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"LC-MS/MS ANALYSIS BASED ON DOUBLE ISOTOPE LABELLING OF HYALURONIC ACID IN COMPLEX MATRICES"

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1. Introduction

Hyaluronic acid (HA) is a high-molecular-weight glycosaminoglycan widely employed in cosmetic formulations owing to its remarkable hydrating properties, anti-aging effects and biocompatibility [1,2]. The growing demand for HA-enriched products has highlighted the need for accurate and reliable analytical methodologies capable of quantifying HA within complex cosmetic matrices, ensuring not only product efficacy and quality, but also regulatory compliance.

Over the years, various analytical methods have been proposed for the quantitation of HA, both as a raw material and as an endogenous component in biological systems [3]. Predominantly, these approaches involve hyphenated methods combining a separation technique (e.g., HPLC, GC, or CE) with detectors such as light scattering, mass spectrometry (MS), UV-Vis, or fluorescence-based systems [4-6]. However, analysing HA presents significant challenges, primarily attributable to its intrinsic chemical properties: it is a non-volatile, high-molecular-weight polymer devoid of chromophore groups and exhibits inherently low ionisation efficiency in mass spectrometry. Consequently, HA in its native form often necessitates specific depolymerisation and derivatisation steps to enable effective detection.

Each available method offers advantages and faces specific limitations. For instance, the direct analysis of intact HA via size exclusion chromatography (SEC) coupled with light scattering or refractive index (RI) detection enables the determination of molecular weight distribution without extensive sample preparation. Nevertheless, such techniques suffer from limited sensitivity. As reported by Suárez-Hernández et al., the achieved limit of detection (LOD) and limit of quantification Moreover, SEC detectors like light scattering and RI are inherently non-selective, responding indiscriminately to a variety of matrix components. Thus, while this strategy may be suitable for analysing raw materials or relatively simple matrices, its application to complex cosmetic formulations remains problematic due to potential interference from numerous ingredients.

At present, the state-of-the-art method for measuring HA in biological matrices involve liquid chromatography coupled with electrospray ionisation mass spectrometry (LC-ESI-MS) [8,9] or

capillary electrophoresis coupled to MS [10], often preceded by extensive sample preparation. Typically, this includes purification steps, enzymatic digestion of HA, derivatisation of the resulting oligomers, and subsequent analysis in multiple reaction monitoring (MRM) mode.
Although highly effective for well-characterised biological matrices—such as serum, synovial fluid, or cell culture media—this approach demands considerable time and resources for method development and validation, rendering it unsuitable for matrices of unknown or highly variable composition.
Cosmetic products, by contrast, are inherently heterogeneous, exhibiting substantial variability in both qualitative and quantitative matrix composition across different formulations. Developing a dedicated method for each cosmetic product would not only be impractical but also economically unviable. Thus, existing LC-ESI-MS methodologies are not suitable for the systematic analysis of HA in cosmetics. Hence, the main challenge in developing a method for the quantitative measurement of HA in cosmetic products lies in the high heterogeneity of the matrix, whose qualitative and quantitative composition varies depending on the formulation. Therefore, the method must be robust against matrix effects, ideally using internal standards that normalise the results and minimise variability due to matrix interference.
Accordingly, the objective of the present study is to develop a generalised incorporating a simple yet effective extraction procedure, suitable internal standards, and the standard addition approach. This methodology is designed to be broadly applicable across a wide range of cosmetic formulations, regardless of their compositional complexity.

2. Materials and Methods

2.1 Chemicals

Formic acid, ammonium formate, acetonitrile (ACN), methyl tert-butyl ether (MTBE), methanol (MeOH) and LC-MS grade solvents were purchased from Sigma-Aldrich (Merck Life Science S.r.l., Milan, Italy). HPLC grade water (18 MΩ cm) was purified with a Milli-Q system (Millipore, Bedford, MA, USA). 700 KDa hyaluronic acid and the isotopic derivatives containing 100% ^{13}C labelled HA (lot M110723) and 50% ^{13}C labelled HA (Lot AG006/24) were synthesised by Fidia Farmaceutici S.p.A, Abano Terme, Padova, Italy). Recombinant hyaluronidase (rHyal-sk, RSK 100 U/mg, 100.696 U/mL) was supplied by Fidia Farmaceutici S.p.A. Bovine Testicular Hyaluronidase (BTH) was purchased by Sigma-Aldrich (Merck Life Science S.r.l., Milan, Italy).

2.2 Sample preparation

In 2 mL low-binding Eppendorf tubes were sequentially added the following aliquots: 50 µL HA standard in aqueous solution or cosmetic serum (in-house and commercial), 50 µL of 100% ^{13}C labelled HA (10 mg/mL, IS1), 110 µL of Milli-Q water, 275 µL cold MeOH, 65 µL MeOH (42.4%) and 1 mL cold MTBE. The samples were vortexed to ensure uniformity in their composition, then centrifuged (15200 rpm) at 4°C for 20 minutes, allowing the separation of any precipitated proteins, along with the upper non-polar phase and the lower polar phase. Aliquots of 25 µL of the aqueous subnatant were collected and added with 75 µL of Milli-Q water, 100 µL of increasing concentration of HA dissolved in buffer pH 5.2 (CH_3COONa 200 mM and NaCl 300 mM). The HA concentration added were: 0, 125, 250 and 375 µg/mL for both the commercial samples.

The hydrolysis was obtained by the addition of 3 µL of a 1:10 diluted solution from enzyme stock solution (RSK) and samples were kept in thermomixer at 37 °C and 500 rpm for 24 hours. The reaction was stopped by adding 10 volumes of cold MeOH; samples were kept for 30 minutes at - 20 °C and then centrifuged at 4 °C for 15 minutes at 15200 rpm. The supernatants were collected and dried under vacuum at 50 °C. Finally, the dry samples were dissolved in 60 µL of a 50:50 (% v/v) mixture (100 mM ammonium formate pH 3/ACN) to which were added 10 µL of IS 2 (previously hydrolysed) before transfer into vials for the analysis. IS 2 was prepared as follows: an aliquot of 500 µL was taken from a stock solution of 50% ^{13}C labelled (10 mg/mL), and added with 500 µL of buffer pH 5.2 and 25 µL BTH (25000 U/mL) to perform the hydrolysis at 37°C and 500 rpm, and incubated overnight. The hydrolysis was stopped by heating to 99° C for 20 minutes, then the samples were centrifuged for 30 minutes at 4°C, 15200 rpm.

2.3 LC-MS/MS conditions

Chromatographic separation was performed using a Hypersil GOLD HILIC column (particle size 3 µm, internal diameter 2.1 mm, length 150 mm, Thermo Scientific), protected by a Hypersil GOLD HILIC precolumn, maintained at 40°C with an Exion LC-100 HPLC system (AB Sciex) and operating at a constant flow of 300 µL/min. An aliquot of 10 µL was injected and eluted with a multistep gradient of mobile phase A (100 ammonium formate, pH 3) and mobile phase B (ACN) as follows: isocratic (80% B) elution (0-0.20 min); gradient elution from 80% B to 30% B (0.21-6.70 min); isocratic (30% B, 6.71-14.70 min and then 80% B, 14.71-23.00 min elutions. A MS/MS method for HA quantitation previously developed in our laboratory (unpublished data) based on the tetrameric oligomer of as representative analyte of HA was used. The tetrameric oligomer is the Δ4-mer (Δ GlcA-GlcNAc-GlcA-GlcNAc, where Δ means the removal of a water molecule from GlcA (glucuronic acid), and GlcNAc (acetyl-N-glucosamine)). The triple quadrupole analyser (API 4000, ABSciex, Milan, Italy) equipped with ESI source (parameters: Charged Aerosol Detector (CAD) gas: 4 a.u., curtain gas: 25 a.u., gas: 1 40 a.u. gas 2: 60 a.u., ion spray voltage: - 4500 V, temperature: 550 °C, entrance potential: -10 V) has been set to MRM (multiple reaction monitoring) acquisition mode working in negative ion mode: the transitions for each analyte are reported in **Table 1**. The analysis of the data and the control of the instrument were performed by means of Analyst software (AB Sciex, Milan, Italy). Standard compounds were previously characterized by LC-HRMS using the same chromatographic conditions and a QTOF X500R (AB Sciex, Milan, Italy), set to acquire full MS spectra with the TOF analyser in negative ion mode at a resolution of 30,000 (FWHM at *m/z* 400), scanning in the 200-1800 *m/z* range and with a build-up time of 0.25.

Table 1. MRM parameters for the quantitative analysis.

Compound	Parent Ion	Product Ion	CE	DP	CXP
Δ4-mer	757	554	-48	-100	-15
Δ4-mer 50% ^{13}C (IS2)	789	199	-48	-100	-15
Δ4-mer 100% ^{13}C (IS1)	785	574	-48	-100	-15

CE = Collison Energy; DP = Declustering Potential; CXP = Collision Exit Potential

3. Results

3.1. General principle of the method

An LC-MS/MS method, originally developed for the bioanalytical quantification of hyaluronic acid (HA) in biological matrices (manuscript in preparation), was applied for the analysis of HA in cosmetic formulations. The method consists of the following steps, detailed in the Materials and Methods section:

i. Matrix purification: HA is purified from the cosmetic matrix using methyl tert-butyl ether (MTBE), which selectively extracts lipid components and precipitates proteins that may be present in the formulation.
ii. Phase separation: the aqueous phase, containing HA alongside other polar ingredients, is separated by centrifugation and collected.
iii. Internal standard addition (IS1): prior to enzymatic digestion, a fully ^{13}C-labelled HA analogue (^{13}C-HA, IS1) is added to the sample. Enzymatic hydrolysis, catalysed by recombinant hyaluronidase (RSK), produces a tetrameric oligomer (Δ4-mer, *m/z* 757.2151) along with its isotopically labelled counterpart.
iv. Internal standard addition (IS2): following hydrolysis, a second internal standard, consisting of a Δ4-mer labelled with 50% ^{13}C atoms, is added to correct the variability in MS detector response.
v. Quantitative analysis: the final sample is analysed by LC-ESI-MS in multiple reaction monitoring (MRM) mode. Peak areas corresponding to the analyte-derived Δ4-mer, the 100%-^{13}C-labelled Δ4-mer, and the 50%-^{13}C-labelled Δ4-mer are recorded.

To address matrix effects and compensate for potential variations in ionization efficiency, quantification is performed using the standard addition method. A detailed rationale for the use of dual internal standards and the standard addition strategy is discussed below.

The addition of fully ^{13}C-labelled HA (IS1) prior to digestion corrects for sample-to-sample variability in enzymatic efficiency, a critical step given that residual polar components, despite MTBE extraction, may interfere with enzymatic hydrolysis. By distinguishing the analyte-derived Δ4-mer from the IS1-derived ^{13}C-Δ4-mer, it becomes possible to accurately adjust for such interferences.

Similarly, IS2 (the 50%-^{13}C-labelled Δ4-mer), prepared through enzymatic hydrolysis of partially labelled HA, corrects for fluctuations in detector sensitivity. Notably, IS2 is synthesised using bovine testicular hyaluronidase (BTH), which, due to its differing enzymatic mechanism, yields oligosaccharides distinct from those generated by RSK, thereby avoiding isotopic overlap. Although the standard addition method provides robust correction for matrix effects, a conventional internal calibration approach could be considered for routine analysis if a suitable blank matrix is available.

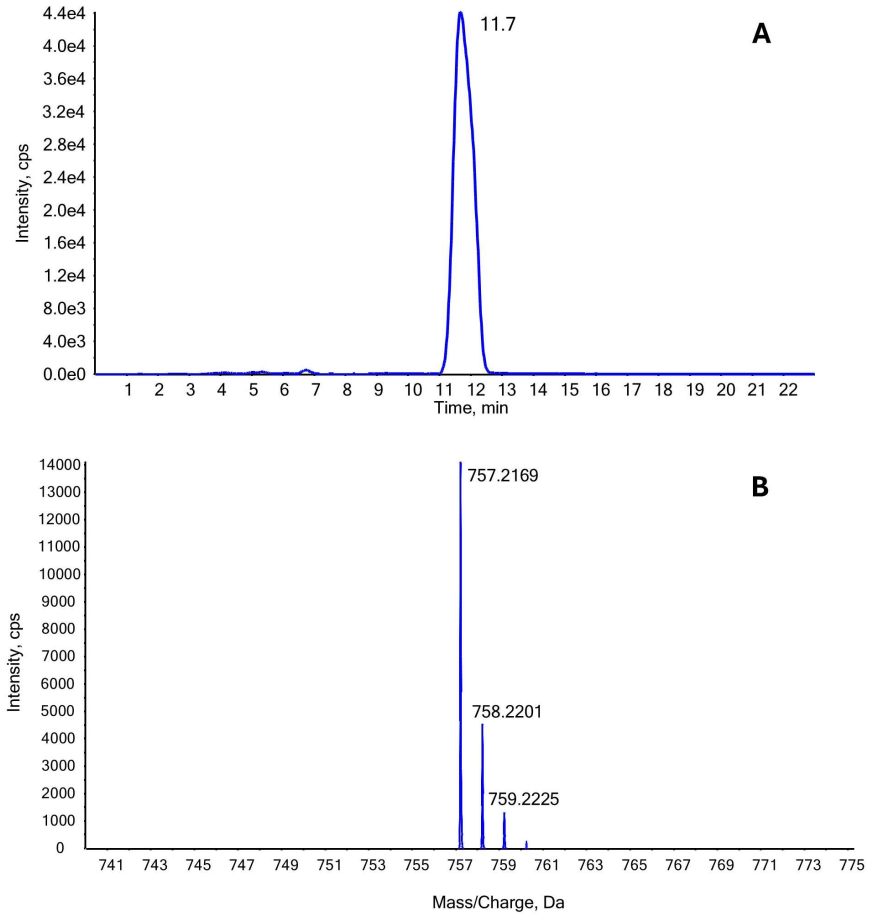
Further details regarding method optimisation and application to two commercial cosmetic products labelled as containing 2% HA are provided in the subsequent section

3.2 Method Set-up

The Δ4-mer oligomer (*m/z* 757.2151), produced predominantly through RSK-catalysed hydrolysis of HA, was selected as the reference analyte for quantification. Figure 1 illustrates the chromatographic profile of the Δ4-mer (panel A) alongside its full-scan high-resolution mass spectrum (panel B).

The internal standard IS1, consisting of fully ^{13}C-labelled HA, is added at the beginning of the sample preparation to normalise for variability in the enzymatic digestion step. Upon hydrolysis, IS1 generates a fully labelled Δ4-mer (Δ4-mer 100%-^{13}C) with a monoisotopic peak at *m/z* 785.3084, as depicted in Figure 1, panel C.

To further normalise the MS response, a second internal standard (IS2), the Δ4-mer containing 50% ^{13}C labelling, is employed. IS2 is obtained via hydrolysis of partially labelled HA using BTH, which differs mechanistically from RSK by not producing unsaturated terminal fragments. Consequently, IS2 oligosaccharides exhibit distinct masses, thereby preventing interference with the native or fully labelled Δ4-mers. Figure 1, panel D, displays the characteristic isotopic distribution of IS2, highlighting the ion at m/z 789.2741, used as the quantitative reference.



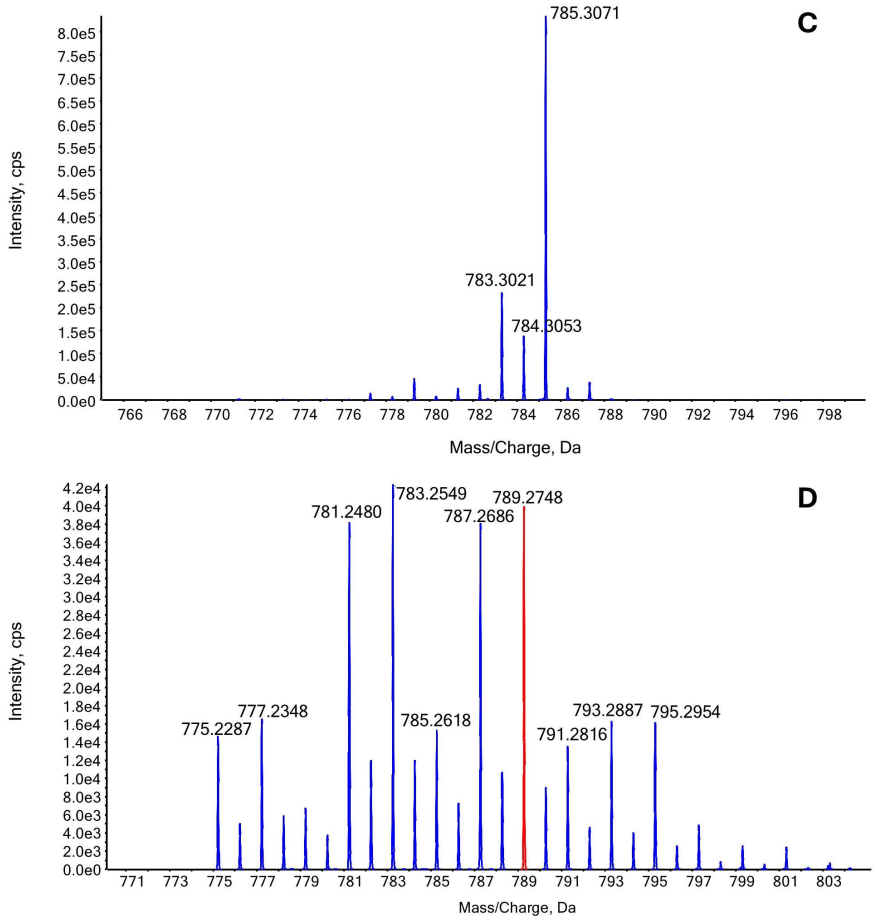


Figure 1. Panel A: Δ4-mer chromatogram and full MS spectrum (panel B) of the peak with RT of 11.6 min; panel C: full HR-MS spectrum of Δ4-mer 100% ^{13}C; panel D: full HR-MS spectrum of Δ4-mer 50% ^{13}C with a characteristic isotopic distribution: the ion *m/z* 789.2748 has been selected as a reference for the quantitative analysis.

3.3. Method validation

Validation was performed using in-house prepared cosmetic serum matrices containing 2.00% (w/w) HA. Evaluated parameters included linearity, limits of detection (LOD) and quantitation (LOQ), matrix effect, recovery, and both intraday and interday precision and accuracy. All criteria fulfilled the predefined acceptance thresholds.

The method exhibited excellent linearity, with coefficients of determination (R^2) consistently exceeding 0.99. The LOD and LOQ were determined to be 0.45 µg/mL and 1.70 µg/mL, respectively. No interfering signals corresponding to the Δ4-mer or its labelled analogues were observed in any of the four blank cosmetic serum formulations tested, confirming the high selectivity of the method.

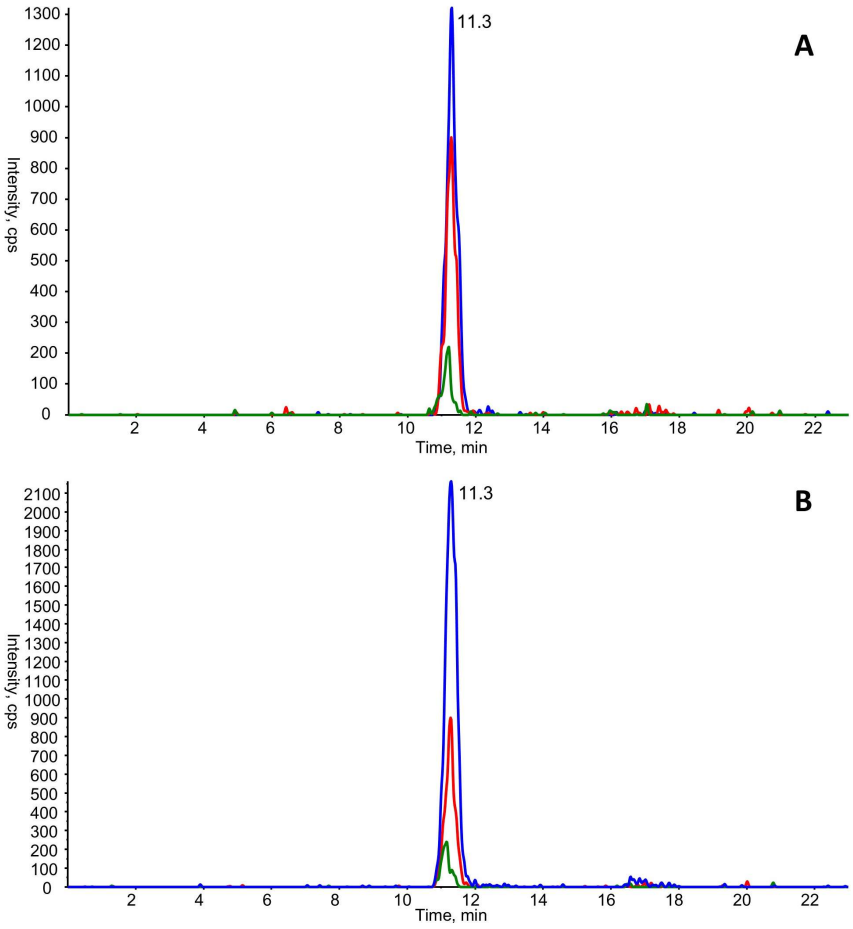
3.4. Method application

The validated method was subsequently applied to quantify HA in two commercially available cosmetic serums, purchased from a retail outlet in Milan and labelled as containing 2% HA (designated Serum A and Serum B). Given the lack of an appropriate blank matrix, quantification was performed using the standard addition method. Sample dilution and HA

spiking levels were optimised to maintain signal linearity without exceeding MS dynamic range. Internal standards were added at concentrations comparable to those of the analyte.

Figure 2 presents representative SRM chromatograms for the Δ4-mer, Δ4-mer 100%-^{13}C, and Δ4-mer 50%-^{13}C following HA addition at varying concentrations (0, 125, 250, and 375 µg/mL) to Serum A.

Table 2 summarises the HA content in both commercial samples, reported as % w/w (mean ± standard deviation).



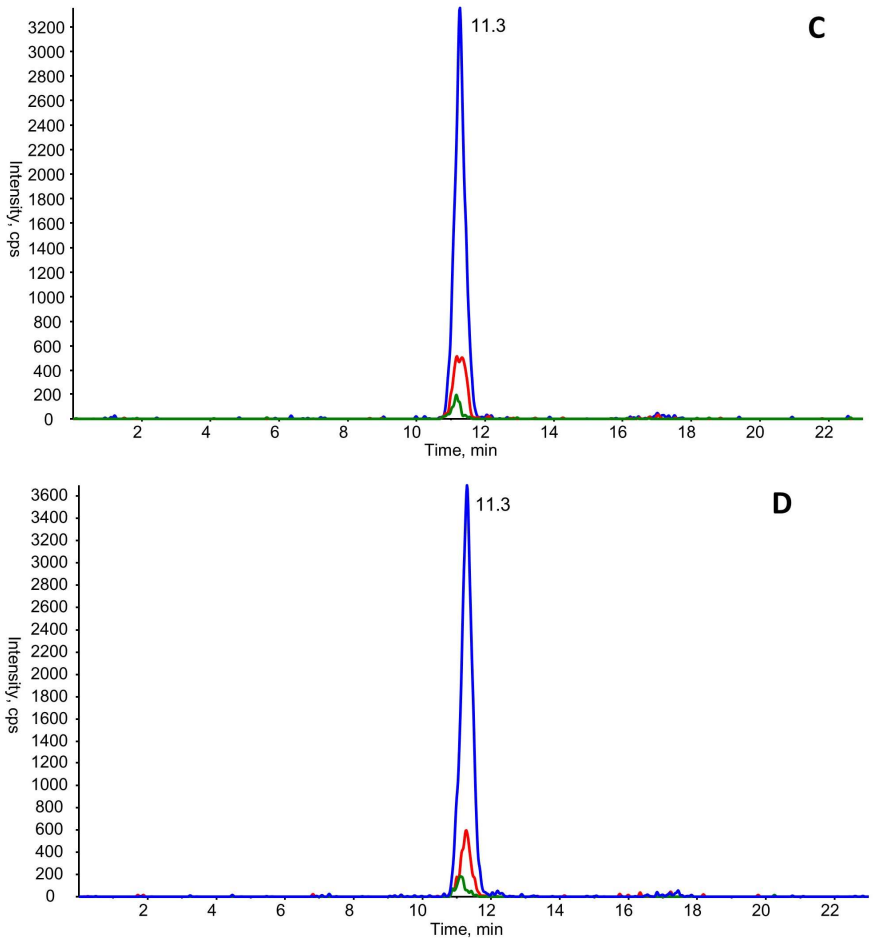


Figure 2. SRM chromatograms of Δ4-mer (blue), Δ4-mer 100% ^{13}C (red) and Δ4-mer 50% ^{13}C (green) with the addition of 0 (panel A), 125 (panel B), 250 (panel C), 375 µg/mL (panel D) of HA to serum A. The CV% relative to the two sample analyses were 7.20 % and 4.85 % indicating the high reproducibility of the method.

Table 2. Concentration (% w/w) of HA in the serum A and B.

Cosmetics	% w/w (mean±SD)
Serum A	1.973 ± 0.142
Serum B	2.109 ± 0.113

4. Discussion

The analytical strategy developed in this study demonstrated the feasibility of employing a highly specific and robust LC-MS/MS method for the quantification of hyaluronic acid (HA) in cosmetic products One of the primary challenges addressed was the inherently complex and variable composition of cosmetic matrices, which frequently undermines the applicability of conventional analytical approaches by introducing substantial matrix effects and compromising selectivity.

The proposed workflow, integrating an MTBE-based extraction protocol, enzymatic hydrolysis mediated by recombinant hyaluronidase, and subsequent isotope dilution LC-MS/MS analysis, proved effective in mitigating these obstacles. Notably, the implementation of two

isotopically labelled internal standards enabled correction for distinct sources of variability: IS1 (100%-^{13}C-HA) addressed enzymatic efficiency, while IS2 (50%-^{13}C-Δ4-mer) accounted for fluctuations in mass spectrometric response. Additionally, the application of the standard addition method facilitated accurate quantification even in the absence of a truly blank matrix, thereby extending the method's applicability to real-world cosmetic formulations with diverse compositions.

The analytical performance of the method was exemplified by excellent reproducibility and linearity, as indicated by the low coefficients of variation (CV%) and satisfactory accuracy values observed when analysing an in-house prepared cosmetic serum containing 2.00% HA. Subsequent application to two commercial serums, both labelled as containing 2% HA, yielded measured concentrations of 1.973 ± 0.142% (serum A) and 2.109 ± 0.113% (serum B), respectively, both of which closely aligned with the declared contents.

5. Conclusion

In summary, the method established in this study provides a robust, versatile, and broadly applicable platform for the quantification of HA across a wide range of cosmetic formulations, eliminating the need for product-specific calibration curves or blank matrices. This renders the approach particularly valuable for routine quality control, product development, and regulatory compliance activities within the cosmetic industry. Future research may focus on further extending the method's validation to additional formulation types—including emulsions, gels, and sprays—and on adapting the approach to monitor parameters such as HA degradation and molecular weight distribution, thereby enhancing its utility in both research and industrial settings.

6. References

[1] Juncan AM et al. Advantages of hyaluronic acid and its combination with other bioactive ingredients in cosmeceuticals, *Molecules* 2021;26:15.

[2] Ratajczak P et al. Directions of hyaluronic acid application in cosmetology, *J Cosmet Dermatol* 2023;22:862-871.

[3] Malaisse J. et al. Hyaluronan metabolism in human keratinocytes and atopic dermatitis skin is driven by a balance of hyaluronan synthases 1 and 3. *J Invest Dermatol* 2014;134:2174.

[4] Soato M. et al. A versatile and robust analytical method for hyaluronan quantification in crosslinked products and complex matrices. *Carbohydrate Research* 2021;503:108314.

[5] Ku¨hn AV et al. Quantification of hyaluronic acid fragments in pharmaceutical formulations using LC–ESI–MS. *JPBA* 2003;30:1531.

[6] Šimek M et al. Analysis of hyaluronan and its derivatives using chromatographic and mass spectrometric techniques. *Carbohydrate Polymers* 2020;250:117014.

[7] Suárez-Hernández LA et al. Validation of an analytical method for the simultaneous determination of hyaluronic acid concentration and molecular weight by size-exclusion chromatography, *Molecules* 2021;26:17.

[8] Volpi N et al. High-performance liquid chromatography-mass spectrometry for mapping and sequencing glycosaminoglycan-derived oligosaccharides, *Nat Protoc* 2010;5:993.

[9] Volpi N. et al. High-throughput glycosaminoglycan extraction and UHPLC-MS/MS quantification in human biofluids. *Nat Protoc* 2025;20:843.

[10] Chindaphan K. et al. Online preconcentration and determination of chondroitin sulfate, dermatan sulfate and hyaluronic acid in biological and cosmetic samples using capillary electrophoresis. *J Sep Sci* 2019;42:2867.