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Quantification of Histidine-Containing Dipeptides in Dolphin Serum Using a Reversed-Phase Ion-Pair High-Performance Liquid Chromatography Method

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Abstract: The quantification of histidine-containing dipeptides (anserine, carnosine, and balenine) in serum might be a diagnostic tool to assess the health condition of animals. In this study, an existing reversed-phase ion-pair high-performance liquid chromatography (HPLC)–ultraviolet detection method was improved and validated to quantify serum anserine, carnosine, and balenine levels in the dolphin. The serum was deproteinized with trichloroacetic acid and directly injected into the HPLC system. Chromatographic separation of the three histidine-containing dipeptides was achieved on a TSK–gel ODS-80Ts (4.6 mm × 150 mm, 5 µm) analytical column using a mobile phase of 50 mmol/L potassium dihydrogen phosphate (pH 3.4) containing 6 mmol/L 1-heptanesulfonic acid and acetonitrile (96:4). The standard curve ranged from 0.1 µmol/L to 250 µmol/L. The average accuracy of the intra- and inter-analysis of anserine, carnosine, and balenine was 97–106%. The relative standard deviations of total precision (RSDr) of anserine, carnosine, and balenine in dolphin serum were 5.9%, 4.1%, and 2.6%, respectively. The lower limit of quantification of these compounds was 0.11–0.21 µmol/L. These results indicate that the improved method is reliable and concise for the simultaneous determination of anserine, carnosine, and balenine in dolphin serum, and may be useful for evaluation of health conditions in dolphins. Furthermore, this method can also be applied to other biological samples.

Keywords: anserine; carnosine; balenine; HPLC; ion pair; UV; imidazole dipeptide; dolphin; serum

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1. Introduction

Carnosine (CAR) and its methylated analogs, anserine (ANS) and balenine (BAL), are representative histidine-containing dipeptides with an imidazole ring skeleton (Figure 1). These imidazole dipeptides (IDPs) are found in the skeletal muscles of a wide range of animal species [1]. In particular, it has been reported that marine mammals, unlike other mammals, have skeletal muscles rich in BAL [2,3]. These compounds appear to have many beneficial effects on physiological functions such as buffering [4], antioxidative activity [5–7], and advanced glycation end-product inhibitory activities [8] and, consequently, may reflect the health condition of animal species. We hypothesized that serum IDPs may be useful indicators of the health of aquarium dolphins and aimed to develop an analytical method to measure the IDP content in the serum of bottlenose dolphins.

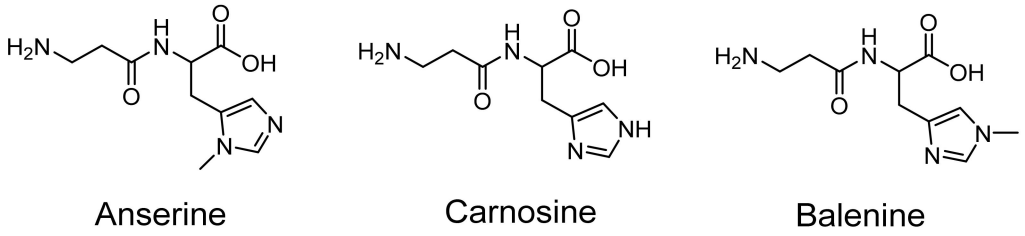

Figure 1. Chemical structures of anserine, carnosine, and balenine.

To quantify IDPs, a methodology involving high-performance liquid chromatography (HPLC) separations with *o*-phthalaldehyde post–column derivatization has been reported [9–12]. Furthermore, a sensitive and specific analytical method using liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS) for IDP analysis has also been reported [13,14]. However, these methods require utilized equipment that may not be readily available in all laboratories, such as derivatization systems, fluorescence detector, and mass spectrometry. Therefore, we aimed to develop a simple method that can be employed with a conventional device for routine analysis. HPLC, followed by ultraviolet (UV) detection, is often used for the analytical separation and detection of IDPs in foods and living organisms. For simple, non-derivative HPLC analysis of IDPs, the hydrophilic interaction chromatography (HILIC) mode [15–17] and reversed-phase (RP) mode [18–21] have both been applied. The HILIC mode is generally inferior in terms of separation and also more difficult to handle than the RP mode. Furthermore, existing HILIC–HPLC–UV methods lack sensitivity to measure trace amounts of anserine in dolphin serum samples. Thus, we aimed to develop a sensitive method that can rapidly separate ANS, CAR, and BAL using the RP–HPLC method. Using an ion-pair reagent is known to be effective for analyzing hydrophilic compounds with low retention in the RP mode. The method by Dunnett and Harris can analyze ANS and CAR in equine plasma samples using isocratic ion-pair RP–HPLC [21]. However, this method did not include BAL detection, which is the main IDP component of dolphin serum samples. To the best of our knowledge, there is no validated RP–HPLC–UV method that can rapidly quantify ANS, CAR, and BAL. In the present study, we report a simple, rapid, and reliable ion-pair RP–HPLC–UV method that can analyze ANS, CAR, and BAL in small volume dolphin serum samples. Furthermore, we examined the applicability of the developed method to other biological samples.

2. Materials and Methods

2.1. Chemicals

L-Carnosine (99.1% purity) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). L-Anserine nitrate (99.0% purity) and potassium dihydrogen phosphate were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). L-Balenine (99.5% purity) was purchased from Hamari Chemicals, Ltd. (Osaka, Japan). 1-Heptanesulfonic acid sodium salt and trichloroacetic acid were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). HPLC-grade acetonitrile was obtained from Merck (Darmstadt, Germany).

2.2. Standard Preparation

Ten millimolar stock standard solutions of CAR (2.26 mg/mL) and BAL (2.40 mg/mL) and a 5 mmol/L ANS stock solution (anserine nitrate; 1.52 mg/mL) were prepared by dissolving the peptides in 10 mL of ultrapure water. These stock standard solutions were mixed and the final concentrations used were 0.10–250 µmol/L for calibration standards. These solutions were stored at −20 °C when not being used.

2.3. Instrumentation

The HPLC system comprised a PU-4180 pump, CO-4060 column oven, UV-4075 UV/Vis detector (JASCO Corporation, Tokyo, Japan), and a 7725i Rheodyne-sampling detector connected to a 20 µL sample loop (Rheodyne, Rohnet Park, CA, USA). ChromNAV Lite software (JASCO Corporation, Tokyo, Japan) was used for data analysis.

2.4. Biological Samples

This experiment using captive dolphins was conducted with the approval of the Research Ethics Committee at the Kagoshima City Aquarium (Approval number 6, 17 November 2020). Blood was collected from dorsal or ventral fin veins in 1 male and 7 female clinically healthy bottleneck dolphins (*Tursiops truncatus*), aged 2 to approximately 30 years they were kept in the Kagoshima City Aquarium. Serum was separated by centrifugation and stored at −80 °C until use. Meat samples including beef thigh (*Bos taurus*), chicken breast (*Gallus gallus domesticus*), pork loin (*Sus scrofa domesticus*), and skipjack tuna (*Katsuwonus pelamis*) were purchased from a supermarket in Kagoshima, Japan.

2.5. Sample Preparation

For the serum analysis, 10 µL of 50% trichloroacetic acid (TCA) was added to 100 µL of serum and vortexed. Next, samples were centrifuged at 12,000 rpm (rt, 3 min), supernatant was filtered and 10 µL of solution was injected onto the HPLC column. For the meat sample analysis, minced samples (0.2 g) were collected in 1.5 mL microtubes and 1.0 mL of 5% TCA was added followed by stirring and extraction using a rotator at room temperature for 10 min (30 rpm). After extraction, the mixture was centrifuged (12,000 rpm, rt, 3 min) and the supernatant was collected in a volumetric flask. This operation was repeated 3 times and the combined test solution was filled up in volume and filtered to prepare an injection sample. A dilution step was performed as needed.

2.6. HPLC Condition

Chromatography was performed on a TSKgel ODS–80Ts column (4.6 mm × 150 mm, 5 µm, Tosoh Corporation, Tokyo, Japan) protected by a guard column (TCI OPTI–GUARD Fit ODS, TCI, Tokyo, Japan). Isocratic elution was carried out using 50 mmol/L potassium dihydrogen phosphate (KH_2PO_4; pH 3.4) containing 6 mmol/L 1-heptanesulfonic acid and acetonitrile (96:4, v/v) at a flow rate of 1.0 mL/min. The column temperature was set to 45 °C. Detection was conducted at a wavelength of 210 nm. After 13 min, the mobile phase was shifted to 50 mmol/L potassium dihydrogen phosphate (pH 3.4) containing 6 mmol/L 1-heptanesulfonic acid and acetonitrile (60:40, v/v) for 5 min to wash the column. The system was then equilibrated again for 17 min to return to the original mobile phase for a total run time of 35 min.

2.7. Linearity of Calibration, Limit of Detection (LOD) and Lower Limit of Quantification (LOQ)

The linearity was evaluated through the coefficient of determination (r^2), interception (y), and slope (s) of the regression line of standard solution (0.10–250 µmol/L). The LOD and LOQ of this method were expressed as: LOD = 3.3 σ/s, LOQ = 10 σ/s (where σ is the standard deviation of 0.10 µmol/L IDPs standard solution (n = 7) injected on different days and s is the calibration curve slope).

2.8. Accuracy

To evaluate accuracy, 10 µL of 0, 20, 200, and 500 µmol/L IDP solution was spiked into 80 µL of the pooled dolphin serum, followed by the addition of 10 µL of 50% TCA solution. The solutions were then mixed by vortexing, centrifuged at 12,000 rpm (rt, 3 min), and the supernatant was filtered and injected into the HPLC column. Accuracy was calculated using Equation (1) [22], and carried out using intra- and inter-day assays.

$$Accuracy\ (\%) = \frac{(Measured\ concentration\ of\ spiked\ sample - endogenous\ concentration)}{Nominal\ concentration} \times 100 \quad (1)$$

2.9. Precision

Precision was evaluated using the relative standard deviation of total precision (RSDr), which was calculated from the intra-day precision and inter-day precision of the pooled dolphin serum ($n = 2, 5$ days). The sample preparation and analytical methods are described above. One-way analysis of variance (ANOVA) was performed for 5 groups and, based on the results of ANOVA, RSDr was estimated using Equations (2) and (3):

$$RSDr\ (\%) = \frac{Total\ precision}{Mean\ of\ IDP\ value} \times 100 \quad (2)$$

$$Total\ precision = \sqrt{(Inter-day\ precision)^2 + (Intra-day\ precision)^2} \quad (3)$$

2.10. Statistical Analysis

ANOVA was performed using Microsoft Excel software (Microsoft, Redmond, WA, USA).

3. Results and Discussion

3.1. Separation Conditions

First, the mobile phase conditions postulated by Dunnett et al. [21] were examined; however, CAR and BAL could not be separated under these conditions (data not shown). On the other hand, the use of a 30 mmol/L KH_2PO_4 (pH 3.4) containing 10 mmol/L 1-heptanesulfonic acid and 50 mmol/L KH_2PO_4 (pH 3.4) containing 6 mmol/L 1-heptanesulfonic acid mobile phase enabled the separation of CAR and BAL [20]. However, this method requires a long analytical time (>60 min) and has not been validated for IDP analysis. Therefore, we modified this condition and succeeded in separating ANS, CAR, and BAL within 11 min using 50 mmol/L KH_2PO_4 (pH 3.4) and 6 mmol/L 1-heptanesulfonic acid containing 4% acetonitrile. The chromatogram (Figure 2) showed good separation of the 3 IDPs. Table 1 shows the retention time (t_R), capacity factor (k'), resolution factor (Rs), and separation factor (α) for this method. Rs values of 4.284 and 1.704 indicate that the method could separate ANS, CAR, and BAL. Furthermore, no significant interfering peaks were observed on the retention time of ANS, CAR, and BAL in the pooled dolphin serum (Figure 3). To the best of our knowledge, this is most rapid method to separate ANS, CAR, and BAL using RP–ion-pair–HPLC.

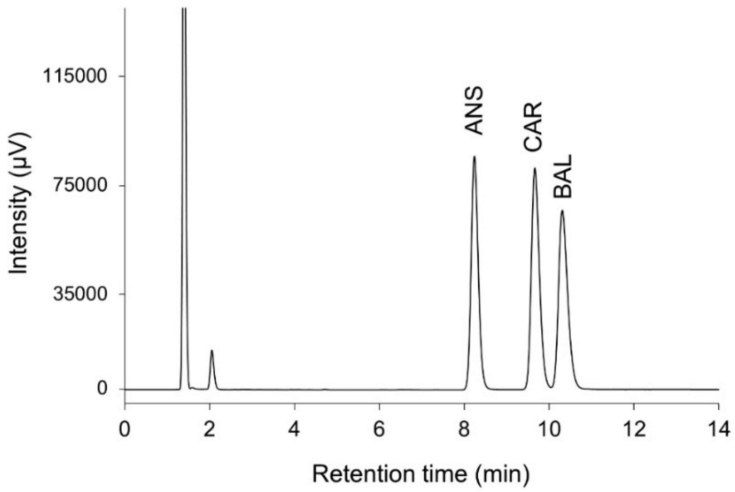

Figure 2. Reversed-phase ion-pair HPLC chromatogram of a mixed standard containing anserine (ANS), carnosine (CAR), and balenine (BAL).

Table 1. Chromatographic parameters of a mixed standard containing anserine (ANS), carnosine (CAR), and balenine (BAL) determined via reversed-phase ion-pair HPLC.

Analyte	t_R (min)	k'	Rs	α
ANS	8.24	4.887	–	–
CAR	9.67	5.905	4.284	1.208
BAL	10.31	6.367	1.704	1.078

IDP standard solution (250 µmol/L) was injected into the RP–HPLC instrument using the method described in this study (n = 5). Retention time (t_R), capacity factor (k'), resolution factor (Rs), and separation factor (α).

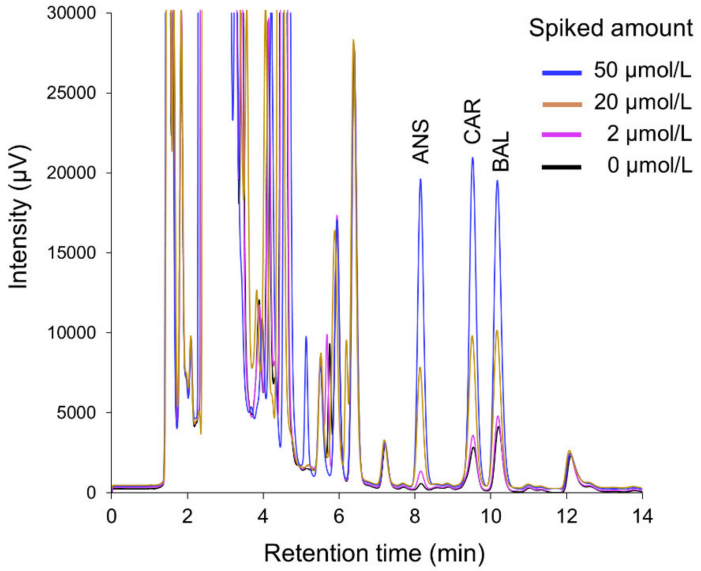

Figure 3. Representative chromatograms of 0, 2, 20, and 50 µmol/L IDP spiked dolphin serum using the developed analytical method.

3.2. Calibration Curves, LOD, and LOQ

The linearity of the IDPs observed with our method is reflected in the calibration curve parameters presented in Table 2. The coefficients of determination for the regression equations were >0.9999. The LOD and LOQ were determined from the results of repeated analysis of standard solutions near the LOQ (0.10 µmol/L). The LODs of ANS, CAR, and BAL were calculated to 0.04, 0.07, and 0.04. The LOQs of ANS, CAR, and BAL were also evaluated as 0.11 µmol/L, 0.21 µmol/L, and 0.12 µmol/L, respectively (Table 2). These LOQs (ANS, 26 ng/mL; CAR, 48 ng/mL; BAL, 30 ng/mL) were lower than those previously reported for the HILIC–HPLC method [15,16]. The quantification values of IDPs in all dolphin sera analyzed in this study exceeded these LOQ values. These results suggested that this method was sensitive enough to measure IDPs in dolphin serum.

Table 2. Calibration curves, LOD, and LOQ of the IDPs.

Analyte	Linearity [a]	Linear Range (µmol/L)	LOD (µmol/L)	LOQ (µmol/L)
ANS	$y = 3938x - 880$ ($r^2 = 0.9999$)	0.10–250	0.04	0.11
CAR	$y = 4220x - 385$ ($r^2 = 0.9999$)	0.10–250	0.07	0.21
BAL	$y = 3839x - 357$ ($r^2 = 0.9999$)	0.10–250	0.04	0.12

[a] y = peak area, x = analyte concentration (µmol/L).

3.3. Accuracy and Precision

The accuracy of this method was evaluated using the spiked samples. The chromatogram of the spiked samples shows good separation and no extraction inhibition of ANS, CAR, and BAL (Figure 3). The quantification results (Intra-day assay) of endogenous concentration of ANS, CAR, and BAL were 1.1, 9.3, and 14.6 µmol/L (Table 3). Inter-day assay results of endogenous ANS, CAR, and BAL were 1.1, 9.6, and 14.4 µmol/L, respectively (Table 4). The accuracy of spiked final dolphin serum extract (2–50 µmol/L) was 97–106% in the intra- and inter-day assays (Tables 3 and 4). The precision of the spiked final dolphin serum extract was 0.2–4.9% RSD in the intra- and inter-day assays (Tables 3 and 4). These results suggested high accuracy and high reproducibility in both the intra and inter assays. Furthermore, we determined the precision of this method by repeated analysis of dolphin serum over 5 days in duplicate analyses (Table 5). ANOVA was performed on the five groups, with the data obtained for each day as a single group. The RSDr was calculated from the ANOVA results. The RSDr values of ANS, CAR, and BAL were 5.9%, 4.1%, and 2.6%, respectively. These results suggested that the accuracy and precision of this method were sufficient for dolphin serum analysis.

Table 3. Intra-day assay accuracy of anserine (ANS), carnosine (CAR), and balenine (BAL) in spiked final dolphin serum extract (n = 5) [a].

Analyte	Spiked Final Dolphin Serum Extract			
	Added Amount (µmol/L)	Measured Amount (µmol/L) [b]	Accuracy (%)	RSD (%)
ANS	0	1.1 ± 0.0	–	1.9
	2	3.0 ± 0.1	97	2.0
	20	21.6 ± 0.3	103	1.4
	50	53.5 ± 1.4	105	2.7
CAR	0	9.3 ± 0.2	–	2.2
	2	11.4 ± 0.1	101	1.1
	20	30.0 ± 0.2	103	0.8
	50	61.4 ± 1.3	104	2.2
BAL	0	14.6 ± 0.1	–	0.6
	2	16.7 ± 0.1	104	0.6
	20	35.2 ± 0.4	103	1.1
	50	67.1 ± 1.1	105	1.6

[a] The values are expressed as µmol/L of the final analyte concentration. Each value corresponds to 5 replicates at each concentration level. The 5 analyses were conducted on the same day. [b] Values are the mean ± SD.

Table 4. Inter-day assay accuracy of anserine (ANS), carnosine (CAR), and balenine (BAL) in spiked final dolphin serum extract (n = 5) [a].

Analyte	Spiked Final Dolphin Serum Extract			
	Added Amount (µmol/L)	Measured Amount (µmol/L) [b]	Accuracy (%)	RSD (%)
ANS	0	1.1 ± 0.1	–	4.9
	2	3.1 ± 0.1	101	2.0
	20	21.5 ± 0.2	102	1.1
	50	54.0 ± 1.1	106	2.1
CAR	0	9.6 ± 0.2	–	2.3
	2	11.6 ± 0.3	100	2.2
	20	30.0 ± 0.3	102	1.1
	50	62.2 ± 1.1	105	1.7
BAL	0	14.4 ± 0.4	–	3.1
	2	16.5 ± 0.4	104	2.3
	20	34.7 ± 0.1	102	0.2
	50	67.3 ± 1.4	106	2.1

[a] The values are expressed as µmol/L of the final analyte concentration. Each value corresponds to 5 replicates at each concentration level. The 5 analyses were conducted on five different days. [b] Values are the mean ± SD.

Table 5. Precision results of the developed analytical method.

	Dolphin Serum (n = 10)	
	Concentration (μmol/L) [a]	**RSDr (%)**
ANS	1.4 ± 0.1	5.9
CAR	12.0 ± 0.5	4.1
BAL	18.4 ± 0.5	2.6

[a] Values are the mean ± SD.

3.4. Stability

The stabilities of the IDPs are listed in Table 6. ANS, CAR, and BAL in the dolphin serum were stable after three freeze/thaw cycles. It has been reported that carnosinase is present in the tissue and serum of mammals [23], which could affect the stability and concentration of dipeptides in serum; however, the IDP content in the samples was not affected after the three freeze/thaw cycles in this study. The ANS, CAR, and BAL contents of the samples treated with 5% TCA did not change, even after storage at 4 °C for 72 h. These results indicate that the dolphin serum was stable under our conditions and that deproteinization allows analysis with an autosampler.

Table 6. Stability of IDPs in dolphin serum.

Conditions	Dolphin Serum (n = 3)			
	Analytes	**Before Stability Test (A) (μmol/L) [a]**	**After Stability Test (B) (μmol/L) [a]**	**B/A**
Freeze/Thaw cycles (Three times)	ANS	2.0 ± 0.0	2.0 ± 0.0	0.97
	CAR	15.1 ± 0.1	15.0 ± 0.2	1.00
	BAL	27.9 ± 0.3	27.9 ± 0.3	1.00
Final Solution72 h (4 °C)	ANS	2.0 ± 0.0	2.1 ± 0.1	1.03
	CAR	15.1 ± 0.1	15.1 ± 0.3	1.00
	BAL	27.9 ± 0.3	28.1 ± 0.4	1.01

[a] Values are the mean ± SD.

3.5. Application to Dolphin Serum Samples

This method has been used for the hematological examination of dolphin samples. Serum samples from eight clinically healthy bottleneck dolphins were analyzed. The analysis revealed that the dolphin serum had a small amount (0.3–2.2 μmol/L) of ANS, a moderate amount (1.7–17.9 μmol/L) of CAR, and a large amount (15.1–29.5 μmol/L) of BAL (Figure 4). The mean ± standard deviation was 1.1 ± 0.6 μmol/L in ANS, 8.3 ± 4.8 μmol/L in CAR, and 22.5 ± 5.4 μmol/L in BAL. It is known that dolphin muscle tissue has the highest amount of BAL among the three IDPs [24]. Although concentrations of 3 IDPs in dolphin serum varied by individual condition, BAL was the main component of all dolphin sera, similar to muscle tissue results. To our knowledge, this is the first report to reveal the concentration of IDPs in dolphin serum. Studies focusing on the composition of dolphin diets and how the concentrations of these IDPs change when the diets change or dolphins are suffering from a disease are currently ongoing. The method reported in this study is useful for monitoring the health conditions of dolphins and may provide important insights into the distribution and role of IDPs, especially that of BAL, in marine mammals.

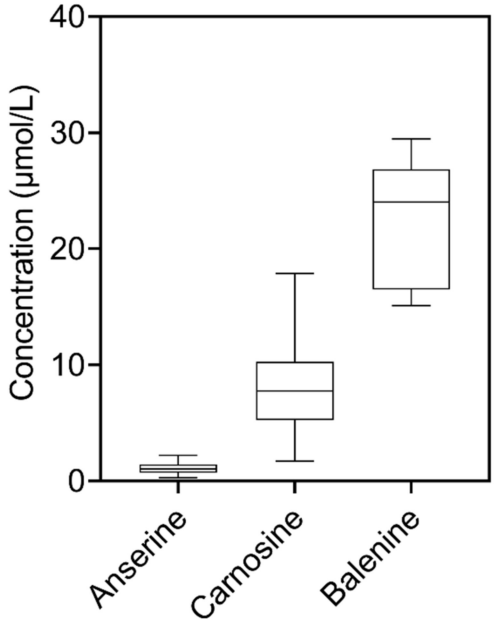

Figure 4. Concentrations of IDPs in the serum of bottleneck dolphins under clinically healthy conditions in an aquarium (n = 8). The horizontal line within the box represents the median value. The whiskers extend to the minimum and the maximum value.

3.6. Application to Other Biological Samples

To confirm the applicability of this method to other biological samples, the IDP content of beef thigh, chicken breast, pork loin, and skipjack tuna (dark muscle and ordinary muscle) was evaluated (Table 7). Beef and pork samples had a large amount of CAR (3.39 and 6.86 mg/g, respectively). On the other hand, the main content of chicken breast and skipjack tuna was ANS (5.65 and 3.20 mg/g, respectively). A small amount of BAL was found in beef thigh, chicken breast, and pork loin (0.02–0.40 mg/g). Some reports have shown that meat species can be distinguished by the ratio of CAR/ANS and the ratio of BAL/ANS [11,12,25]. In this study, the CAR/ANS ratios of beef, chicken, and pork were 4.5, 0.19, and 26, respectively, and the BAL/ANS ratios of beef, chicken, and pork were 0.030, 0.025, and 1.5, respectively. These data were almost similar to those previously reported ratios of three meat species [11,25]. The ANS content of ordinary muscle in skipjack tuna was higher than that in dark muscle. IDPs quantification results of the above five samples were consistent with previously reported data [11,14–16]. Therefore, although the sample matrix effect needs to be considered individually, this method can be used to quantify the IDPs in various biological samples. In addition, analysis of these samples did not show a peak after 20 min on the chromatogram, allowing us to eliminate the column wash process. This method could analyze these samples in 15 min.

Table 7. Quantitative analytical results of IDPs in beef, chicken, pork, and skipjack tuna [a].

	ANS	CAR	BAL
Beef Thigh	0.75 ± 0.01	3.39 ± 0.09	0.02 ± 0.00
Chicken Breast	5.65 ± 0.03	1.05 ± 0.00	0.14 ± 0.00
Pork Loin	0.26 ± 0.00	6.86 ± 0.02	0.40 ± 0.00
Skipjack Tuna (Dark Muscle)	0.53 ± 0.05	0.02 ± 0.00	nd
Skipjack Tuna (OrdinarMuscle)	3.20 ± 0.06	0.17 ± 0.01	nd

[a] Each value represents the mean ± SD (mg/g of wet meat) of three samples from the same meat cut. nd = not determined.

4. Conclusions

An existing RP–HPLC–UV method was improved and validated in terms of LOQ, accuracy, and precision to quantify ANS, CAR, and BAL in aquarium dolphin serum.

The LOQ and accuracy range were adequate for the quantification of IDPs in dolphin serum. The advantages of this method are sensitivity, rapid analysis time (dolphin serum, 35 min; meat samples, 15 min) and the small sample volume required. This method was successfully used to determine the IDPs in dolphin serum and could therefore be useful for monitoring dolphins' health conditions in aquariums. Moreover, this method can be applied to other biological samples.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the research ethics committee of the Kagoshima City Aquarium (Approval number 6, 17 November 2020).

Data Availability Statement: All data are contained within the article.

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Conflicts of Interest: The authors declare no conflict of interest.

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