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Characterization of suspected dermal fillers containing hyaluronic acid

Celine Vanhee,* Bart Desmedt, Sébastien Baudewyns, Angélique Kamugisha, Mélissa Vanhamme, Jean-Luc Bothy, Sona Kovackova, Patricia Courselle and Eric Deconinck

Hyaluronic acid (HA) has important applications in the field of orthopedics, ophthalmology and cosmetics. This polymer can be present in cosmetic products or can be used as a dermal filler. The latter frequently contains cross-linked HA. Today's methods to quantify the amount of cross-linked HA are not very specific. In this paper the development and validation of a specific identification and quantification method is described. The proposed method utilizes an essential pre-extraction step prior to identification by infra-red spectroscopy followed by an identity confirmation and quantification with the carbazole micro-assay. The method was successfully validated using the "total error" approach in accordance with the validation requirements of ISO-17025. During the validation a variety of formulations including those present in legal HA containing dermal fillers were taken into consideration. As a proof of principle, legal and suspected illegal dermal fillers intercepted in 2016 were assessed. All legal samples and 2 out of 14 suspected illegal samples were conform for the amount of HA claimed and present in the sample. Most of the suspected illegal samples contained much less than what was claimed on the package. Taken together, the newly developed and validated method can be used for the identification and quantification of HA in legal and suspicious HA-containing dermal fillers. Furthermore, this method doesn't require the use of expensive instrumentation or reagents and can therefore be executed in any laboratory equipped with a centrifuge, micro-plate reader and heating block.

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1 Introduction

The pursuit to comply with today's beauty standards has led to an increasing demand for cosmetic and aesthetic procedures. The yearly revenue of this business amounts to hundreds of billions of dollars and is estimated to increase even more in the future. For instance, the total expenditure of Americans on nonsurgical cosmetic procedures was more than $5 billion in 2015.[1] In addition to creams, serum and lotions, soft tissue fillers or dermal fillers are also gaining popularity. Apparently in the United States of America (USA), injections with hyaluronic acid (HA) containing temporal dermal fillers were the second most frequently performed cosmetic procedure after BOTOX® injections.[2] HA injections are being used to create a smoother and/or fuller appearance in the face, including the nasolabial folds, cheeks and lips. The effect of this macromolecule is thought to last for about six months. Moreover, in the USA and in the European Union (EU) the procedure can only be performed by a dermatologist or a plastic surgeon. Depending on the origin of hyaluronic acid, either from recombinant bacterial production technologies or by extraction from animals, these dermal fillers are respectively classified as class IIb or class III medical devices.[3] Consequently, they must display the appropriate CE marking, including the four-digit number reflecting the notified body, on their sterile packaging. Moreover, these products must also contain an accompanying leaflet or link to a digital leaflet. In addition to the product directive, health care professionals residing in the USA must limit their use of these products to only Food and Drug Administration (FDA)-approved injectable wrinkle fillers which have been listed.[4] Unfortunately no such list exists in the EU, however, efforts are being made to generate such a list.[5] This shortcoming makes it very difficult to know exactly what is legally on the market. Nevertheless, similar to medicines, illegal HA-containing products can be bought online and are being intercepted by customs or during routine inspections at the different shipment companies. In Belgium, this task is, in collaboration with law enforcement agencies and customs, allotted to the Federal Agency for Medicines and Health Products (FAMHP). Samples seized by the FAMHP, including suspicious medicines, food supplements and dermal fillers are routinely analysed by our official medicines control laboratory (OMCL). Last year, a demand was raised by the inspectorate to analyze suspicious HA-containing dermal fillers. HA, also known as hyaluronan or hyaluronate, is a glycosaminoglycan occurring

Division of Food, Medicines and Consumer Safety, Section Medicines and Health Care Products, Scientific Institute of Public Health (WIV-ISP), J. Wytmanstraat 14, B-1050 Brussels, Belgium. E-mail: Celine.Vanhee@wiv-isp.be; Tel: +32 642 51 42

naturally throughout the human body. Glycosaminoglycans (GAGs) are linear polysaccharides comprised of different repeating galactose or uronic acid–hexosamine disaccharides. HA can be several thousands of disaccharide units long. The quantification method, currently used by at least two HA-based dermal filler producing companies is based on the method described in the European Pharmacopoeia for the determination of the amount of HA present in raw materials.[6] In this colorimetric method, carbazole interacts with uronic acid, obtained post acid hydrolysis of HA, and generates a violet color that absorbs at 530 nm. It stands to reason that this assay is not very specific and will interact with all uronic acid containing molecules, including heparin, chondroitin sulphate and dermatan sulphate. Furthermore, this assay also generates positive results for many carbohydrates.[7] Moreover, it has also been reported that the co-occurrence of phosphate or salts and HA can lead to an overestimation of the amount of HA present in the sample.[8] Therefore alternative methods, to identify and quantify linear natural HA, have been developed. These methods include HPLC based methods with either a Photo Diode Array Detector (PDA), light scattering, fluorescence or mass spectrometry (MS) detector.[8–12] Additionally, also enzyme-linked immunosorbent assay (ELISA)-based methods and turbidimetric methods have been developed.[13,14] However, only the turbidimetric[14] and carbazole methods have been reported to be compatible with the quantification of cross-linked HA, which is often the preferred form in which this molecule is present in dermal fillers. The first method, similar to the carbazole assay, also suffers from possible interference from other GAGs. Therefore, it is pivotal for the public health sector to develop reliable, efficient, low cost and easily applicable cross-linked HA identification and quantification strategies for routine analysis of suspected HA containing dermal fillers. Here, we present such a specific and selective HA identification and quantification method, where an additional extraction step, prior to the carbazole micro-assay, is able to resolve possible interference issues that could be encountered when dealing with suspicious illegal samples. Interestingly, this methodology doesn't require the use of expensive instrumentation or reagents and labeled standards, and can therefore be executed in any laboratory equipped with a centrifuge, microplate reader and heating block.

2 Materials and methods

2.1 Standards and reagents

The reference standards for low M_w (8–15 kDa, lot BCBQ5909V) and high M_w Na-hyaluronic acid (2–2.4 MDa, lot BCBJ2311V) were purchased from Sigma-Aldrich (St Louis, USA). Sodium chloride (batch K45393104), gelatin (lot K32373978), starch (lot F1721952-218), calcium chloride dehydrate (lot A431982), and analytical grade formic acid were bought from Merck (Darmstadt, Germany). D-Glucuronic acid (lot SLBM3408V), chondroitin-6 sulphate (lot 067K095), chondroitin-4 sulphate (lot 075K1869), sucrose (lot SLBD2965V), melanotan II acetate salt (lot 091M4715V), polyethylene glycol 4000 (batch BCBM7029V), glutathione (lot 083K0684), sodium carboxymethyl cellulose (lot 058K0112), cetyl pyridinium chloride (lot SLBC7204V) and carbazole (lot BCBP5577V) were purchased from Sigma-Aldrich (St Louis, USA). Lidocaine hydrochloride monohydrate (lot 15C16-B05-311031), mannitol (lot 13A14-B02-281665), and glycerol (lot 15H26-B06-316079) were obtained from Fagron (Waregem, Belgium) while dermatan sulphate, oversulphated chondroitin sulphate (lot 2) and sodium heparin (lot 3.1) were obtained from EDQM (European Directorate for the Quality of Medicines). Carbopol 974P (lot M719031) was bought from Asta Medica (Essen, Germany), the copper peptide was acquired from Peptide Sciences (https://www.peptidesciences.com/) and BOTOX® (lot STD015) was a kind gift from Allergan (Dublin, Ireland). UPLC-MS grade acetonitrile and absolute ethanol were purchased from Biosolve (Valkenswaard, the Netherlands). Water was obtained using a Milli-Q Gradient A10 system (Millipore, Billerica, MA, USA). The 10 × concentrated tris buffer saline, abbreviated as 10 × TBS (lot 200006787), was obtained from Bio-Rad (Hercules, CA, USA).

2.2 Sample set of legal and suspected illegal hyaluronic acid containing medical devices

The legal sample set consisted of 8 different products that were kindly provided by two popular dermal filler producing companies. The sample set with suspected samples was taken by inspectors from the FAMHP. These suspected samples do not include, at least to our knowledge, counterfeited samples of the legal samples used in this study or counterfeits of the FDA-approved injectable wrinkle fillers. Collection of these 14 samples took place in 2016. Due to the confidential nature of inspection data it is not possible to give precise information about each sample individually.

2.3 Sample preparation

2.3.1 Calibration standards. Standard stock solutions of both low M_w and high M_w HA (2 mg ml^{-1}) were made in pure water and placed overnight at 2–8 °C to hydrate the macromolecule. In order to generate the calibration curves for quantitative analysis, standard stock solutions were diluted into 8 different concentrations in pure water and were also placed overnight at 2–8 °C prior to analysis. The selected concentration intervals (0.1–1 mg ml^{-1}) for the validation of the quantitative method correspond to 10 to 200 times dilutions of concentrations that can often be found in these dermal fillers.

2.3.2 Standard mock solutions. The standard stock solutions of the different mocks, carboxymethyl cellulose (CMC), chondroitin-6 sulphate (CS-6), chondroitin-4 sulphate (CS-4), heparin, dermatan sulphate (DS), lidocaine, glucuronic acid (GlcA), gelatin, melanotan II, copper peptide (Cu-peptide), starch and gluthatione (GSH) (10 mg ml^{-1}), were made in pure water. BOTOX® (100 IU) was resuspended in 0.5 ml pure water and a 50% (v/v) glycerol, a 5% (w/v) sucrose, a 10% (w/v) PEG and a 10% (w/v) mannitol solution was also prepared with pure water.

2.3.3 Sample set. The injectables containing or suspected to contain HA were diluted with pure water and left overnight at 2–8 °C prior to analysis. For quantification purposes, the

samples were diluted until a concentration within the interval of the calibration line was obtained.

2.4 General methodology

2.4.1 Extraction method. The extraction protocol comprises several sequential precipitation steps/salting out steps that are listed in Fig. 1. The initial step serves to remove most of the small molecules from the larger macromolecules as has been described for the purification of HA from fish eyeballs.[15] Briefly, 100 μl of a 3 M NaCl solution was mixed with a 100 μl sample, followed by an addition of 800 μl ethanol prior to a short mix and 2 hours of overnight incubation on ice. Next, the sample was centrifuged for 5 minutes at 20 238 × *g* and the supernatants were discarded prior to a wash with 70% ethanol. The resulting pellet was dried at 50 °C, followed by an overnight reconstitution in 100 μl pure water at 2–8 °C. Next, CMC was removed by salting out with the addition of $CaCl_2$ (to a final concentration of 0.1 M) and ethanol (to a final concentration of 20% (v/v)) as mentioned in Lali *et al.*, 2000.[16] Subsequently, sulphated GAGs were salted out by the sequential addition of 7 μl of 1 M HCl and 50 μl of 5% (w/v) CPC, prior to a swift vortex and incubation for 5 minutes at room temperature.[17] After the incubation, the samples were centrifuged for 5 minutes at 20 238 × *g* and the supernatants were again centrifuged for 2 minutes at 20 238 × *g*. The upper phase was transferred to a fresh tube where 200 μl of 3 M NaCl and 800 μl ethanol were added to remove all remnant sulphated GAGs. The mixture was again centrifuged for 5 minutes at 20 238 × *g* and the soluble phase was used for subsequent precipitation of HA under alkaline conditions. These conditions were obtained by the sequential addition of 110 μl of 10 × TBS solution and 10 μl of 5 M NaOH to the sample, followed by a minimum of 2 hour incubation on ice. Next, the sample was centrifuged for 5 minutes at 20 238 × *g*, the supernatants were discarded and the pellet was washed with 70% ethanol and dried at 50 °C.

2.4.2 Identification by ATR-FTIR. The pellet obtained by the extraction was then further analyzed by means of Attenuated Total Reflectance-Fourier Transform InfraRed spectroscopy (ATR-FTIR). Briefly, all spectra were recorded in absorption mode using a Nicolet iS10 FT-IR spectrometer (Thermo Scientific, Madison, USA) that accommodated a Smart iTR accessory, equipped with a single bounce diamond crystal, for ATR sampling and a deuterated triglycine sulfate (DTGS) detector. Each spectrum was measured at a spectral resolution of 4 cm^{-1} and consisted of 16 co-added scans. Recording of the spectrum was performed in the range of 4000 to 650 cm^{-1}. Spectral data were obtained using the OMNIC Software version 8.3 (Thermo Scientific, Madison, USA). After each measurement, the crystal was cleaned using a soft tissue soaked with methanol or water and left to dry in ambient air. Before each sample a blank was measured to check the crystal for contamination and carryover using the absorbance limits for contamination defined by the European Directorate for the Quality of Medicines and HealthCare (EDQM).[18] Spectral queries were performed with the OMNIC software for the fingerprint region ranging from 1800 to 650 cm^{-1}.[19]

2.4.3 Identification and quantification by the carbazole micro-assay. The previously obtained pellet was reconstituted in 100 μl pure water and left to hydrate overnight at 2–8 °C prior to quantification by means of the carbazole micro-assay, developed by Frazier *et al.*, 2008.[8] The absorbance at 530 nm was measured using an iMark Microplate reader (Bio-Rad, Hercules, CA, USA). GlcA was used as an external standard for generation of the standard curves of the HA standard while the HA standard was used to generate the calibration curves for the mock samples and the quantification of HA in the legal and suspected illegal samples. The different curves were derived by plotting the absorbance against the concentration of either GlcA or HA.

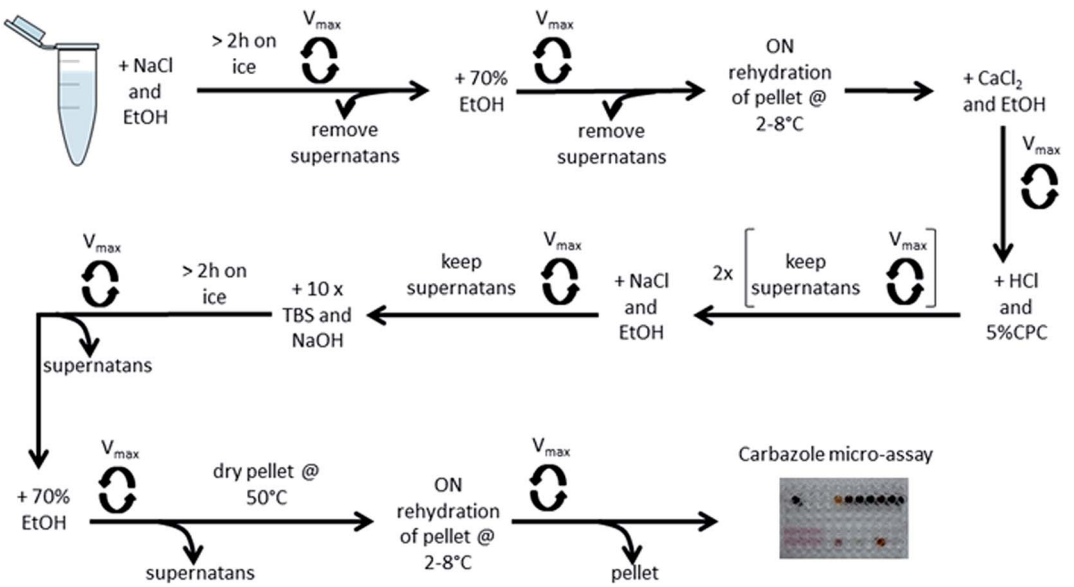

Fig. 1 Schematic overview of the extraction protocol consisting of several sequential precipitation steps/salting out steps which will result in a pellet that can be directly analyzed by ATR-FTIR (identification) and carbazole micro-assay (identification and quantification).

2.5 Validation of the identification and quantification method

An identification method should reliably identify and distinguish HA from components possibly present in the suspected samples. Furthermore, the method should also generate as little as possible false positives and false negatives. For this reason we utilise the combined screening with ATR-FTIR and the carbazole micro-assay. Only when both methods are in agreement with the presence of HA, the sample is considered positive for HA. To ensure selectivity, colorimetric measurement, which could be affected by the interacting compounds, was also performed on different possible interferents alone (referred to as mock samples) and on these mock samples spiked with 0.5 mg ml^{-1} low M_w HA. For an identification method there are no requirements with regard to linearity and recovery according to method validation criteria.[20] However, a screening detection limit (SDL), the lowest concentration for which it has been demonstrated that a given analyte can be detected in at least 95% of the samples, is required. The concentration used for validation of the identification method, concurrent with an SDL of 0.1 mg ml^{-1}, was chosen to be 10 to 200 times lower than the amount typically present in the commercially available products and still results in a detectable pellet for ATR-FTIR analysis.

The present quantification method was validated according to ISO-17025 applying accuracy profiles, which are based upon the "total error" approach, reflecting an estimation of the highest error inherent to an analytical method. Three independent replicates of the different dilutions (0.1, 0.5 and 1 mg ml^{-1}) of either the low M_w HA or the high M_w HA were made daily and analyzed for four consecutive days. The corresponding concentrations were back-calculated using the calibration lines generated during each experiment. These calculated concentrations were then used to determine the linearity of the results, trueness, precision (repeatability and intermediate precision) and accuracy. To ensure that this method was capable of analyzing different types of formulations, a recovery study for five additional formulations was performed. Formulation 2 consisted of HA in PBS with 3 mg ml^{-1} lidocaine, formulation 3 contained formulation 2 supplemented with 5 mg ml^{-1} CMC, formulation 4 again contained formulation 2 supplemented with 2 mg ml^{-1} CS-4, formulation 5 contained formulation 2 with the addition of 5 mg ml^{-1} gelatin while formulation 6 consisted of 50% (v/v) glycerol in PBS and 3 mg ml^{-1} lidocaine.

3 Results and discussion

3.1 Visual inspection of the legal and suspected illegal HA containing dermal fillers

Prior to chemical analysis the samples were subjected to inspection of their conformity to the appropriate CE markings (CE label and 4 digit code for the identification of the notified body) and the presence of an accompanying leaflet (paper or link to the digital version). The medical devices directive states that the appropriate CE-marking is required on the sterile packaging. However, the CE-label is not required on the syringe itself if this device is part of sterile packaging.[21] From the results, represented in Table 1, it's clear that only 2 of the 8 legal samples had the appropriate CE-markings on the sterile packaging (see Table 1 and Fig. 2A). Half of the legal samples did not respect the dimensions of the CE-marking and often showed the misleading China export

Table 1 CE-labelling of legal and suspected illegal samples

Sample	Sterile package	Syringe	Accompanying leaflet
1	Dim	+	+
2	Dim	+	+
3	Dim	+	+
4	Dim	+	+
5	–	+	+
6	–	+	+
7	+	+	+
8	+	+	+
9	Wrong	–	–
10	Wrong	–	–
11	Wrong	–	–
12	Wrong	–	–
13	+	–	+
14	–	–	–
15	–	–	–
16	–	–	–
17	–	–	+
18	–	–	–
19	–	–	–
20	–	–	–
21	Wrong	–	–
22	Wrong	–	–

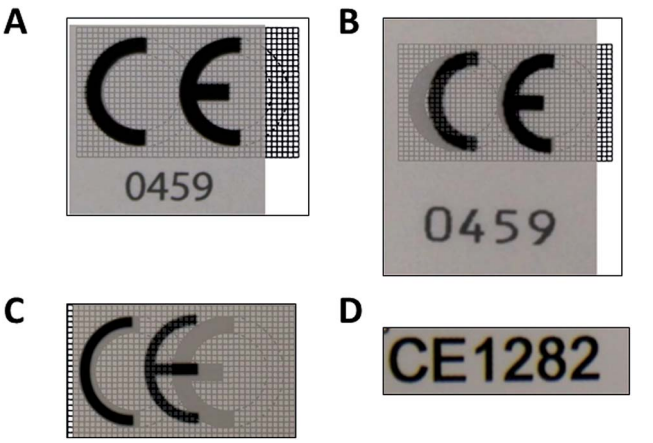

Fig. 2 Prior to chemical analysis the samples were subjected to inspection of their conformity to the appropriate CE markings on the sterile packaging (CE label and 4-digit code for the identification of the notified body) and the presence of an accompanying leaflet (paper or link to digital version). The CE-labels of the products were graphically overlaid on the official CE-template available online.[26] Conformity to the CE-label present on legal sample 7 (A), CE-label found in legal sample 1 but with aberrant dimensions (B) and CE-labels present on suspected illegal sample 9 (C) and sample 21 (D).

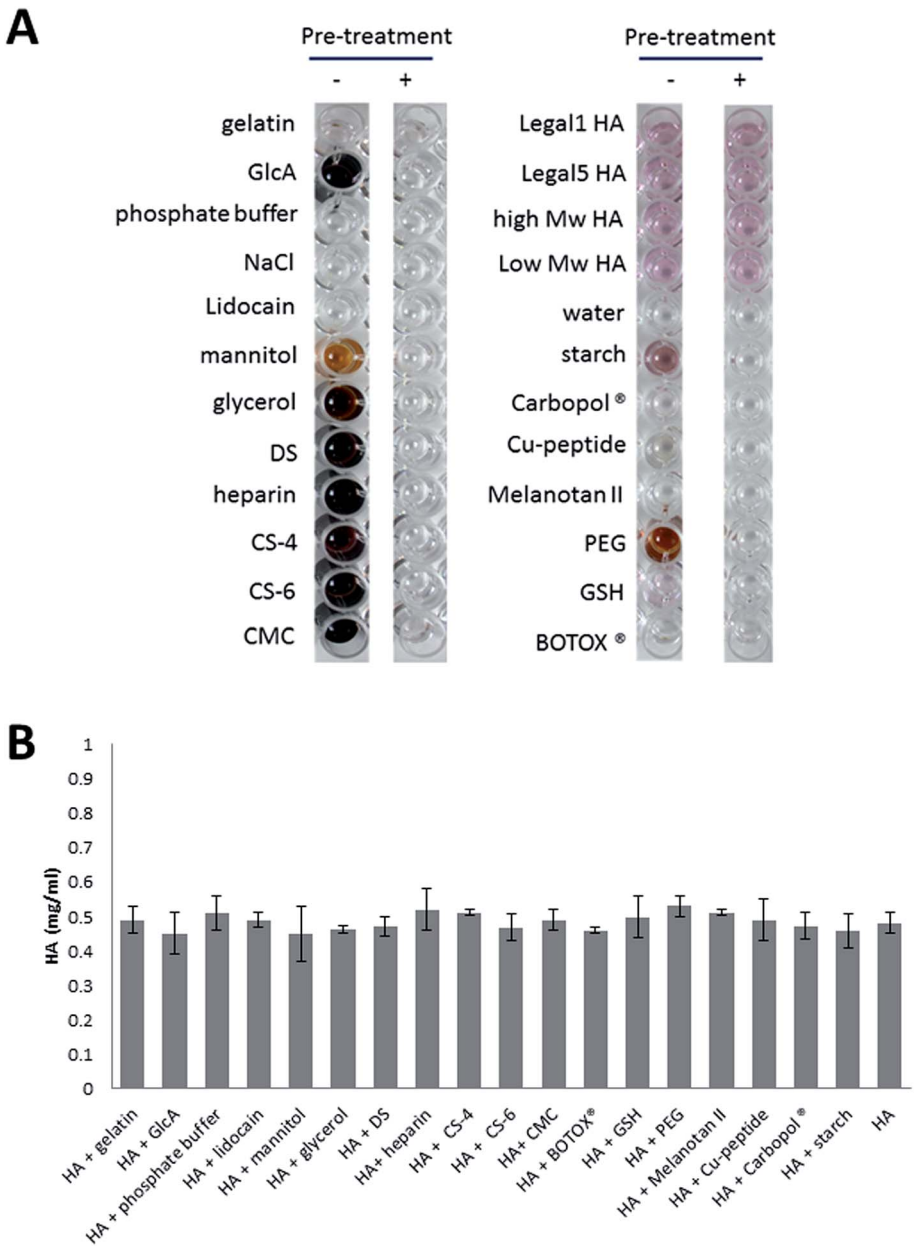

Fig. 3 Representative carbazole micro-assay performed on mock samples and HA containing samples (A) and quantification of 0.5 mg ml^{-1} HA present in those different mock samples (B). Statistical analysis was performed by using Student's t-test ($\alpha = 0.05$).

logo (see Fig. 2B). From the suspected illegal samples only one sample conformed to the CE-marking present on the sterile packaging. Startlingly, this sample, sample 13, originated from the same suspicious company as sample 14, which lacked any CE-labelling. Also the 6 other suspected illegal samples (samples 9–12 and samples 21 and 22), contained CE-labels, however these labels were aberrant (see Table 1 and Fig. 2C and D). Moreover, only 2 of the suspected illegal samples had a mandatory accompanying leaflet, while all legal samples enclosed such a leaflet. Taken together based on the above-mentioned visual inspection, startlingly only 2 legal and one suspected illegal sample conformed to their CE-labelling.

3.2 Development and validation of the identification method

Highly specific determinative procedures including chromatography coupled to mass spectrometry are very selective. In contrast, colorimetric measurements can be affected by the presence of chemicals with similar properties to those of the analyte. Therefore, we have analyzed the behavior of potential interferents in the carbazole assay. These interferents include a list of substances that are known to be present in legal samples (mannitol, NaCl, phosphate buffer and lidocaine), possible interferents that generate a gel-like appearance (gelatin, Carbopol, PEG and CMC), other GAGs (heparin, CS-4, CS-6 and dermatan sulphate), glycans (GlcA and sucrose),

Table 2 Trueness, precision, accuracy and uncertainty of the quantification method for low and high M_w HA

Compound	Concentration (mg mL^{-1})	Linearity R^2	Trueness (relative bias (%))	Precision		Accuracy (β-expectation tolerance limits (%))	Uncertainty (relative expanded uncertainty (%))
				Repeatability (RSD)	Intermediate precision (RSD)		
Low M_w HA	0.1	0.9995	−5.57	7.67	7.73	[−19.6; 8.5]	16.0
	0.5		−1.44	4.23	4.9	[−10.7; 7.8]	10.3
	1.0		0.33	2.62	7.03	[−16.7; 17.4]	15.6
High M_w HA	0.1	0.9999	−4.00	4.24	7.01	[−19.1; 11.1]	15.2
	0.5		2.60	2.90	3.67	[−4.6; 9.8]	7.8
	1		0.83	2.83	4.33	[−8.3; 9.9]	9.4

other molecules that can be used in aesthetic procedures (BOTOX®, melanotan, Cu-peptide and GSH) and a very popular compound amongst malignant entities, starch. Stock solutions of these mock samples underwent the extraction method and were subsequently analyzed by the carbazole micro-assay. The results shown in Fig. 3A demonstrate that the method is specific for HA. Subsequently we have also analysed the potential influence of the above-mentioned substances on the extraction efficiency of HA. Therefore, the mock samples were spiked with 0.5 mg ml^{-1} low M_w HA and underwent the extraction methodology and carbazole micro-assay. From the results, depicted in Fig. 3B, it is clear that no statistical difference could be found between the HA determination in pure water and the HA determination in any of the spiked mock samples.

Next, based on the methods described by the International Conference on Harmonization (ICH), the limits of detection (LODs) were calculated as the analyte concentration corresponding to the mean value obtained for the blank samples plus three times the standard deviation of these samples.[22] Based on the results obtained for 12 blanks, the LOD of the method corresponds to 0.02 mg ml^{-1} of HA. However, this value does not result in a visual pellet for ATR-FTIR experiments. Therefore, we set our SDL to 0.10 mg ml^{-1}. Therefore, it can be stated that the method is sufficiently sensitive for the detection of HA in legal and suspected illegal dermal fillers, since these samples contain or claim to contain at least 10 mg ml^{-1} of HA.

The pellet, obtained post extraction, was also analysed by ATR-FTIR to independently identify a compound. The obtained fingerprint region (from 1800 to 650 cm^{-1}) was queried against our database containing 5000 spectra. The sample is regarded as positive for HA if the fingerprint region matches with at least 90% of that of the reference standards of HA that underwent the same extraction treatment, recorded on the instrument. None of the mock samples gave a match of 90% while all of the HA containing samples did (results not shown).

It stands to reason that fully desulfated GAGs cannot be distinguished from HA with our methodology. However, since the generation of these chemically desulfated GAGs is much more cumbersome and expensive than the simple purchase of HA powder, we assume that the occurrence of such compounds in illegal dermal fillers is unlikely.

3.3 Validation of the quantification method

3.3.1 Selectivity. Once the presence of HA is confirmed by ATR-FTIR and the carbazole micro-assay, the quantification is done by the latter.

3.3.2 Linearity of the calibration standards. Calibration curves for low and high M_w HA were obtained using ordinary least squares linear regression and linearity was confirmed

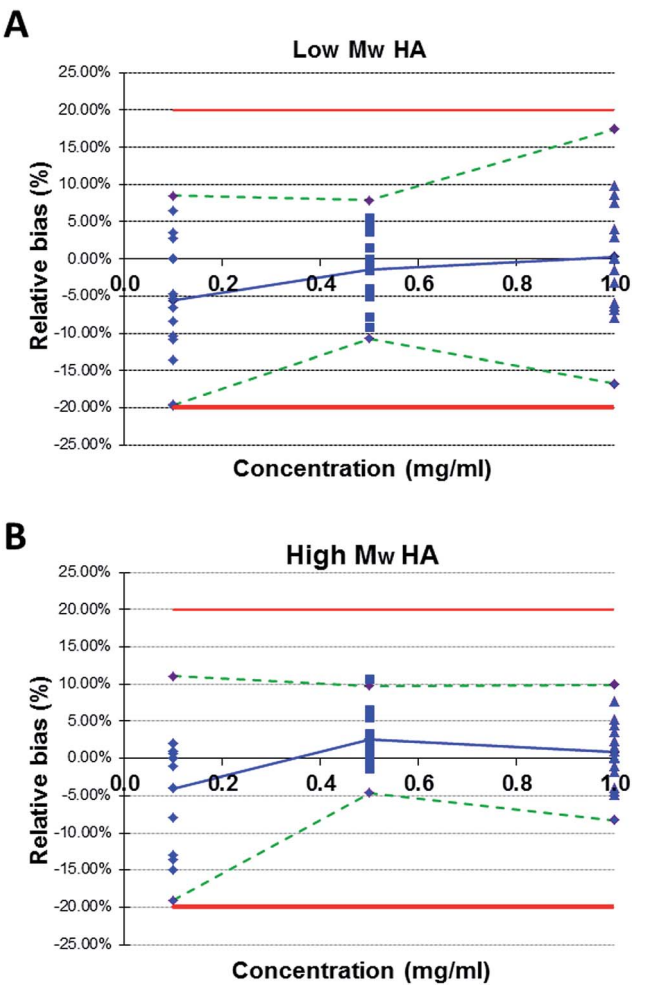

Fig. 4 Accuracy profiles of low M_w HA (A) and high M_w HA (B). Relative bias (···), β-expectation tolerance limits (- - -), acceptance limits (——) and relative back-calculated concentrations (▲).

using R^2 values and the Lack Of Fit (LOF) test as has been described.[20] With the R^2 values of both curves being above 0.99, it becomes clear that the calibration lines adequately describe the observed relationship, within the chosen concentration ranges.

3.3.3 Linearity, trueness, precision, accuracy and uncertainty assessment. The present method was validated according to ISO-17025 applying accuracy profiles which are based upon the "total error" approach.[23] The results are given in Table 2 and Fig. 4.

The linear relationship described in this section describes the linearity between the theoretical and measured concentration which is mandatory. Furthermore, the linearity of the results obtained is acceptable as R^2 values are above 0.999.[20]

Accuracy takes into account the total error of the test results and is represented by the β-expectation tolerance limits. There are, to our knowledge, no predefined limits for the quantification of HA in dermal fillers. Therefore we chose to use the acceptance limits [−20%, 20%] previously used for the quantification of cosmetic substances in gel-like formulations.[24] As shown in Table 2, the β-expectation tolerance limits do not exceed the acceptance limits, which means that 90% of the future measurement of unknown samples will be included within the tolerance limits.

The trueness is a measure of the systematic error of the method and is expressed in terms of relative bias. From Table 2 it can be concluded that the relative bias is limited between −5.57 and 2.60%. These values are lower than the values previously obtained for the quantification of cosmetic substances in gel-like formulations[24] and can therefore be considered acceptable. The precision of a method is a measure of the relative errors of the method and is expressed using relative standard deviations (RSDs). From Table 2 it can be seen that the obtained repeatability and the intermediate precision of the method can be considered acceptable since the highest RSD equals 7.73 (<RSD = 13.6 (ref. 24)).

3.3.4 Limits of quantification. Based on the methods described by the International Conference on Harmonization (ICH),[22] the limits of quantification (LOQs) coincide with the analyte concentration that corresponds to the mean value obtained for the blank samples plus ten times the standard deviation of these samples. Based on the results obtained for 12 blanks, the LOQ of the method corresponds to 0.07 mg ml^{-1} of HA. However, as already mentioned in Section 3.2 our SDL equals 0.1 mg ml^{-1} and therefore this value was taken as the LOQ. The upper limit of quantification (ULOQ), reflecting the endpoint of the linear relationship obtained in 3.3.2, was found to be equal to 1 mg ml^{-1}.

Table 3 Recovery of low M_w HA in different formulations

	Recovery for 0.1 mg ml^{-1}	Recovery for 0.5 mg ml^{-1}	Recovery for 1.0 mg ml^{-1}
Formulation 2	87.7 (±6.9)	95.5 (±4.9)	100.6 (±3.1)
Formulation 3	90.3 (±1.8)	97.2 (±6.4)	96.6 (±4.4)
Formulation 4	96.8 (±5.6)	97.5 (±1.1)	103.9 (±6.2)
Formulation 5	90.7 (±3.9)	98.5 (±6.0)	104.6 (±4.0)
Formulation 6	89.8 (±6.5)	101.2 (±3.6)	99.5 (±4.2)

3.4 Recovery

Validation has been based upon low and high M_w HA in water, therefore we also examined the recovery of low M_w HA in 5 different matrices for which some correspond to the formulations present in legal samples. The five additional formulations were analyzed in triplicate at a concentration of 0.1, 0.5 and 1 mg ml^{-1} HA and the results are shown in Table 3. The obtained recoveries were all between 80% and 120%, which were considered sufficient for this type of products.

3.5 Identification and quantification of legal and suspected illegal HA containing dermal fillers

The described identification and quantification methodology was applied to analyse the 8 legal samples and the 14 suspected illegal samples. In the sample set, all samples, except sample 15, were positive for the presence of HA while all samples claimed to contain HA. This one sample was then further investigated and was found to contain collagen, as also mentioned on the packaging. Although it is also possible that the amount of HA present in the sample was less than 0.1 mg ml^{-1}, online it is stated that HA is the main component of this product. Alternatively, it is also possible that the HA present in the sample had a M_w lower than 8–15 kDa, which could result in pro-inflammatory responses upon injection.[25] However, we don't think that high concentrations of very low M_w HA are present in the sample since analysis of the intact sample, after

Table 4 Overview of the quantification results of the legal and suspected illegal samples

Sample	Amount of HA declared (mg ml^{-1})	Amount of HA measured (mg ml^{-1})	% HA measured compared to the declared dose
1	17.8	15.7 (±1.3)	*88.2*
2	24.7	28.5 (±2.1)	*115.4*
3	14.8	13.2 (±1.1)	*89.2*
4	20.3	23.4 (±0.8)	*117.0*
5	20	16.3 (±2.9)	*81.5*
6	25.5	20.7 (±2.6)	*81.2*
7	20	18.7 (±1.5)	*93.5*
8	23	23.6 (±0.7)	*102.6*
9	20	3.2 (±0.6)	16.0
10	20	4.1 (±0.5)	20.5
11	20	5.1 (±0.4)	25.5
12	20	4.9 (±0.5)	24.5
13	8	3.1 (±0.4)	38.8
14	14	3.2 (±0.2)	22.9
15	n.a.	<LOD	0
16	n.a.	32.2 (±1.0)	n.a.
17	20	3.9 (±0.7)	19.5
18	20	5.5 (±1.1)	27.5
19	20	8.5 (±0.8)	42.5
20	n.a.	3.7 (±0.2)	n.a.
21	25	28.5 (±2.1)	*114.0*
22	25	28.3 (±1.8)	*113.2*

lyophilisation, by ATR-FTIR was negative for HA. However, the spectra obtained did correspond very nicely to the spectrum of glycerol (data not shown), which could be responsible for the viscous characteristics of the sample.

Next, we have also quantified the amount of HA detected in the samples. Table 4 lists the amount of HA mentioned on the packaging, the amount of HA measured by applying our methodology and the percentage of HA measured compared to the declared dose. It's clear that for all legal samples the percentage of HA measured, taking into account the allowed recovery error of ±20%, did correspond to the amount mentioned on the packaging. This was also true for suspected illegal samples, samples 21 and 22, while for most of the other suspected illegal samples, the percentage measured ranged from 16 to 42.5% of what was mentioned. Furthermore, also some dermal fillers didn't mention any concentration of HA, which is also in violation with the regulatory aspects of these types of products. In summary, we can state that most suspicious HA samples were underdosed and probably result in less-lasting cosmetic effects.

4 Conclusion

The classical, widely used methodology for the quantification of HA present in dermal fillers is not selective at all. The extraction method and subsequent assay, presented here, is able to distinguish HA from interfering substances like sulfated GAGs, small molecules sometimes present in those formulations, starch, other compounds that can be present for other aesthetic procedures and low-budget chemicals that could be used by malignant entities to generate a gel-like appearance. Furthermore, the validated quantification method was considered to be suitable for the quantification of legal and putatively illegal HA containing samples. Indeed, during this study, 8 legal and 14 suspected illegal samples were subjected to our methodology. The obtained study results revealed that many of these suspected illegal samples did not conform to their HA content. Although this does not present a health threat as such, it will probably result in a less long-lasting effect, which in turn might lead to more skin penetrating dermal procedures and their inherent risks. Alternatively, it is also possible that these samples contain a large fraction of low M_w HA, which is known to induce pro-inflammatory responses upon injection. Unfortunately, the minimal amount of the low M_w HA that is allowed to be present in these dermal fillers is not set, nor are the M_w requirements for this molecule. Furthermore, almost all analyzed suspected illegal samples did not comply with the medical devices directive for appropriate CE-labeling and the accompanying leaflet. Startlingly, it appears that many legal samples, utilized in this study, also were not conforming to the directive, making it even more difficult for the inspectors to discriminate between a legal and counterfeit or illegal sample.

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