent. Only after oxidation of these amino-acids can they react with OPA [25]. Oxidation of these amino-acids in the standard mixture or in the tissue extracts was not carried out in the present study because this would result in a rather long sample pre-treatment procedure and would abolish the advantage of developing a simple and fast LC procedure for the analysis of free amino-acids. Furthermore, quantification of these secondary amino-acids did not seem essential for the phenomena being studied.

Analytical variables

In order to evaluate the developed procedure, some relevant parameters were calculated, including the reproducibility of retention times and absorbance signal, as well as the linearity of the method.

Reproducibility. Calculation of the reproducibility of the retention times was based on five consecutive runs; the absolute retention times had a mean relative standard deviation (RSD) of 0.63% ± 0.14% (Table 2). Even for Lys, with a retention time of

Table 1
Peak areas (in arbitrary units) and means ±SD of 19 amino-acids with different derivatization times

	Derivatization time					
	15 s	30 s	1 min	2 min	3 min	4 min
His	5.688	5.609	5.579	5.650	5.751	5.681
Asn	5.638	5.636	5.609	5.646	5.731	5.716
Ser	4.815	4.760	4.718	4.721	4.738	4.753
Gln	4.532	4.509	4.409	4.455	4.392	4.440
Asp	5.021	5.177	5.116	5.238	5.183	5.349
Arg	4.542	4.537	4.528	4.572	4.614	4.640
Gly	3.467	3.359	3.363	3.385	3.376	3.388
Thr	5.126	4.966	4.971	5.003	4.992	5.008
Glu	5.676	5.625	5.645	5.667	5.787	5.792
Ala	4.838	4.650	4.643	4.559	4.592	4.563
Tyr	5.156	5.107	5.104	5.148	5.240	5.276
IS	5.742	5.686	5.688	5.668	5.726	5.710
Met	5.692	5.663	5.712	5.728	5.818	5.792
Val	6.295	6.207	6.359	6.283	6.372	6.293
Phe	5.207	5.256	5.162	5.184	5.233	5.220
Trp	4.538	4.520	4.528	4.489	4.539	4.520
Ile	6.720	6.658	6.701	6.734	6.704	6.717
Leu	5.580	5.536	5.549	5.597	5.631	5.607
Lys	5.726	5.537	5.084	4.596	4.110	4.174
Means	5.263	5.210	5.183	5.175	5.186	5.192
±SD	0.734	0.734	0.755	0.767	0.816	0.799

Table 2
Retention times (means ±SD) and RSD

	Absolute retention time (s)	RSD (%)
His	233.8 ± 1.6	0.68
Asn	248.0 ± 1.4	0.56
Ser	315.0 ± 1.9	0.60
Gln	358.4 ± 1.8	0.50
Asp	393.0 ± 3.3	0.84
Thr	626.4 ± 2.7	0.43
Glu	685.8 ± 3.1	0.45
Ala	1069.4 ± 4.3	0.40
Tyr	1214.6 ± 7.2	0.59
IS	1374.6 ± 8.4	0.61
Met	1449.4 ± 8.4	0.60
Val	1554.4 ± 9.9	0.64
Phe	1625.2 ± 11.4	0.70
Trp	1644.8 ± 11.7	0.71
Ile	1816.4 ± 13.9	0.77
Leu	1873.8 ± 14.5	0.77
Lys	2814.2 ± 24.9	0.88

Mean = 0.63; $n = 5$.

about 40 min, the RSD was <1%. This high reproducibility is probably due to the standardization of the entire derivatization and chromatographic procedure.

The within-day reproducibility of the total procedure (measuring peak areas and subsequently calculating concentrations) was measured by the analysis of five 1.25 mM samples of the 19 individual amino-acids. The mean RSD was 1.8%. The day-to-day

reproducibility of the procedure was measured by the analysis of the same solutions on five consecutive days and the mean RSD was 3.8%. These data are shown in Table 3.

Linearity. The calibration curves of all of the amino-acids showed good linearity and the correlation coefficients (r) ranged from 0.9804 for OPA–Arg to 0.9997 for OPA-Trp. Correlation coefficients of all the amino-acids are shown in Table 4. Also included in this table are the slopes and intercepts of the equations for the calibration of all the amino-acids.

Table 3
Reproducibility of the amino-acid quantification. Means (±SD) and RSD of five runs (injected concentration 1.25 mM) on 1 day (A) and five runs on 5 consecutive days (B)

	A	RSD	B	RSD
His	1.24 ± 0.02	1.6	1.25 ± 0.04	3.2
Asn	1.25 ± 0.02	1.6	1.25 ± 0.05	4.0
Ser	1.25 ± 0.06	4.8	1.25 ± 0.06	4.8
Gln	1.25 ± 0.02	1.6	1.25 ± 0.06	4.8
Glu	1.25 ± 0.03	2.4	1.26 ± 0.04	3.2
Ala	1.25 ± 0.01	0.8	1.26 ± 0.11	8.7
Tyr	1.24 ± 0.01	0.8	1.25 ± 0.03	2.4
Met	1.25 ± 0.01	0.8	1.25 ± 0.02	1.6
Val	1.24 ± 0.01	0.8	1.25 ± 0.02	1.6
Phe	1.26 ± 0.02	1.6	1.25 ± 0.05	4.0
Trp	1.25 ± 0.01	0.8	1.26 ± 0.06	4.8
Ile	1.25 ± 0.01	0.8	1.25 ± 0.03	2.4
Leu	1.25 ± 0.01	0.8	1.25 ± 0.04	3.2
Lys	1.31 ± 0.07	5.3	1.25 ± 0.06	4.8

Means: A, 1.8%; B, 3.8%.

Table 4
Linear-regression data for calibration curves. Slopes, y-intercepts and correlation coefficients (r) of all the amino-acids after analysing five samples in duplicate

	Slope	y-Intercept	r
His	4.2	36.7	0.9851
Asn	3.9	11.4	0.9956
Ser	3.1	13.7	0.9916
Gln	2.3	6.3	0.9907
Asp	3.1	5.7	0.9990
Arg	4.1	12.6	0.9804
Gly	2.5	4.3	0.9919
Thr	3.8	4.5	0.9944
Glu	4.9	11.3	0.9970
Ala	3.4	9.5	0.9947
Tyr	4.2	14.6	0.9972
Met	4.7	14.3	0.9978
Val	5.3	17.1	0.9970
Phe	4.6	14.0	0.9978
Trp	3.4	8.7	0.9997
Ile	5.7	11.5	0.9982
Leu	4.8	5.1	0.9989
Tau	3.5	7.3	0.9988

Application of free amino-acid analysis on goldfish muscle tissues

The LC procedure described was used to quantify the free amino-acids in white and red muscle of the teleost fish *Carassius auratus* L. (goldfish).

The results of these measurements are depicted in Table 5, where the amounts of 19 free amino-acids (in μmol g^{-1} of wet weight tissue) are presented. The figures represent the means, and the corresponding standard deviations (SD), of five separate runs. Significant differences in amounts of amino acids between both muscle types are indicated by the arrows. No correction is made in this table for the recoveries of the individual amino-acids in the extraction procedure. Based on five determinations, these recoveries were calculated for a mixture of 12 amino-acids; all the amino-acids in this mixture had the same concentration so that the recoveries of co-eluting Gly and Thr could be determined from their known individual UV-responses. The results (means ±SD) are: His, 82 ± 8%; Asn, 36 ± 2%; Ser, 82 ± 1%; Gln, 60 ± 5%; Glu, 44 ± 2%; Ala, 71 ± 3%; Met, 65 ± 1%; Val, 53 ± 2%; Ile, 54 ± 2%; Leu, 77 ± 1%; Gly, 92 ± 20%; Thr, 65 ± 5%.

In the present paper the amounts of Gly and Thr in goldfish muscles are based on estimations of peak areas in the chromatograms. A Gly/Thr ratio can be calculated from these amounts. In goldfish white and red muscles these ratios are 2.5 and 0.8, respectively. For white and red muscles as a whole, the Gly/Thr ratio is 1.7. The values of the Gly/Thr ratio calculated from data previously described in the literature, agree well with those just stated. Thus the ratio of Gly/Thr for anchovy (*Engraulis japonicus*) white muscle is 1.1, and for red muscle, 0.6 [36]; in that study amino-acids were determined by chromatography on Amberlite CG-120 columns. In four species of Pacific salmon (*Oncorhynchus nerka*, *O. tshawytscha*, *O. kisutch* and *O. gorbuscha*) a mean value of 2.1 for the Gly/Thr ratio can be calculated [37], depending on the fish species, sex, and sampling location in the muscle (muscle type not specified); in that work [37] an

Table 5
Amounts of free amino-acids in white and red muscle of goldfish (*C. auratus* L.) in μmol g^{-1} wet wt. Means ±SDs ($n = 5$). Significant differences between both muscle types are indicated by arrows (Student's *t*-test)

	White muscle		Red muscle	
His	15.36 ± 2.41	→	11.62 ± 2.64	$P < 0.025$
Asn	0.80 ± 0.41		0.92 ± 0.28	
Ser	0.42 ± 0.16		0.44 ± 0.11	
Gln	1.92 ± 0.59		1.54 ± 0.38	
Asp	0.14 ± 0.05	→	0.24 ± 0.09	$P < 0.025$
Arg	<0.1		<0.1	
Tau	21.46 ± 3.10	→	28.46 ± 2.47	$P < 0.005$
Gly	2.74 ± 0.29	→	0.72 ± 0.35	$P < 0.005$
Thr	1.08 ± 0.23		0.94 ± 0.23	
Glu	0.72 ± 0.11	→	1.84 ± 0.31	$P < 0.005$
Ala	1.18 ± 0.62		0.78 ± 0.37	
Tyr	<0.1		<0.1	
Met	0.14 ± 0.09		<0.1	
Val	0.18 ± 0.04	→	0.12 ± 0.04	$P < 0.05$
Phe	<0.1		<0.1	
Trp	<0.1		<0.1	
Ile	0.14 ± 0.05	→	0.22 ± 0.04	$P < 0.025$
Leu	0.22 ± 0.04		0.18 ± 0.04	
Lys	2.30 ± 0.72		1.92 ± 0.43	

automatic amino-acid analyser was used to measure the amounts of free amino-acids. The Gly/Thr ratio in mackerel (*Scomber japonicus*) and yellowtail (*Seriola quinqueradiata*) white and red muscle can be calculated from published data [38]. In mackerel, the white muscle Gly/Thr ratio is 1.3, and in red muscle the Gly/Thr ratio is 1.4. For yellowtail these ratios are 1.1 and 0.8, respectively; a modified automatic amino-acid analyser was used in that study.

As can be seen from Table 5, both muscle types contain the same free amino-acids. The white muscle was significantly richer in histidine (His), Gly and valine (Val), whereas the red muscle was significantly richer in taurine (Tau), glutamic (Glu) and aspartic (Asp) acid, and isoleucine (Ile).

The conclusion may be that His and Tau are the dominant free amino-acids in both muscle types of the goldfish. The contents of His are up to 15 μmol g^{-1} in the white muscle and up to 12 μmol g^{-1} in the red muscle. About twice as much Tau is present in both muscle types: 22 μmol g^{-1} in white muscle and 29 μmol g^{-1} in red muscle.

The large amounts of His in carp muscles may serve for several purposes; the most important seems to be buffering of protons that are generated during bursts of swimming activity [31, 32]. This is of special importance in anaerobically-working white muscle, being a glycolytic tissue [33, 34]. Indeed, goldfish white muscle is shown in the present study to have significantly larger amounts of free His than has red muscle.

The other quantitatively important free amino-acid in both muscle types is Tau. This non-proteinaceous amino-acid derivative is a sulphonate-containing product of methionine (Met) metabolism [28]. The only adequately documented metabolic reaction in which Tau participates is conjugation with bile acids in the liver of mammals [29]. Therefore, the large amounts of this amino-acid found in both muscle types of goldfish is particularly intriguing. Because Tau biosynthesis cannot be observed in fish, it is probably taken up by the fish with the food [30]. The Tau content of the food was found to be 4 mM kg^{-1}. This amount would be sufficient to explain the high levels found in both muscle types. Recently, the biochemistry of Tau, its rôle in nutrition and disease, and its biological functions were reviewed [35].

Conclusions

The conclusion is that the RPLC procedure described allows the quantification of most of the proteinaceous, free amino-acids with sufficient selectivity, sensitivity and reproducibility. Furthermore, the method is rapid, easy to perform and only requires basic LC instrumentation. Application of the method in establishing the type and amounts of free amino-acids in different types of muscle tissue in a teleost fish resulted in accurate evaluation of these metabolites. A critical evaluation of all of the biochemical data will be presented in a separate study.

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