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

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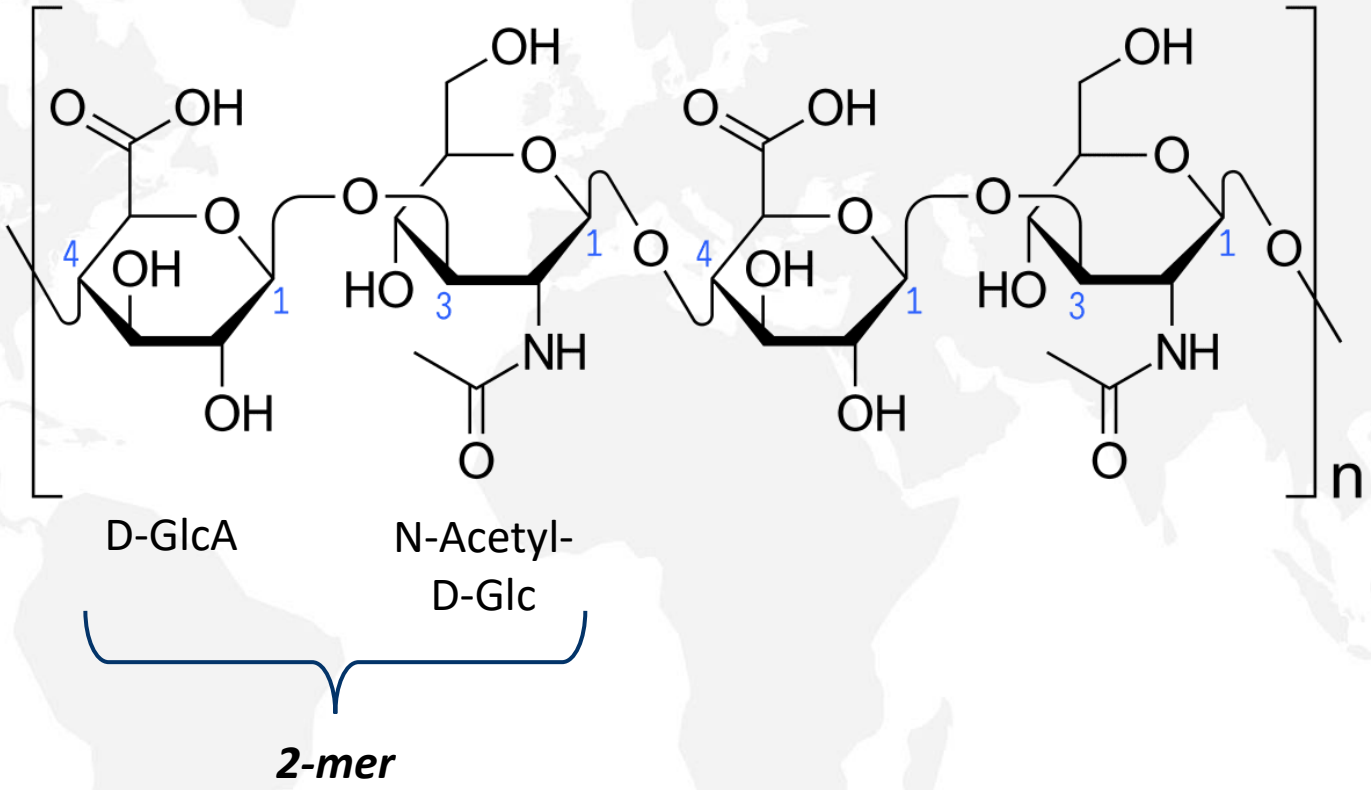
BACKGROUND

Hyaluronic acid (HA) is a non-sulphated polysaccharide whose disaccharide unit is composed of D-glucuronic acid (**D-GlcA**) and N-Acetyl-D-Glucosamine (**N-Acetyl-D-Glc**) linked by alternating β1→3 and β1→4 glycosidic bonds.

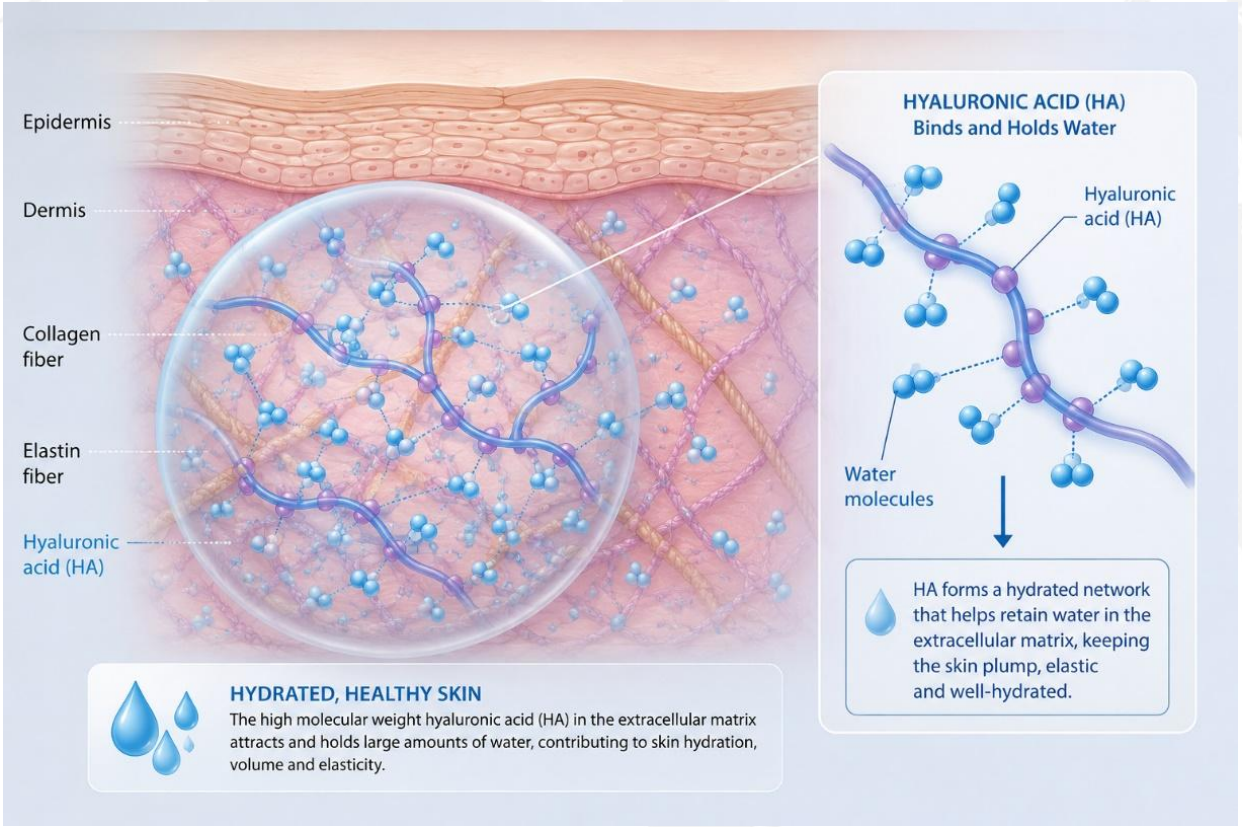


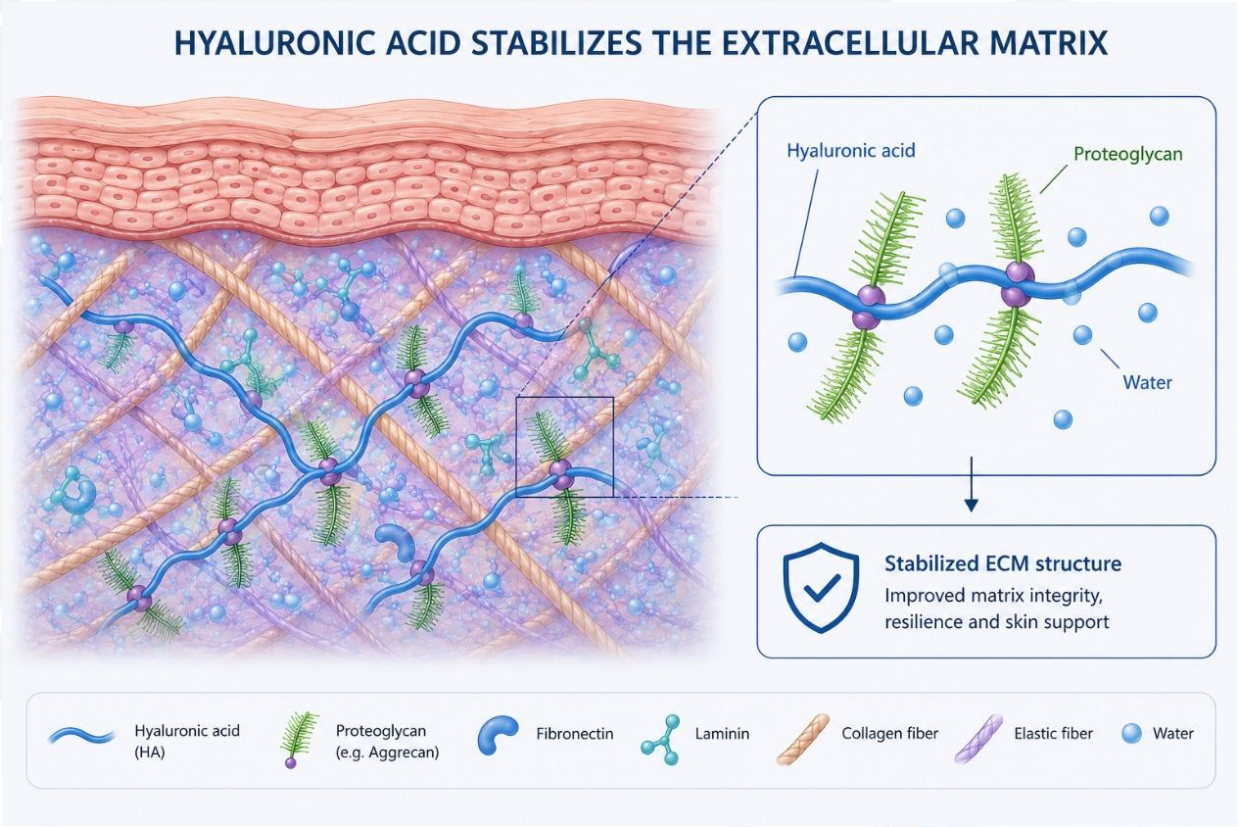
Within biological systems, HA plays a multitude of biological roles that can vary significantly depending on the size of the polysaccharide or the specific location in which it exerts its function.

BACKGROUND

HA exerts its activity through a **physical** and a **ligand-based** mechanism.

The **physical mechanism**: high molecular weight HA (HMW-HA)

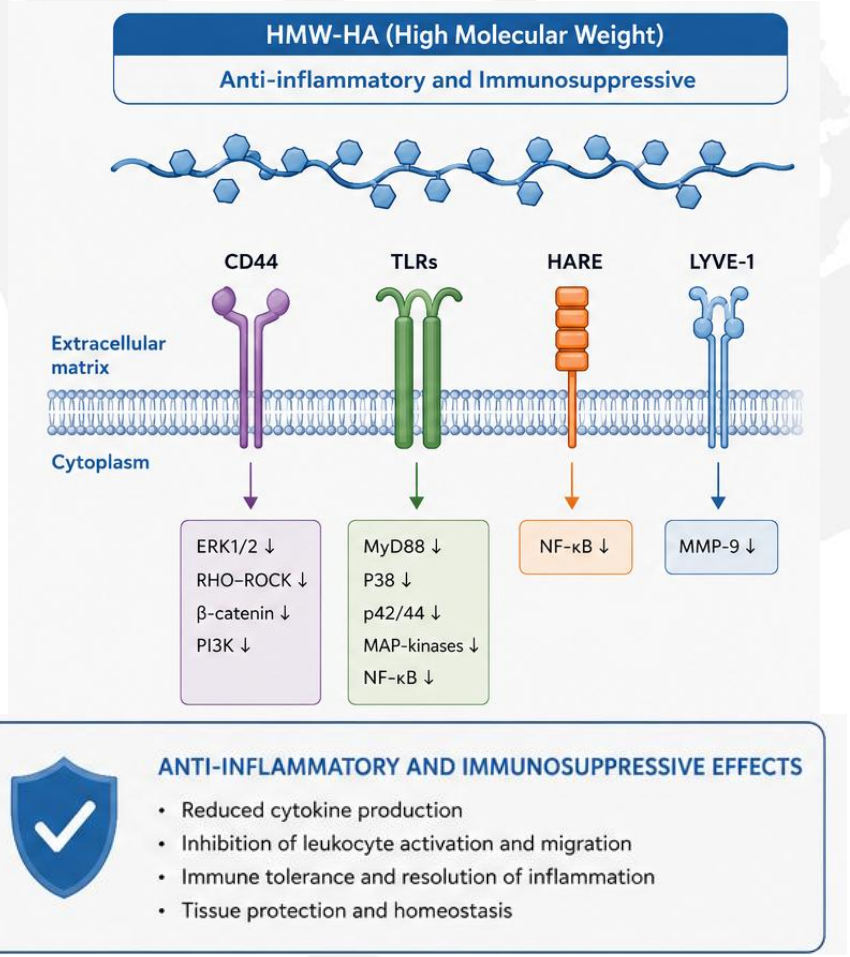


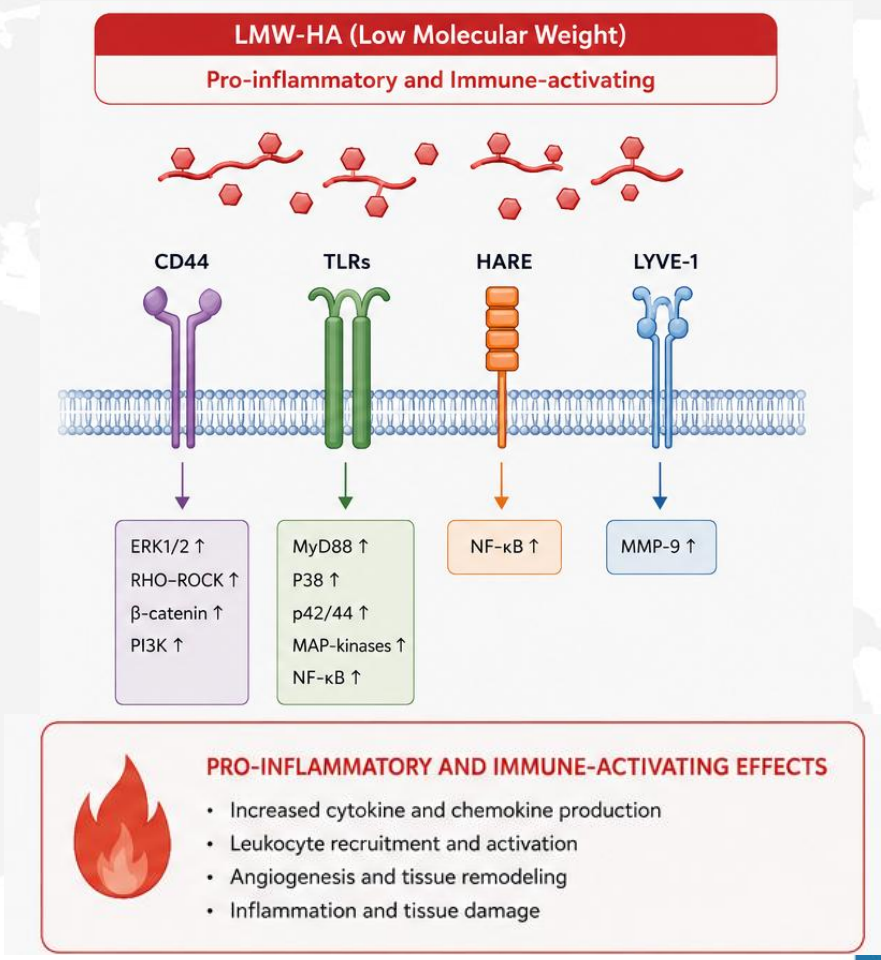


BACKGROUND

HA exerts its activity through a **physical** and a **ligand-based** mechanism.

The **ligand-based mechanism**: high molecular weight HA (**HMW-HA**) and low molecular weight HA (**LMW-HA**)

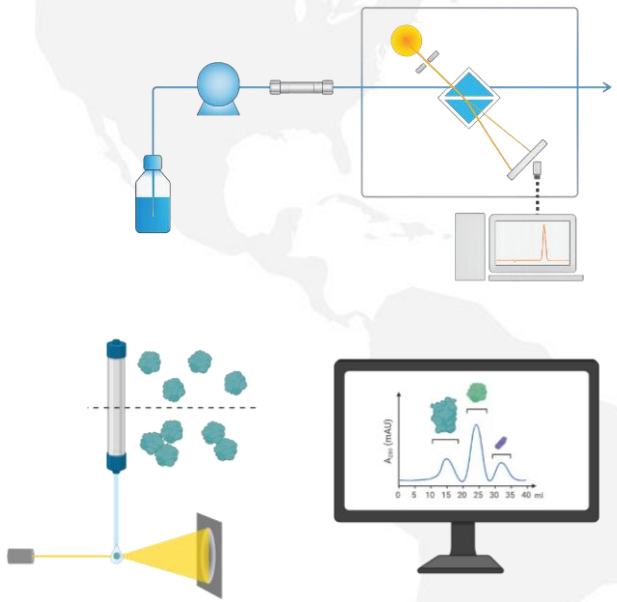




BACKGROUND

The analysis of HA is generally challenging due to the chemical nature of the analyte: it is a high-molecular-weight, non-volatile polymer lacking chromophore groups and exhibiting negligible ionization efficiency in mass spectrometry. As a result, HA is not easily detectable in its native form without specific depolymerization and derivatization.

SEC + RI or LS



Quantification of intact HA

✅ **Advantages**

- Direct analysis of native HA
- No sample preparation required
- Determination of molecular weight distribution

⚠️ **Limitations**

- Low sensitivity
- Limited selectivity
- RI and light scattering detectors are non-specific
- Interference from matrix components
- Suitable mainly for raw materials and simple matrices
- Challenging application to complex cosmetic formulations

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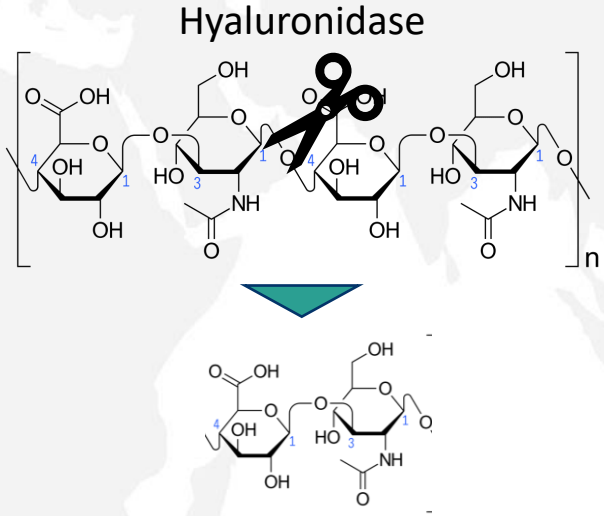
✅ **Advantages**

- High sensitivity
- High selectivity
- Accurate quantification
- Applicable to known biological matrices

⚠️ **Limitations**

- Labor-intensive workflow
- Multiple sample preparation steps
- Matrix-dependent method validation
- Limited transferability to complex cosmetic matrices

HPLC-MS



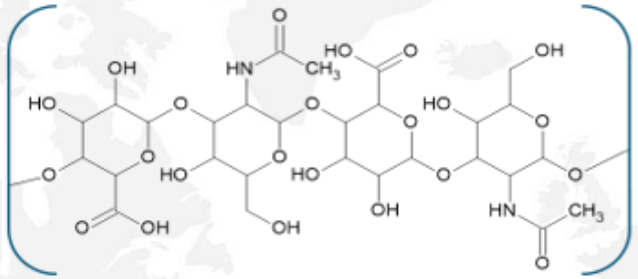
Hydrolysis of HA and oligomers quantification

AIM

The aim of the present study is to develop a **LC-ESI-MS/MS** method based on a general extraction procedure combined with the use of **suitable internal standards** and the **standard addition method** for quantitative analysis.

This approach is designed to be applicable across a broad range of cosmetic formulations, regardless of their specific composition.

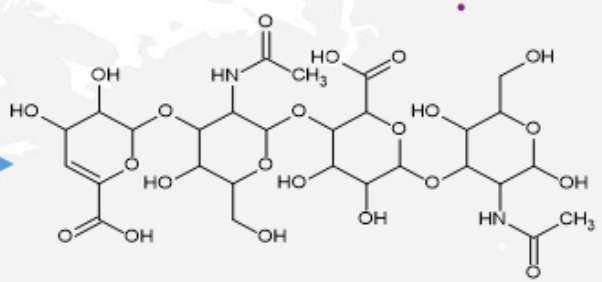
ANALYTICAL METHOD



Hyaluronic Acid

RSK

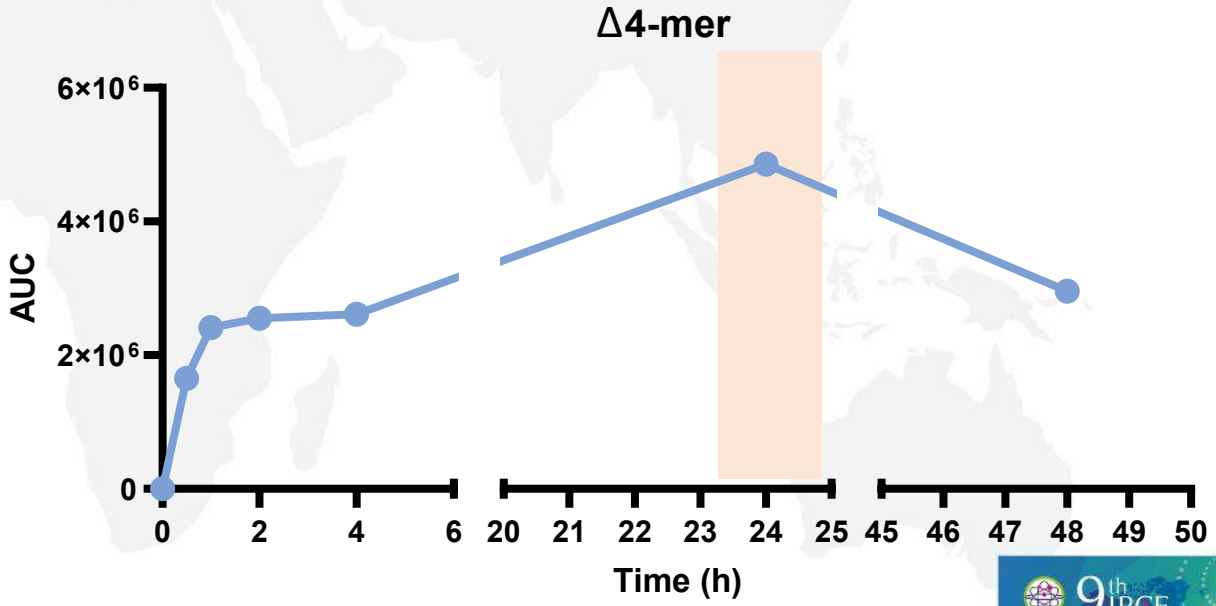
n



Δ4-mer

Hydrolysis protocol:

- *HMW-HA (20 mg/mL)*
- *Recombinant hyaluronidase from Streptomyces koganeiensis (RSK) in acetate buffer (100 mM, NaCl 150 mM, pH 5.2)*
- *Incubation up to 48h, 37 °C 350-400 rpm*
- *Cold methanol addition, at -20 °C for 30 min*
- *Centrifuged for 15 min, 4°C, 15200 rpm*
- *Dried under vacuum*
- *Resuspended in mobile phase*



ANALYTICAL METHOD

1 HA ^{13}C 100% IS

Acetate buffer pH 5.2

RSK 24h 37°C

HA ^{13}C 100% (3.4 mg/mL)

^{13}C 100% Δ4-mer

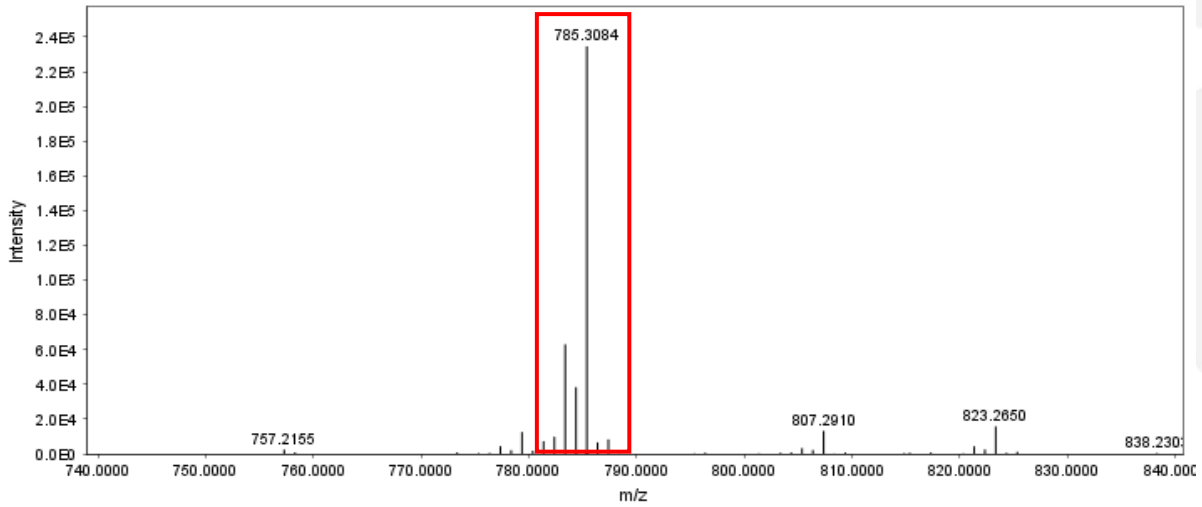
2 HA ^{13}C 50% IS

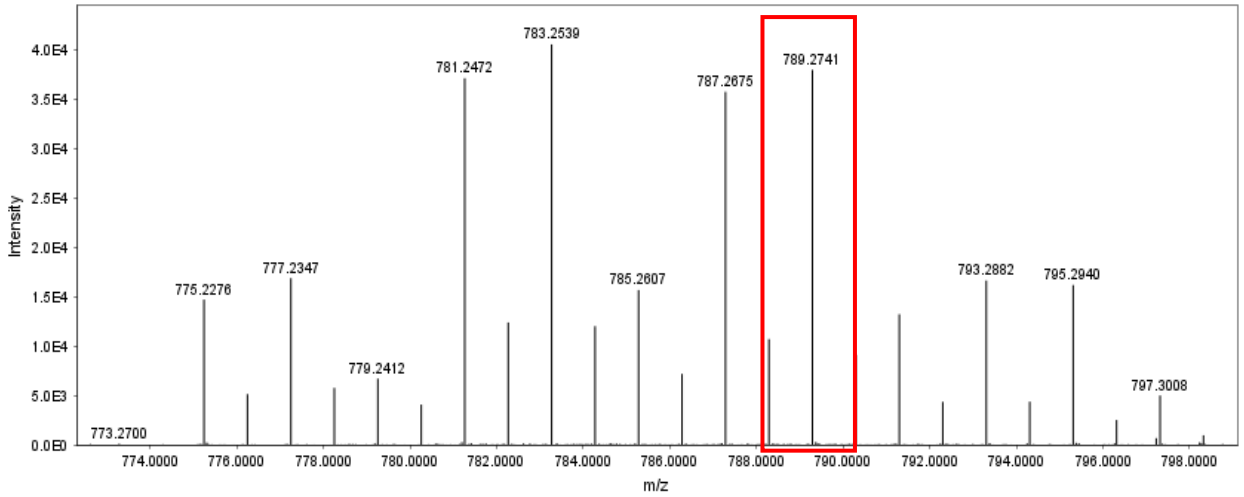
Acetate buffer pH 5.2

BTH overnight 37°C

HA ^{13}C 50% (10 mg/mL)

^{13}C 50% 4-mer





1 SAMPLE PREPARATION NORMALIZATION

INSTRUMENTAL RESPONSE NORMALIZATION 2

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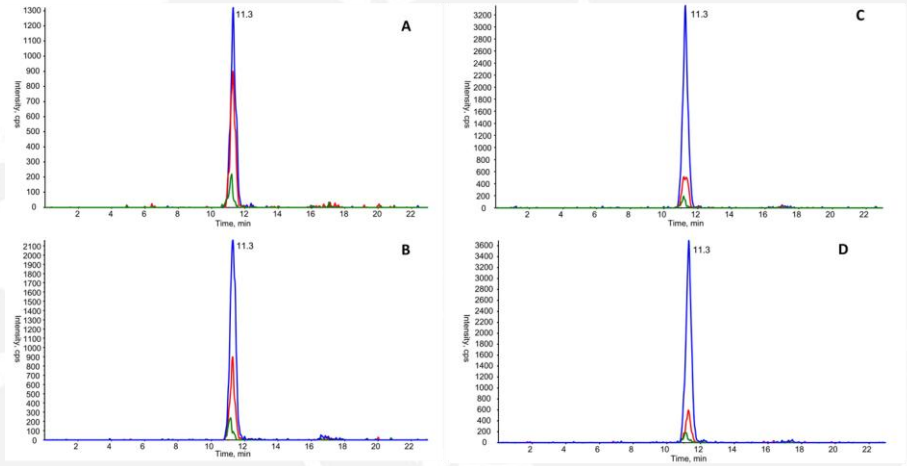
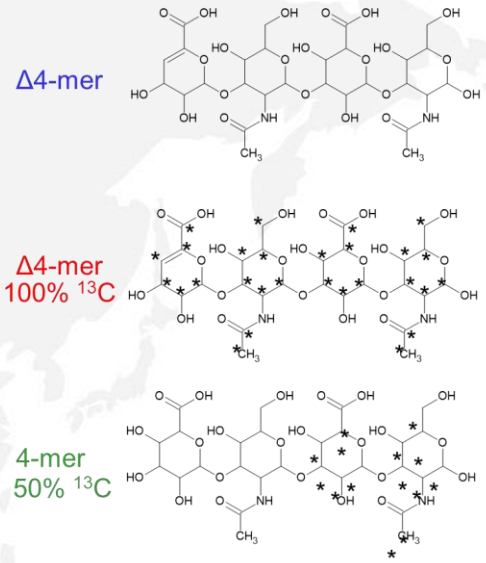
ANALYTICAL METHOD

Chromatographic conditions:

- *Gradient of NH_4COOH 100 mM pH 3) and CH_3CN*
- *Hypersil GOLD HILIC (3 µm, 2.1 x 150 mm), 40°C*
- *Constant flow rate 300 µL/min*
- *HPLC Exion LC100 (AB Sciex)*

Standard addition method





MRM PARAMETERS

Compound	Parent ion	Product ion	CE (volt)	DP	CXP
Δ4-mer	757	554	-48	-100	-15
4-mer 50% 13C	789	199	-48	-100	-15
Δ4-mer 100% 13C	785	574	-48	-100	-15

5500 Triple Quadrupole (Sciex)

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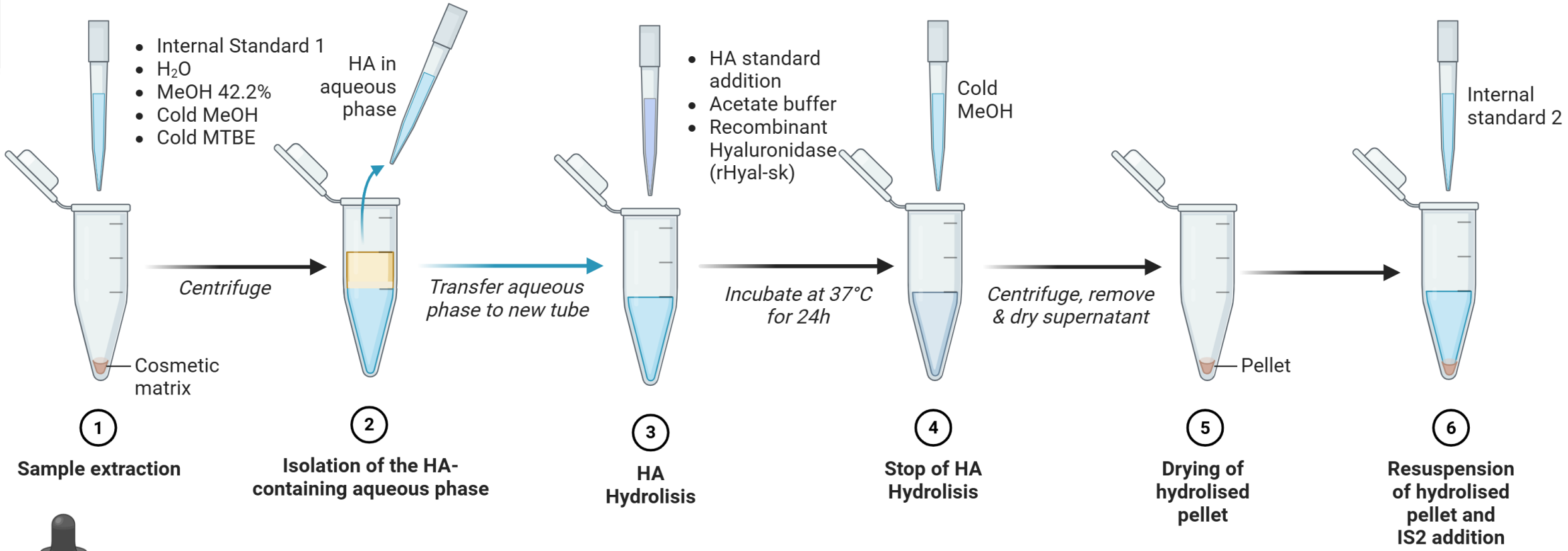
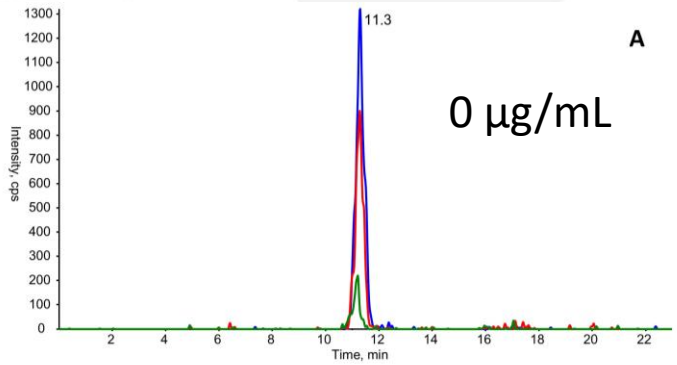
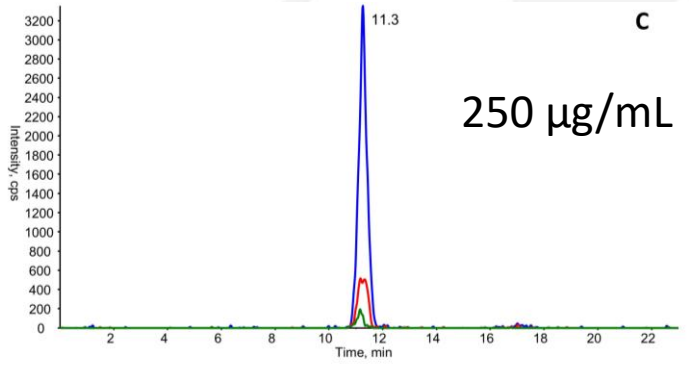
SAMPLE PREPARATION

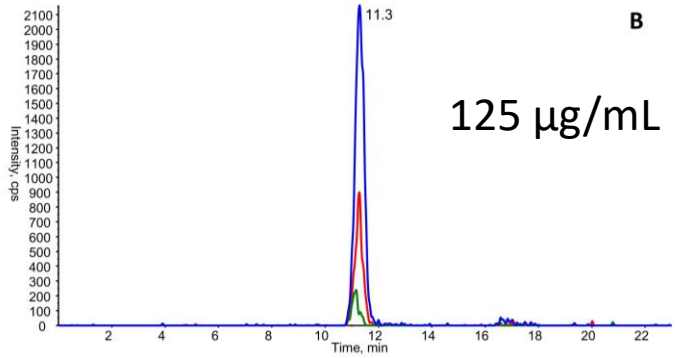


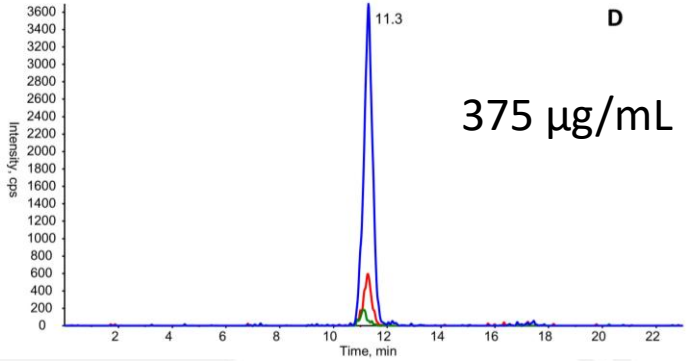
1. **Sample extraction**
2. **Isolation of the HA-containing aqueous phase**
3. **HA Hydrolisis**
4. **Stop of HA Hydrolisis**
5. **Drying of hydrolised pellet**
6. **Resuspension of hydrolised pellet and IS2 addition**

APPLICATION TO SERUM SAMPLES









Cosmetic	% w/w (mean±SD)	CV%
Serum A	1.973 ± 0.142	7.20
Serum B	2.109 ± 0.113	4.85

CONCLUSIONS

The method developed offers a robust and versatile platform for the quantitation of HA in a wide range of cosmetic formulations, without requiring product-specific calibration curves or blank matrices.

This makes it a valuable tool for quality control, product development, and regulatory compliance in the cosmetic industry. Future work may focus on further validating the method for other types of formulations (e.g., emulsions, gels, sprays), as well as expanding the approach to monitor HA degradation or molecular weight distribution.

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