Analytical Methods

ROYAL SOCIETY OF CHEMISTRY

TECHNICAL NOTE

View Article Online
View Journal | View Issue

Cite this: *Anal. Methods*, 2023, **15**, 5901

Assessment of collagen content in fish skin – development of a flow analysis method for hydroxyproline determination†

Maria M. P. Melo, Raquel B. R. Mesquita, Ezequiel R. Coscueta, Manuela E. Pintado and António O. S. S. Rangel *

This work describes the development of a flow injection method to determine hydroxyproline (HYP), one of collagen's most abundant amino acids. Collagen is a protein with several applications and high nutritional value. Evaluating the feasibility of using collagen from fish skin over its mammalian source is essential. The determination of HYP requires the pre-treatment and hydrolysis of the fish skin to break down collagen into its amino acids, and the HYP value quantified relates to the collagen content. The determination was based on the HYP oxidation with permanganate in an alkaline medium and the consequent decrease of colour intensity registered. Under optimal conditions, the developed method enables the determination of the HYP within the dynamic range of 23.8 to 500 mg L^{-1}, with a limit of detection (LOD) of 2.6 mg L^{-1} and a limit of quantification (LOQ) of 23.8 mg L^{-1}. Different samples were processed, and the digests were analysed by the proposed method and with the conventional procedure with good correlation (relative error < 7%). Moreover, the analyte quantification is performed faster, simpler, and more accurately, with less toxic solutions. The reproducibility of the developed method was also evaluated by calculating the relative standard deviation of the calibration curve slope (RSD < 1%).

Received 8th September 2023
Accepted 10th October 2023

DOI: 10.1039/d3ay01589k

rsc.li/methods

Universidade Católica Portuguesa, CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Rua Diogo Botelho 1327, 4169-005 Porto, Portugal. E-mail: arangel@ucp.pt

† Electronic supplementary information (ESI) available. See DOI: https://doi.org/10.1039/d3ay01589k

Maria Melo completed, in November 2022, her MSc in Biomedical Engineering (final grade of 18/20) in Escola Superior de Biotecnologia (Universidade Católica Portuguesa) and, in the same institution, she received her BSc in Bioengineering (final grade of 17/20), in 2020. Maria is a researcher at Centro de Biotecnologia e Química Fina, after winning a research grant in the project FISHCOLBOOSTER. During the course of her master's degree, she attended an Erasmus+ program during one semester at Univesità degli studi di Verona (Italy) and had presented her work in an international conference. Throughout her academic path, she also received merit awards and participated in the organisation of conferences and other events, as well as in other dynamic activities, such as in a students' union and volunteering.

Raquel Beatriz Ribeiro de Mesquita completed her PhD in Biotecnologia in 2007 at Universidade Católica Portuguesa Escola Superior de Biotecnologia (UCP-ESB), bachelor's degree in Microbiologia in 1999 at UCP-ESB and master's degree (MSc) in Biotechnology in 2000 at De Monfort University. She is a contracted researcher at UCP-ESB, has published 61 articles in peer reviewed journals and has been in the organizing committee of two international conferences. She has been invited to lecture in scientific symposiums (#8) and in seminars (#3) and presented/co-authored her work in both national and international scientific conferences. She supervised 5 MSc dissertations and co-supervised 3 PhD theses. She has received over 10 awards and/or honors including a FIA Award for younger researchers by the Japanese Association for Flow Injection Analysis (JAFIA) committee and an Endeavour Research Fellowship by the Australian Government Department of Education. She works in the areas of Exact Sciences with emphasis on Chemical Sciences and Analytical Chemistry, applied to Environmental Sciences and Medical and Health Sciences.

1. Introduction

Collagen is one of the most valuable proteins, as it is used in various applications, such as in food, biomedical and cosmetic fields.[1,2] Despite being extracted from natural sources, this polymer has other advantageous properties, such as its low toxicity, weak antigenicity, high nutritional value, and flexibility, as well as its biodegradability and biocompatibility.[3,4]

Its extraction from mammalian sources, *i.e.*, bovines, is decreasing due to health and environmental problems,[5] and, therefore, fish has become a good alternative collagen source. In fact, the decrease in collagen extraction in land animals can be explained by the possible human infections resulting from the outbreak of diseases in stock animals. Moreover, religious reasons are another limiting factor for using mammalian collagen in some industries.[3,6]

The extraction of this protein from fish reflects the need to value fish sub-products and utilise the unused biomass of its production, making extraction possible from fish skin, cartilage, scales, and bones. Marine collagen shows remarkable properties such as the capacity to retain water, higher absorption capacity and "no irritant potential".[1,7]

Collagen has a triple helix structure formed by its amino acid sequence (19 amino acids), where the most significantly present are glycine, hydroxyproline and proline.[2,8,9] One way to quantify the collagen present in fish skin to obtain high-value fractions is the determination of hydroxyproline (HYP). The analysis of this molecule from fish skin requires pre-treatment, with prior proteolytic digestion and hydrolysis of a skin section, to break down collagen into its amino acids. The quantified value is compared to the amount of pure collagen studied for each type of fish and was previously assessed to be around 38 μg of HYP per mg of pure collagen.[9,10]

To quantify the amount of HYP present, its oxidation is necessary. The most typically used methods are based on the oxidation of the amino acid to form a chromophore with *p*-dimethylaminobenzaldehyde, with chloramine T or hydrogen peroxide.[11–13] However, another redox reaction of HYP with permanganate in an alkaline medium uses a smaller number of reagents that have the advantage of being less toxic and harmful.[14]

The main goal of this work was to develop a faster and simpler way to quantify HYP in fish skin samples and to automate this determination; with this goal, a flow injection analysis (FIA) method is proposed. Flow-based techniques, combined with spectrophotometric detection, allow the miniaturisation of chemical analysis, minimal operator intervention, reduced volume of samples and reagents, and low waste production.[15] This kind of system includes plastic tubes and a peristaltic pump that aspirates the solutions (carrier and reagents) along the defined system manifold.[16] The reaction time is determined by the tube lengths and peristaltic pump speed, and the sample is inserted into the carrier stream through an injection valve. In

Ezequiel R. Coscueta is a junior researcher at CBQF and Invited Assistant Professor at ESB-UCP. He has a PhD in Biological Sciences (National University of Rosario, 2018) and BSc in Biotechnology (National University of the Littoral, 2013). He is a technology developer within the circular economy approach with expertise in green chemistry, peptidomics, and nanotechnology for the food and nutraceutical industries. His career consists of 8 years in Food Sciences, Biotechnological Engineering, and Nanotechnology, particularly in the isolation, purification, analysis, and delivery of bioactive compounds. He's specialized in the development of novel green technology for the valorisation of agri-food by-products, in a sustainable and efficient way. Likewise, his current interests align with the development of alternative matrices and ingredients for the next generation of food and nutraceutical products, and the resilience of food systems. He is an author of 25 international peer-reviewed articles, one book, and five chapters. He is also an editor of 4 issues in high-impact international journals and a co-author of 1 patent.

Maria Manuela Estevez Pintado has a BSc in Pharmacy from the Faculty of Pharmacy of the University of Porto (1991) and PhD in Biotechnology from the Portuguese Catholic University (UCP) (1999). She is Associate Professor, Director of CBQF – State Associate Laboratory and Associate Director of the College of Biotechnology of UCP. She is the leader of the Biobased and Biomedical products group and coordinates the Bioactives and Bioproducts Research Laboratory. She has established strong interaction with industry mainly in the food sector. Her main research interests include development and characterization of bioactives and by-product valorization through bioprocesses and extraction processes aiming to obtain high-added value products to be applied to food and other industries. She has co-authored ca. 400 papers in international journals, is a creator of 20 patents, has been involved in the supervision/co-supervision of 34 concluded PhD theses and has coordinated/co-coordinated ca. 90 externally funded projects.

António Osmaro Santos Silva Rangel is Full Professor at Universidade Católica Portuguesa (ESB-UCP), Porto, Portugal. He is President of the Scientific Board of ESB and member of the Direction Board of the Research Center. He holds a degree in Chemistry (Univ. Porto, 1985) and a PhD degree in Biotechnology (UCP, 1991). He has a 35 year teaching activity involving general chemistry and analytical chemistry courses. His research interests are focused on flow-based methods for the automation of (bio) chemical assays in the environmental, food, and biomedical sectors. He has supervised 20 PhD theses and is co-author of more than 200 papers.

the detection unit, the signals obtained for a determination with a FIA method are peaks proportional to the analyte concentration for a given determination,[17] in this case, HYP.

2. Materials and methods

2.1. Reagents and solutions

The solutions were prepared with analytical grade chemicals and Milli-Q® Water (resistivity > 18.2 MΩ cm, Millipore, Bedford, MA, USA).

The permanganate solution was prepared with boiled water by diluting a stock solution of 0.1 M to a final concentration of 5 mM. A stock solution of 0.1 M permanganate was prepared by dissolving 1.58 g of sodium permanganate in 100 mL of water.

A 1.5 M sodium hydroxide stock solution was prepared, dissolving 30 g of sodium hydroxide (PanReac, Spain) in 500 mL of water.

A HYP stock solution of 1000 mg L^{-1} was prepared by dissolving 10.0 mg of L-hydroxyproline (Sigma Aldrich, Germany) in 10.0 mL of water. The working standards within a range of 25.0 mg L^{-1} to 500 mg L^{-1} were prepared from the stock solution.

2.2. Flow injection manifold and procedure

The flow injection manifold (Fig. 1) was composed of a Gilson Minipuls 3 peristaltic pump (PP in Fig. 1) equipped with three PVC tubes (F117938), a miniature CCD detector (Flame, Ocean Insight) connected to a halogen lamp light source (UV-VIS-NIR, Mikropack, DH-2000-BAL), and a 1 cm light path flow cell (Hellma 178.710-QS). The three channels of the flow manifold consisted of Gilson polytetrafluoroethylene (PTFE) tubes (inner diameter 0.8 mm, 008T16-080-20) attached to a four-channel confluence and connected to the flow cell. For sample/standard insertion, a six-port injection valve (Omnifit 1106 rotary valve) was placed at one of the three channels, the water stream (carrier, C in Fig. 1).

A 0.65 mL min^{-1} flow rate for the HYP determination was set for all the channels. The sample was inserted into a water carrier (C) stream, mixing with both permanganate (R_1) and hydroxide (R_2) at the confluence. Then, the mixture went through a heated 2 m reaction coil (L_1) to promote the reaction. The L_1 reactor was connected to a water bath set at 30 °C. Afterwards, a 52 cm reaction coil (L_2) was placed before the flow cell to allow the mixture to cool down prior to detection. An absorbance signal decrease, caused by a loss of colour in the permanganate solution, was observed because of the permanganate reduction by hydroxyproline. The signal was acquired with a computer equipped with SpectraSuite® software.

2.3. Sample treatment and hydroxyproline determination

The dermal tissue of the blue shark, which emerged as a secondary product during the filleting operations by Brasmar-Trade Food Ltd for human dietary intake, was utilised as the primary research substrate. Before they were ready for HYP determination, the by-products were processed using an industrial grinder. These cryogenically preserved samples were conserved under optimal conditions, awaiting subsequent treatments. All specimens underwent further procedural interventions by ETSA – SGPS, Ltd (Empresa Transformadora de SubProdutos Animais) under conditions bound by industrial confidentiality. Then, an acidic hydrolysis was performed to break down collagen into its amino acids. HYP was determined in the extract, and the collagen quantity was assessed through a previously established correlation of HYP content in pure collagen (38 μg of HYP per mg of pure collagen).

To maximise the available extract volume, a tenfold dilution was made before insertion in the developed flow injection system.

2.4. Conventional procedure – comparative method

The same samples were analysed in the developed FI method and with the conventional determination based on the HYP

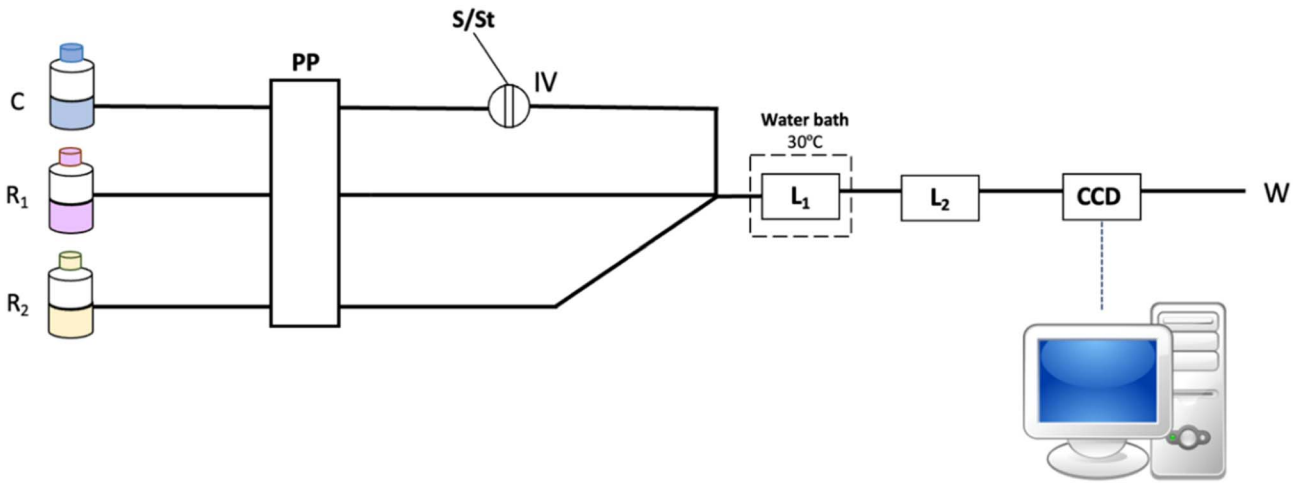

Fig. 1 Flow injection manifold developed for the HYP determination in fish skin. PP, peristaltic pump; S/St, sample/standard; C, water; R_1, 5 mM permanganate solution; R_2, 1.5 M sodium hydroxide solution; L_1, reaction coil 2 m length; L_2, reaction coil 52 cm length; IV, injection valve; CCD, detector (L = 526 nm); W, waste.

oxidation by chloramine T,[13] and the results were compared for method validation.

3. Results and discussion

3.1. Preliminary studies

The first approach was to develop a FI based on the conventional determination with chloramine T, previously adapted to a flow approach.[18] This reaction would have the advantage of observing colour formation by chloramine reduction. However, that determination requires heating at 85–90 °C, which results in signal interference due to the formation of air bubbles. Additionally, more reagents were involved with higher toxicity, namely perchloric acid (70%) and propanol (about 30%). Consequently, the automation based upon the chloramine T reaction was abandoned, and the alternative hydroxyproline oxidation by permanganate was chosen for developing the FI system.

The oxidation of HYP with permanganate[14] in an alkaline medium results in the loss of colour of the reagent with the consequent decrease of absorbance (at 526 nm). When the reaction was carried out in the flow injection approach, it was observed that the baseline values had slight variations before each peak (ESI Fig. 1†), so a subtraction of the baseline value from the peak value was considered. Two calibration curves were established to evaluate if subtracting the baseline values would affect the determination sensitivity (calibration curve slope), one with subtraction of the baseline and the other without (ESI Fig. 2†). Despite the change from negative to positive (due to subtracting the higher value of the baseline), there was no difference between them with a deviation of the calibration curve slopes of 1.5%, so it was set to subtract the baseline.

3.2. Chemical parameters – reagent concentrations

Since NaOH is used to ensure an alkaline medium, the influence of its concentration on the determination sensitivity (calibration curve slope) was studied. Calibration curves were established using different concentrations of sodium hydroxide (R_2), 0.5, 1.0 and 1.5 M. The one that provided a higher sensitivity was 1.5 M, so this concentration was chosen (ESI Fig. 3†).

Then, the impact of MnO_4^- concentration on the determination sensitivity was also assessed, and four concentrations were compared, 1 mM, 2 mM, 5 mM, and 7 mM. The concentration of 5 mM was chosen as it resulted in a higher sensitivity (ESI Fig. 4†).

The following studies were conducted with 1.5 M hydroxide and 5 mM permanganate.

3.3. Reactor length – reaction extension

Different reactor lengths were studied to optimise the reaction extension and, subsequently, the method's sensitivity, 1.1 m, 1.6 m, 2 m, 2.5 m, and 2.75 m (Fig. 2A). The results led to the conclusion that the sensitivity increased with the increase of the reactor length (higher reaction extension). However, the calibration curve slope increase comparing the reactor length of 2.5 m and 2.75 m was insignificant (RD < 10%), so the reactor length chosen was 2.5 m.

3.4. Sample volume

The influence on the calibration curve slope of increasing the sample/standard volume was assessed to improve the method sensitivity further.

Three volumes were tested, 130 μL, 380 μL and 630 μL (Fig. 2B), and although higher volumes provided higher sensitivity, the difference between 380 μL and 630 μL was not significant (relative deviation of the slope < 10%). For this reason, the volume of 380 μL was chosen.

3.5. Temperature influence

The influence of temperature was evaluated by immersing the reaction coil in a water bath and testing different temperatures. Calibration curves were established for each of the temperatures tested, namely, 20 °C (room temperature), 25 °C, 30 °C, 35 °C and 40 °C (Fig. 3A). As expected, the increase in temperature increased sensitivity (calibration curve slope). However, at 40 °C, too many air bubbles were formed inside the reaction coil and interfered with the measurement, so the calibration curve at this temperature was discarded. When comparing the

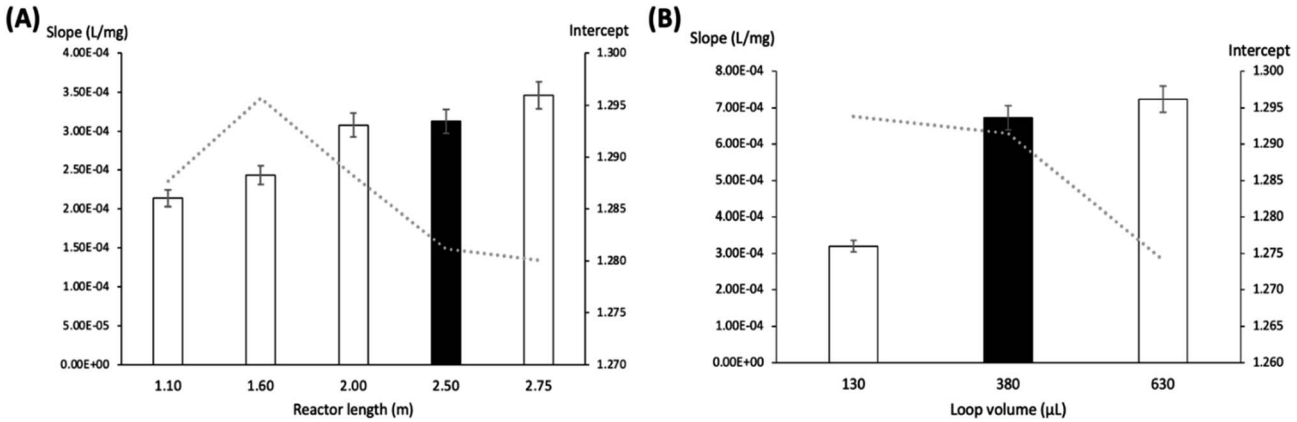

Fig. 2 Sensitivities achieved with (A) different reactor lengths and (B) different loop volumes; the respective intercept values are represented by the dotted line; error bars represent a 10% deviation.

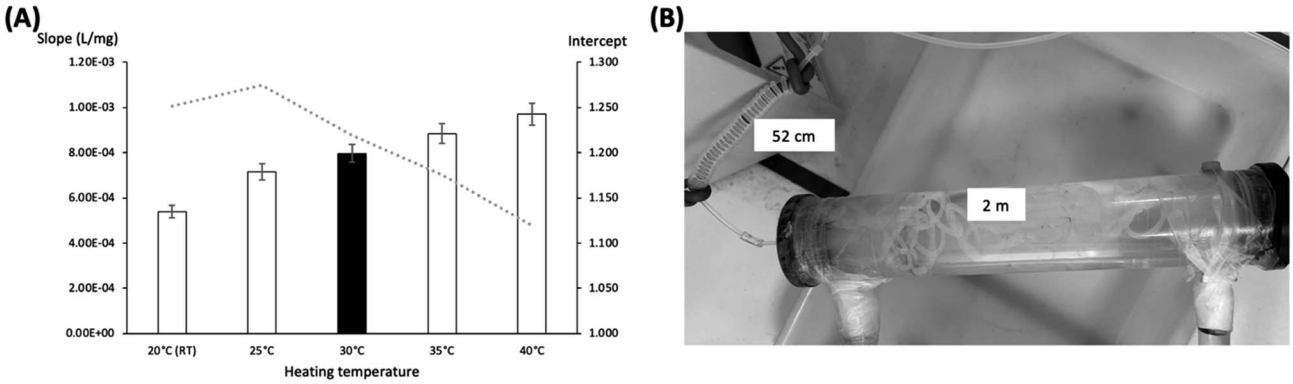

Fig. 3 A) Sensitivities achieved with different heating temperatures and the respective intercept values are represented with the dotted line (error bars represent a 10% deviation); (B) reactor arrangement and heating module.

calibration curves at temperatures of 30 °C and 35 °C, it was possible to conclude that the difference between them was not significant (relative deviation between slopes < 10%) and, so, 30 °C was the chosen temperature for the water bath.

As the reactor length previously chosen was 2.5 m, a reactor of 2 m was placed inside this hot bath. A 52 cm long reactor was placed outside the heating module (Fig. 3B), allowing the solutions to cool down slightly before reaching the detector.

3.6. System manifold

The mixture of the solutions was compared using two different manifolds: with one confluence, where the solutions were all mixed together before reaching the reactor (Fig. 4A), and the other with two confluences, where the sample inserted was first mixed with NaOH and only later with permanganate. In the last case, three reactor lengths to mix the sample and NaOH were studied (Fig. 4B).

Looking at Fig. 4C, it is possible to observe that, with one confluence, the sensitivity obtained was slightly higher, and it is easier to mix all the solutions with no extra confluence and no extra reactor. Due to this fact, the manifold using just one confluence was chosen.

3.6.1. Permanganate solution flow rate. The flow rate of the permanganate solution was also studied to see how it would affect the reaction proportion. Equal flow rates of 0.65 mL min^{-1} in the three channels were compared with having a lower flow rate of the permanganate channel of 0.48 mL min^{-1}. It was concluded that, when using the 0.48 mL min^{-1} channel, the linearity of the calibration curve decreased, as well as the baseline value, so the flow rate of 0.65 mL min^{-1} was maintained.

3.7. Features of the FIA system

Under optimal conditions, the features of the developed method are presented in Table 1.

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated, following IUPAC recommendations,[19] respectively as the concentration equivalent to three times and

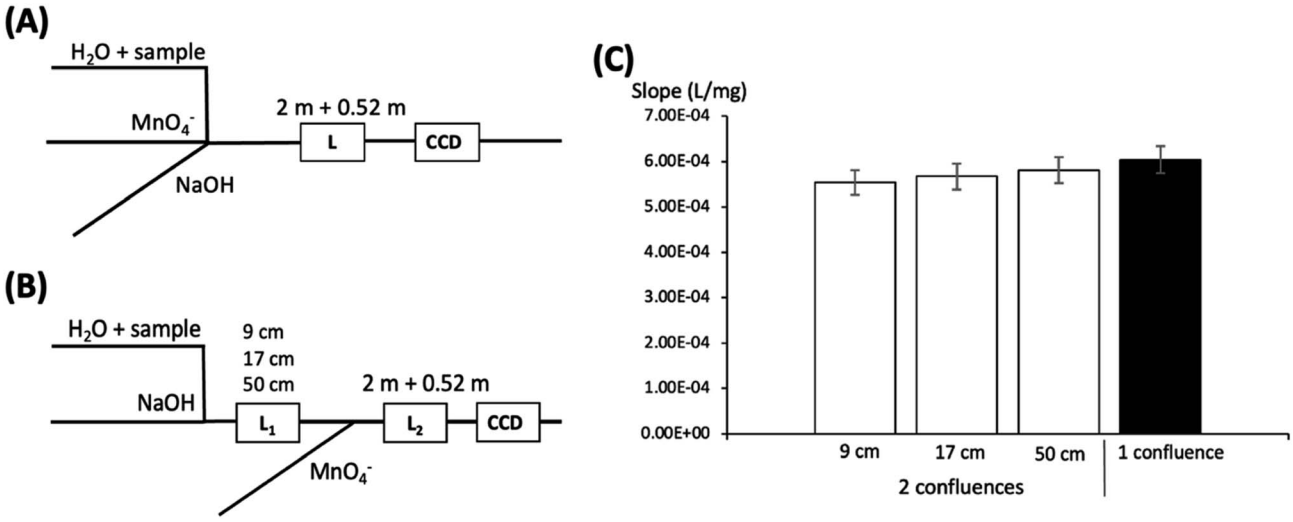

Fig. 4 System manifold study. (A) Manifold with one confluence and one reactor (L); (B) manifold with 2 confluences and 2 reactors (L_1, the sample is mixed with NaOH first; L_2, the solution is mixed with MnO_4^-); (C) Sensitivities obtained with different manifolds in the FIA system. 9 cm, 17 cm and 50 cm represent the different reactor lengths studied for mixing the first the sample with NaOH. (Error bars represent a 10% deviation.)

Table 1 Features of the developed FIA system for hydroxyproline quantification

Dynamic range	23.8–500 mg L^{-1}
LOD	2.6 mg L^{-1} (0.52 μg HYP per mg pre-treated fish skin powder)
LOQ	23.8 mg L^{-1} (4.76 μg HYP per mg pre-treated fish skin powder)
RSD sample[a]	1.8% (35.2 mg L^{-1})
	0.3% (285 mg L^{-1})
Sample consumption[b]	380 μL (1140 μL/3 replicas)
Reagent consumption[b]	0.39 mg of MnO_4^-, 39 mg of NaOH
Calibration curve[c]	$A = 1.03 \times 10^{-3}$ [HYP] ($\pm 1 \times 10^{-5}$) + 2.1×10^{-2} ($\pm 1 \times 10^{-3}$)

[a] $n = 13$. [b] Per determination. [c] $n = 5$.

Table 2 Composition of the most significantly present amino acids in shark skin gelatin collagen.[8]

Amino acid	Content in shark skin (%)
Hydroxyproline	10.9
Alanine	11.2
Glutamic acid	10.3
Glycine	26.5
Proline	13.9

Table 3 Interference assessment of some relevant compounds from the shark skin in the absorbance signal; the tested amounts correspond to the expected amount in the skin, added to a HYP standard of 250 mg L^{-1}

Tested interferent	Added amount (mg L^{-1})	Interference percentage (%)
Alanine	250	3
Glutamic acid	250	1
Glycine	500	3
Proline	250	8

ten times the standard deviation of the intercept. The superior limit of the dynamic range was set at 500.0 mg L^{-1} since higher concentrations did not align with the established linear regression. The method's precision was assessed by analysing the RSD of two different samples (#13 measurements). The consumption of reagents and samples was calculated based on HYP determination, corresponding to one injection.

3.8. Interferences

The collagen molecule has 19 amino acids in its constitution and, therefore, the potential interferences of the most significantly present amino acids in shark skin (Table 2) were studied.

A HYP standard solution of 250 mg L^{-1} was prepared to evaluate the potential interference with and without the potential interfering compounds. Analysing the percentage of each amino acid, the alanine, glutamic acid, and proline content is similar to the hydroxyproline content in shark skin; therefore, the amount of these amino acids added was also 250 mg L^{-1}. The percentage of glycine is approximately double that of hydroxyproline, and 500 mg L^{-1} of this component was added. The results are shown in Table 3.

As seen in the previous table, no interferences were verified when considering the content in shark skin. Furthermore, higher concentrations of the possible interferents were added, and a minimal percentage was verified when adding 400 mg L^{-1} alanine and glutamic acid, 600 mg L^{-1} glycine and 273 mg L^{-1} proline (ESI Table 1†).

3.9. Application to samples

3.9.1. Accuracy assessment. To evaluate the accuracy of the developed FIA manifold for HYP determination in fish samples, blue shark skin samples (#8) were directly analysed with the system, and the results were compared to a classic method using microplates. The concentration of HYP in each sample was determined, and the relative error between both methods ($[HYP]_{FIA}$ and $[HYP]_{\mu Plate}$) was calculated, as shown in Table 4.

A linear relationship between the two methods was established. The obtained equation (where the values in brackets represent the 95% confidence interval), $[HYP]_{FIA} = 1.05$

Table 4 Comparison of the results obtained with the developed flow system (FIA) and comparative microplate method (μPlate), by calculating the relative error percentage (RE); SD, standard deviation

Sample ID	$[HYP]_{FIA} \pm SD$ (mg L^{-1})	$[Collagen]_{FIA}$ (g L^{-1})	$[HYP]_{\mu Plate} \pm SD$ (mg L^{-1})	$[Collagen]_{\mu Plate}$ (g L^{-1})	RE (%)
1	328 ± 11	8.63	345 ± 10	9.07	−4.8
2	368 ± 20	9.68	377 ± 3	9.93	−2.6
3	436 ± 43	11.5	417 ± 6	11.0	4.6
4	411 ± 14	10.8	427 ± 18	11.2	−3.8
5	557 ± 15	14.7	530 ± 2	14.0	5.1
6	334 ± 17	8.79	320 ± 28	8.42	4.4
7	463 ± 34	12.2	461 ± 27	12.1	0.5
8	341 ± 24	8.96	319 ± 3	8.40	6.7

Table 5 Recovery studies performed with the developed FIA system for HYP determination; SD, standard deviation

Sample ID	Initial concentration (mg L^{-1}) ± SD	Added concentration (mg L^{-1})	Found concentration (mg L^{-1}) ± SD	Recovery percentage (%)
A	176 ± 2	45.5	227 ± 6	116
		90.9	269 ± 2	104
B	203 ± 3	45.5	253 ± 2	109
		90.9	299 ± 3	106
C	183 ± 1	45.5	229 ± 2	101
		90.9	274 ± 3	99
D	193 ± 2	45.5	238 ± 1	100
		90.9	303 ± 5	121
E	208 ± 1	45.5	252 ± 1	95
		90.9	305 ± 1	106
F	201 ± 3	45.5	245 ± 2	95
		90.9	297 ± 2	105
G	302 ± 1	45.5	349 ± 4	103
		90.9	415 ± 5	125
H	193 ± 1	45.5	238 ± 1	98
		90.9	286 ± 1	103

(±0.224) $[HYP]_{\mu Plate}$ − 13.1 (±90.9), proved that the developed method is not significantly different from the comparative method since neither the slope nor the intercept was statistically different from 1 and 0, respectively.[20]

3.9.2. Recovery studies. To further evaluate the accuracy, recovery studies were also performed adding two different amounts of HYP to the extract samples (Table 5).

Analysing the results, it is possible to observe some outliers (the recovery percentage for the higher concentration added in samples D and G). The average recovery percentage obtained without these outliers was 103%, and the standard deviation was 4%. Considering the outliers, the average value obtained was 105%, with a standard deviation of 9%, indicating that there were no matrix multiplicative interferences.[20]

4. Conclusion

In this work, a flow injection system was developed to quantify hydroxyproline in fish skin samples, specifically in blue shark skin, to assess the present collagen content. This represents a simple, faster, and more automated way to determine HYP compared to classic methods that are more complex and time-consuming. Furthermore, the developed FIA manifold uses fewer, less harmful and less toxic reagents. It is less operator-dependent since the operator only has to inject the sample into the system.

To determine HYP, the sample injected reacts with NaOH and permanganate, resulting in a signal decrease (loss of permanganate characteristic purple colour) in the detection unit, proportional to the HYP concentration increase. A sample analysis takes about three minutes, including obtaining 3 peaks for each sample. Usually, the samples must be diluted (about 10-fold dilution) due to the high content observed in blue shark skin. The dynamic range of HYP quantification is 23.8–500 mg L^{-1}, has an RSD smaller than 2% for samples, and enables the determination with no significant interferences of other shark skin components.

Conflicts of interest

There are no conflits to declare.

Acknowledgements

M. M. P. Melo is thankful for the grant POCI-01-0247-FEDER-049636_BI. We thank André Almeida and Ana Rosa of the ETSA Group for providing the processed collagen material. We also acknowledge the scientific collaboration of CBQF under the FCT project UIDB/50016/2020. This work was funded by project "FISHCOLBOOSTER – Development of collagen peptides from fish in an integrated system to obtain high-value fractions for human consumption, aquaculture and cosmetics" supported by the European Regional Development Fund (FEDER) through Programa Operacional Competitividade e Internacionalização (POCI).

References

1 C. G. Sotelo, M. Blanco Comesaña, P. Ramos Ariza and R. I. Pérez-Martín, *J. Aquat. Food Prod. Technol.*, 2016, **25**, 388–399.
2 A. Sionkowska, K. Adamiak, K. Musial and M. Gadomska, *Materials*, 2020, **13**(19), 4217, DOI: 10.3390/MA13194217.
3 M. Shalaby, M. Agwa, H. Saeed, S. M. Khedr, O. Morsy and M. A. El-Demellawy, *J. Polym. Environ.*, 2020, **28**, 166–178.
4 E. Martins, R. L. Reis and T. H. Silva, *Mar. Drugs*, 2023, **21**(2), 57, DOI: 10.3390/md21020057.
5 K. Dopelt, P. Radon and N. Davidovitch, *Int. J. Environ. Res. Public Health*, 2019, **16**(8), 1359, DOI: 10.3390/IJERPH16081359.
6 S. Geahchan, P. Baharlouei and M. A. Rahman, *Mar. Drugs*, 2022, **20**(1), 61, DOI: 10.3390/MD20010061.
7 H. Jafari, A. Lista, M. M. Siekapen, P. Ghaffari-Bohlouli, L. Nie, H. Alimoradi and A. Shavandi, *Polymers*, 2020, **12**, 1–37.

8 A. M. Holwerda and L. J. C. Van Loon, *Nutr. Rev.*, 2022, **80**, 1497–1514.
9 Y.-S. Lim, Y.-J. Ok, S.-Y. Hwang, J.-Y. Kwak and S. Yoon, 2019, **17**, DOI: **10.3390/md17080467**.
10 J. Liu, M. Shibata, Q. Ma, F. Liu, Q. Lu, Q. Shan, T. Hagiwara and J. Bao, *J. Food Biochem.*, 2020, **44**(8), e13265, DOI: **10.1111/jfbc.13265**.
11 R. Kuttan and A. N. Radhakrishnan, Chapter 5 - Biochemistry of Hydroxyprolines, *Advances in Enzymology - and Related Areas of Molecular Biology*, ed. A. Meister, 1973.
12 D. S. Miyada and A. L. Tappel, *Anal. Chem.*, 1956, **28**, 909–910.
13 S. Brown, M. Worsfold and C. Sharp, *Biotechniques*, 2001, **30**, 38–42.
14 S. A. Farokhi, A. K. Kini and S. T. Nandibewoor, *Inorg. React. Mech.*, 2002, **4**, 67–76.
15 M. A. Segundo and A. O. S. S. Rangel, *J. Flow Injection Anal.*, 2002, **19**, 3–8.
16 A. Cerdà and V. Cerdà, An introduction to flow analysis, *SCIWARE, S.L.*, Palma de Mallorca, 1st edn, 2009.
17 V. Cerdà, L. Ferrer, J. Avivar and A. Cerdà, *Flow Analysis*, Elsevier Science, 2014, pp. 1–42.
18 J. Möller and A. Sjödin, *Anal. Chem.*, 1988, **329**, 732–734.
19 L. A. Currie, *Pure Appl. Chem.*, 1995, **67**, 1699–1723.
20 J. N. Miller and J. C. Miller, *Statistics and Chemometrics for Analytical Chemistry*, Pearson Education, Essex, England, 6th edn, 2010.

