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CTAB turbidimetric method for assaying hyaluronic acid in complex environments and under cross-linked form

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ABSTRACT

The cetyltrimethylammonium bromide turbidimetric method (CTM) has been developed to quantify the hyaluronic acid (HA) in complex media to overcome the lack of selectivity and specificity of the standard carbazole method. The objective of this work is to assess the potential application of CTM to determine HA concentration. Factors such as duration of incubation, linearity range, HA size and form (natural linear HA or cross linked HA), pH and ionic environment impact were investigated. The incubation time was set to 10 min and the calibration curve was linear up to 0.6 g L^{-1}. The quantitative method was relevant whatever the HA size and form, and also for a wide range of conditions. The robustness of the CTM added to its high specificity and simplicity demonstrated that the CTM is a valuable method that would be an interesting substitute to the carbazole assay for HA quantification.

1. Introduction

Hyaluronic acid (HA) is a linear and unbranched mucopolysaccharide, composed of N-acetylglucosamine and glucuronic acid joined alternately by β(1-3) and β(1-4) glycoside bonds. This molecule exhibits a wide range of molecular weights between ten thousand Da and several million Da. HA is abundant in the synovial fluid of articulation joints and in the intercellular space of the epidermis. It plays many physiological functions, notably in cell proliferation and differentiation. Furthermore, its rheological properties make HA an attractive biomaterial for medical-related applications like pharmacology and drug delivery, orthopedic surgery and rheumatology, dermatology and plastic surgery, ophthalmology and otolaryngology (Kogan, Šoltés, Stern, & Gemeiner, 2007).

For biomedical applications, at first, HA was extracted and purified from rooster combs. Nowadays, HA production by microbial culture is preferred since it allows the standardization of molar mass distribution (Huang, Chen, & Chen, 2008; Kim, Lee, & Moon, 2006; Shiedlin et al., 2004; Yamada & Kawasaki, 2005). After the microbial culture step, HA has to be separated from cells and purified from the various molecules composing the complex culture medium. To do so, tangential flow filtration (micro- and ultrafiltration) (Carlono & Magnette, 2000; Zhou, Ni, Huang, & Zhang, 2006), ethanol (Pires, Macedo, Eguchi, & Santana, 2010) or isopropanol (Reddy, Karunakaran, & Rao, 2011) precipitation are often used. Then, HA can be further processed by chemical cross-linking (for rheological properties improvement) (Jeon et al., 2007) or heat treatment (for sterilization). In this context, the quantification of HA in complex and variable mixtures or under different reticulation states is a current issue.

The conventional method for quantifying HA is the so-called carbazole method (Bitter & Muir, 1962; Cesaretti, Luppi, Maccari, & Volpi, 2003). The principle of this method lies on a full hydrolysis of HA, a conversion of released monosaccharides into furfural derivatives (under highly acidic conditions and temperature of 95 °C) and a chemical complexation with carbazole reactant to form a violet chromophore that can be assayed by spectrophotometry. However, to be applicable, the carbazole method requires the HA solution to be pure since any other ose or oside (like glucose, sucrose or starch) present in solution would also react and strongly interfere with the assay (Chen, Chen, Huang, & Chen, 2009; Pires & Santana, 2010; Song, Im, Kang, & Kang, 2009). Other quantification methods include gel permeation chromatography–light scattering–refractive index (GPC–LS–RI), which is difficult to perform in complex media because of many operating problems occurring, such as the seal of the chromatography column or the RI signal interference. As a consequence, this method is usually not

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convenient to follow HA production in microbial culture broth or HA quantification along the purification process. Taken as a whole, this led us to focus on the development of an alternative method for HA quantification.

It is well known that polyanionic polymers (like HA) are precipitated by organic ammonium cations such as cetyltrimethylammonium bromide (CTAB) or hexadecyltrimethylammonium chloride (HTMACl). The turbidity resulting from the precipitation is related to polymer concentration and can be titrated by spectrophotometry. Interesting results for HA quantification were obtained with CTAB (Scott, 1960). The CTAB turbidimetric method (CTM) was even demonstrated to be more accurate, sensitive and specific than the carbazole method (Chen & Wang, 2009; Song et al., 2009). Thanks to its high specificity, CTM allows HA quantification from microbial broth directly. Consequently, this makes CTM a possible and convenient alternative to carbazole assay. However, further investigation on the impact of the environment of HA, its molecular weight or its reticulation state on CTM assay has to be carried out in order to broaden the possibility of use of the assay and to assess its robustness. This study investigates the effect of HA environment (pH, ionic strength and microbial culture media), HA form (butanediol-diglycidyl ether cross-linked or linear HA) and size, assay incubation duration and the linearity range on the CTM assay.

2. Materials and methods

2.1. Material

Freeze-dried HA (70 kDa, 0.5 MDa, 2 MDa and 4 MDa) were obtained from HLT biotechnology (Javene, France). Cross-linked HA came from Teoxane laboratories (Geneva, Switzerland). CTAB, carbazole, brain heart infusion (BHI) and hyaluronidase (HAase) were purchased from Sigma Corporation (St-Quentin Fallavier, France). Na_2HPO_4, KH_2PO_4, H_2SO_4, $Na_2B_4O_7$, KCl, NaCl and NaOH were purchased from Carlo Erba (Val-de-Reuil, France).

2.2. Methods

2.2.1. Carbazole method

According to the method reported by Kosakai and Yosizawa (1979), 1 mL of HA solution was introduced into glass tubes filled with 5 mL of 25 mM $Na_2B_4O_7$ in H_2SO_4. The tubes were incubated for 15 min at 95 °C, then cooled in ice for 2 min. 200 μL of 0.125% (w/v) carbazole in absolute ethanol were introduced and the tubes were incubated for 15 min at 95 °C. After cooling, the absorbance was read at 550 nm (spectrophotometer Thermo Fisher Scientific, Vantaa, Finland). The equation of the standard curve used to determine the HA concentration was: absorbance = 6.2 [HA] with HA concentration in $g\,L^{-1}$ (equation obtained experimentally). Each assay was performed in triplicate.

2.2.2. GPC–LS–RI method

50 μL of HA solution at 0.5 $g{\cdot}L^{-1}$ was injected into a GPC–LS–RI apparatus (Malvern, Orsay, France). The GPC–LS–RI apparatus was constituted of a pre-column, two columns (A6000M and A7000, Malvern) connected in series, a light scattering (LS) detector and a Refractive Index (RI) detector. The eluent was phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4) pH 7.4 at a flow rate of 0.35 $mL.min^{-1}$. The universal calibration was performed with PEO-19 kD (Malvern). The Dn/Dc used to determine the HA concentration was 0.147 $ml\,g^{-1}$ (experimentally determined value).

2.2.3. CTM assay

The CTAB reagent (2.5 g) was dissolved in 100 mL of 2% (w/v) NaOH according to (Di Ferrante, 1956). 50 μL of HA standard solutions were introduced into 96 well plates filled with 50 μL of 0.1 M phosphate buffer pH 7. The plates were incubated at 37 °C for 15 min in the UV–vis spectrophotometer (Multiskan Go, Thermo Scientific, Gometz-le-Châtel, France). Then, 100 μL of CTM reagent at 37 °C was added to each well and the plate was incubated for 10 min at 37 °C. The plate was shaken for 10 s at the beginning and at the end of this incubation. Absorbance was read at 600 nm against the blank (HA solution replaced by 0.1 M phosphate buffer pH 7) and plotted against HA concentrations. The slope of the standard curve was obtained by the linear regression for HA concentration range between 0 and 0.6 $g{\cdot}L^{-1}$ (concentration over which the curve was no longer linear).

2.2.4. Quantification of cross linked HA

1 g of butanediol-diglycidyl ether cross-linked HA (BDDE-cross-linked HA) gel was dissolved in 20 mL 0.1 M phosphate buffer pH 7 under sonication (10 cycles of 30 s at 22 W) (Vibra-cell Ultrasonic Processor, Sonics & Materials, France). Then, the HA solution was further diluted in 50 mL final volume in 0.1 M phosphate buffer pH 7. The solution was quantified by CTM, the carbazole method and the GPC–LS–RI method.

3. Results and discussion

3.1. CTM assay for solubilized microbial HA

3.1.1. Kinetic stability

In order to assess the impact of incubation duration in the presence of the CTM on the quantification method, assays were performed with various durations. 2 MDa HA was chosen to represent the HA size range. Indeed, HA mostly produced by microbial culture presents molecular weights from 70 kDa to 6 MDa (Im, Song, Kang, & Kang, 2009). Solutions with 2 MDa HA in 0.1 M phosphate buffer were used at concentrations ranging from 0 to 0.6 $g{\cdot}L^{-1}$. Absorbance at 600 nm was automatically measured every minute for 1 h. These are reported in Fig. 1A. The absorbance is due to the turbidity induced by the formation of HA/CTAB precipitate occurring in the course of the reaction. Fig. 1A shows that absorbance increases in the very first moments of the reaction (before 5 min) then slowly decreases. This variation with time is more noticeable for the highest HA concentrations. The HA precipitation triggered by CTAB probably results from an intermolecular association and dissociation reaction. The very slow decreasing of absorbance after 50 min, obviously going on after 60 min (since non constant value was reached) indicated that the equilibrium between association and dissociation was probably not reached even after 60 min incubation. Then, the HA/CTAB precipitate formed is not very stable. From the absorbance values displayed in Fig. 1A, the slope of the HA standard curves were calculated and plotted as a function of the CTM reaction duration (Fig. 1B). As expected, the slope values clearly decrease from 5 to 60 min duration meaning that the sensitivity of the assay is optimal for a low duration of reaction (between 0 and 5 min). However, in this duration range, the precipitates are the most unstable and too short a duration might lead to manipulation issues. Thus, the reaction duration for the protocol was arbitrarily set to 10 min in order to balance sensitivity and ease to handle the reaction duration.

3.1.2. Linearity range

The next step of the study was to determine the range of concentrations for which the assay was valid. 2 MDa HA was assayed at concentration ranging from 0 to 1 $g{\cdot}L^{-1}$ in 0.1 M phosphate buffer pH 7 in order to check the linearity range of the standard

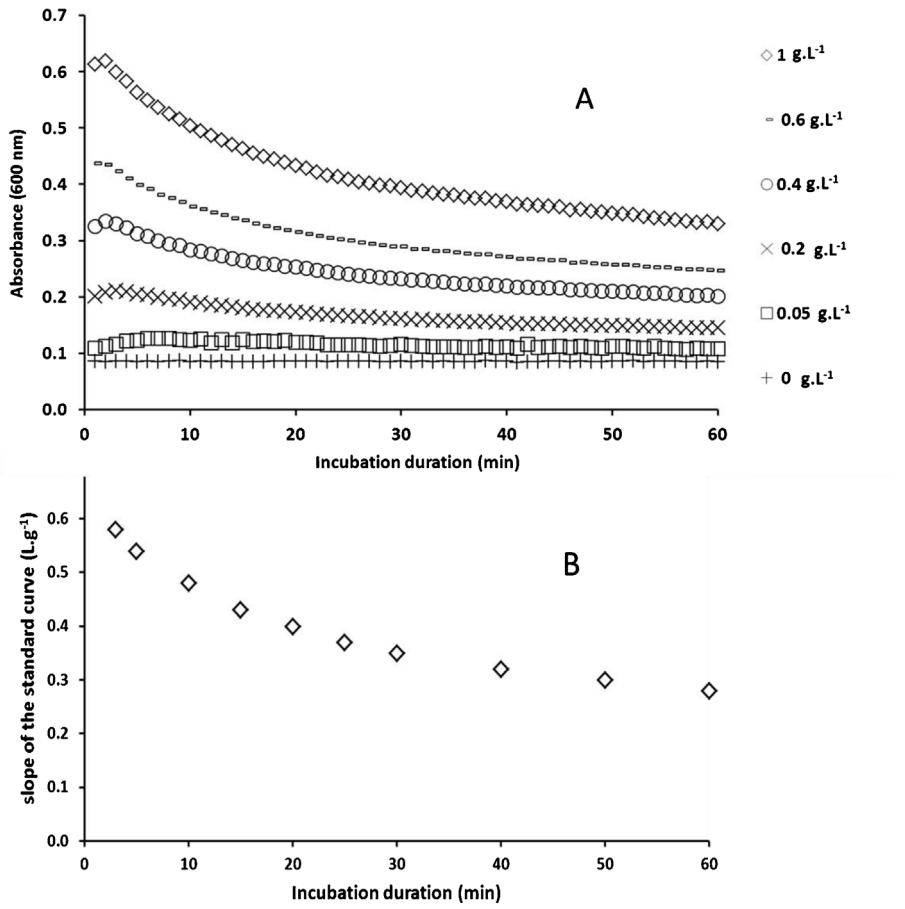

Fig. 1. (A) Effect of the reaction duration on CTM sensitivity: evolution of absorbance at 600 nm at various HA concentrations. (B) Slope of the standard curves plotted versus incubation time.

curve (Fig. 2A). Linear regressions were drawn using four sets of concentration (0 $g{\cdot}L^{-1-1}$ to 0.5, 0.6, 0.8 and 1 $g{\cdot}L^{-1}$). Correlation coefficients (R^2) of the regressions are presented in Fig. 2B. R^2 value is satisfactory and constant up to 0.6 $g{\cdot}L^{-1}$, and it decreases as the highest HA concentration increases. This is a particularly good result since a previous study showed a good linearity only up to 0.16 $g{\cdot}L^{-1}$(Chen & Wang, 2009).

The lack of linearity observed beyond 0.6 $g{\cdot}L^{-1}$ can be due either to a poor mixing efficiency caused by increase of sample viscosity at high HA concentration or to a saturation of CTAB by HA. The slope of HA assay (from 0 to 0.6 $g{\cdot}L^{-1}$) in the conditions of Fig. 1 (using a 10 s shaking duration) was 0.49 $L{\cdot}g^{-1}$ (R^2 = 0.990). The same experiment was carried out using a shaking duration of 20 s (data not shown). In these conditions, the slope observed was 0.50 $L{\cdot}g^{-1}$ (R^2 = 0.980). The weak influence of shaking tends to demonstrate that the problem of linearity at high HA concentration is not due to a lack of mixing efficiency.

3.1.3. Impact of HA size

The first part of this study concerning (i) the influence of the incubation duration and (ii) the linearity range assessment was performed using HA with a 2 MDa molecular weight. The following step of the study focused on the influence of the HA size on the CTM method. The slope values of CTM standard curves established for HA concentration between 0 and 0.6 $g{\cdot}L^{-1}$ with molecular weights of 70 kDa, 0.5 MDa, 2 MDa and 4 MDa were close to 0.50 $L\,g^{-1}$ with regression coefficients of 0.99 (Fig. 3). Accordingly, the HA size did not affect the CTM. This result appeared surprising. Indeed, a reinforcement of HA-CTAB interactions would be expected with a high molecular size HA since more negative charges would be exposed at the surface of the polymer. As a consequence, larger complexes should be formed impacting the precipitation reaction. The secondary and tertiary structures of HA in aqueous solution proposed by (Scott, Cummings, Brass, & Chen, 1991) might explain this absence of size dependence. According to these authors, in aqueous solution, the HA would constitute a complex network of interwoven polymer chains. So, as a matter of fact, the CTAB reaction would most likely occur with pieces of HA network rather than with individual HA molecules, thus limiting the effect of the polymer molecular weight on CTM. This result proved to be particularly interesting since it indicates that the standard curve established with a particular HA molecular weight will apply for assaying HA in a wide range of molecular size.

Furthermore, the constant slope and R^2 values shown whatever the molecular weight of HA sample confirm that the shaking conditions used provide an efficient mixing and tends to confirm that the lack of linearity observed in Fig. 2 is not related to the sample viscosity.

3.1.4. Effect of the HA sample environment on CTM

HA can be found in many different environments: in the microbial culture medium of HA production, in solution with various salinities or pH during the purification steps, or in pure water. The following study investigates the robustness of the assay for various HA solutions.

3.1.4.1. Effect of sample pH. The pH of the final solution (after all reactants were added) needs to be high enough to achieve

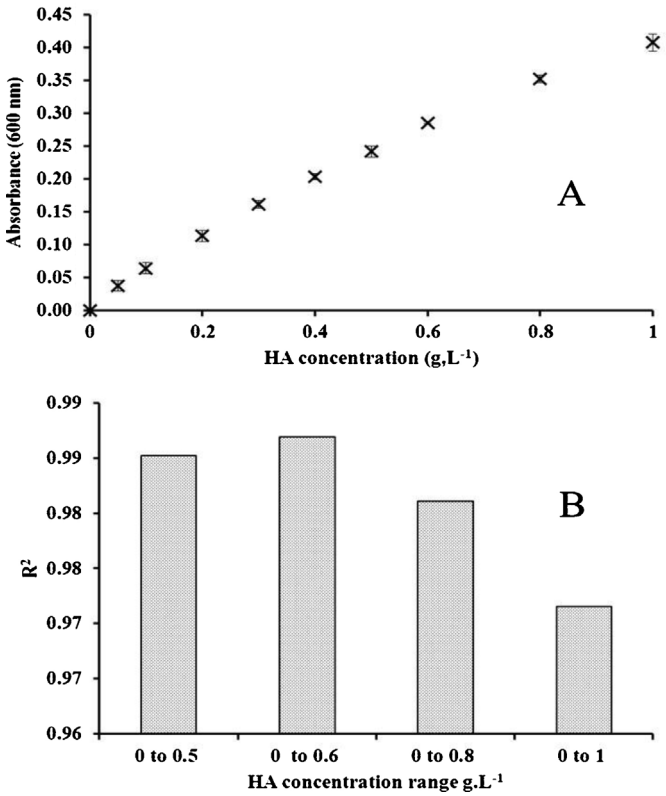

Fig. 2. (A) Absorbance of CTM assays in relation to HA concentration. (B) R^2 of the standard curve established for various HA concentration ranges. 2 MDa HA was used. The incubation duration prior absorbance measurement was 10 min.

precipitation of the HA. Indeed, carboxylic acid functions of glucuronic acids must be in a negatively charged form. Thus, if the added reagents do not buffer the HA sample enough, alterations of the assay might occur. The impact of sample pH was then investigated. 2 MDa HA samples (from 0 to 0.6 g·L^{-1}) were adjusted either at pH 2 and at 4 (using 1 M H_2SO_4) or at pH 8 and at 10 (by 5 M NaOH) prior to the addition of assay reagents. Fig. 4 clearly shows that the slopes of the standard curves obtained for the various pH samples are constant, indicating that the assay conditions guarantee a high enough pH to allow a precise quantification whatever the HA sample pH. This can be due either to the presence of NaOH or phosphate buffer in CTM reactants. The same set of experiments was achieved without adding NaOH in the CTM reactant (data not shown). In this case, slopes obtained for HA samples with pH ranging from 4 to 10 were all close to 0.5 L g^{-1} while no HA-CTAB precipitation occurred

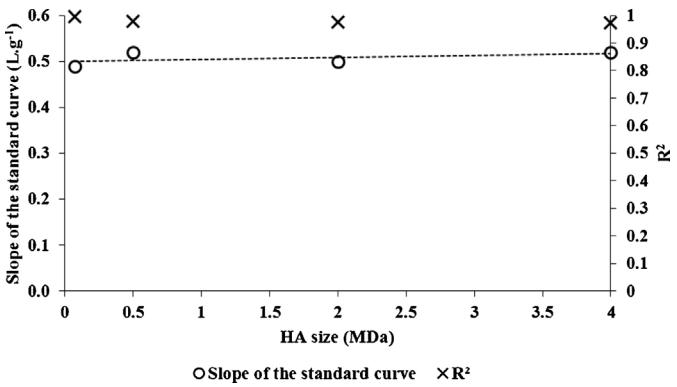

Fig. 3. Impact of HA size on the HA quantification by CTM. The regression coefficients (R^2) and the slope of the standard curves were plotted against the HA size.

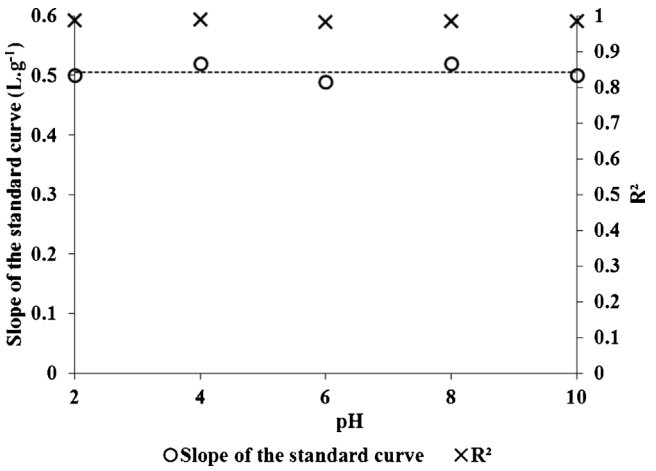

Fig. 4. No pH effect on the CTM sensitivity. The standard curves and regression coefficients were established with 2 MDa HA solutions in 0.1 M phosphate buffer. pH was adjusted by NaOH or H_2SO_4.

with pH 2 samples. So, when the sample pH value is equal to or higher than 4, the overall pH solution is high enough to allow precipitation. This is not the case for samples whose pH values stand below 4: NaOH in the reagent is therefore needed to achieve the precipitation.

The study of the pH impact confirmed that the interaction between HA and CTAB is an ionic interaction. As a consequence, it might be dependent on the ionic strength.

3.1.4.2. Effect of sample salt content. To study the effect of the salt environment on the assay, HA samples were adjusted to KCl concentrations ranging from 0 to 0.50 M. Fig. 5 exhibits the evolution of standard curve slopes obtained as a function of KCl concentration. This figure shows a linear decrease in slopes with increasing KCl concentrations. After regression, the following equation between KCl concentration in sample and CTM HA standard curve slope was proposed:

$$\text{Slope of standard curve} = -0.93\,[\text{Chloride ion concentration}\,(M)] + 0.51 \quad (1)$$

This phenomenon could result from a negative influence of the overall ionic strength on HA-CTAB association constant or a direct competition of chloride anion with HA for CTAB interaction. Fig. 6 exhibits assay curve slopes obtained with HA samples in phosphate buffer, NaCl or KCl with 0.3 M of ionic strength. It reveals that modifying ionic strength through phosphate addition has no

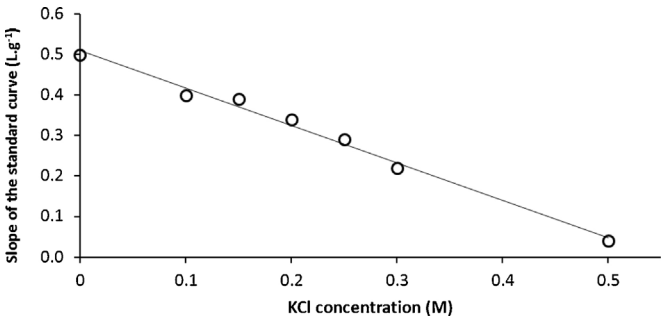

Fig. 5. Impact of KCl concentration on the slope of the standard curve established by CTM. KCl was added to HA solution to obtain KCl final concentration between 0 and 0.5 M. The standard curve was established for each KCl concentration and the slope is plotted versus the KCl concentration.

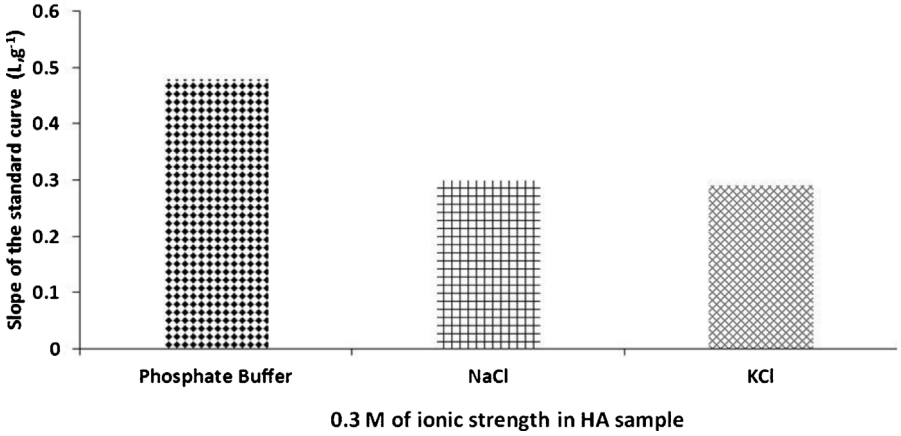

Fig. 6. Competition of chloride anion with HA for CTAB interaction. The slope of the standard curve of 2 MDa HA in 0.3 M of ionic strength of phosphate buffer was compared to the standard curves at 0.3 M of ionic strength obtained by addition of NaCl and KCl.

effect whereas increasing ionic strength through KCl or NaCl addition induces a significant slope decrease. The decrease with Na or K as counter ion is of the same extent when the anion used is Cl^-. The observed salt interference is thus probably related to the chloride anion. This could explain why when regarding the use of CTAB in the field of carbohydrate purification, carbohydrate – CTAB is usually dissolved by chlorinated salts (mainly $CaCl_2$ and NaCl) (Anderson & Smith, 1977; Bartoloni, Norelli, Ceccarini, Rappuoli, & Costantino, 1995).

3.1.4.3. Effect of a complex biochemical environment. Standard curves of 2 MDa HA (from 0 to 0.6 g·L^{-1}) in 0.1 M phosphate buffer pH 7 and in Brain Heart Infusion (BHI) were compared (Fig. 7A). BHI is a complex mixture commonly used as culture medium for microbial cultures. It is composed of proteins, peptides, amino acids, glucose and salts. HA standard curve in BHI (pH 7) remains linear up to 0.6 g·L^{-1}, indicating that CTM can be used for direct HA quantification in samples from production or purification steps. The observed slope is lower in BHI than in phosphate buffer. So, BHI matrix modified the assay by limiting HA-CTAB aggregate formation though. BHI contains many different biomolecules (like peptides, amino acids, nucleic acids, etc.) and is rather rich in salts which were demonstrated to influence the CTM. According to the manufacturer, the concentration of NaCl in BHI is 0.125 M. Eq. (1) was used to calculate the slope of HA standard curve in phosphate buffer at the corresponding concentration of NaCl. From the calculated slope (0.4 L·g^{-1}), absorbances of CTM assay at HA concentration from 0 to 0.6 g·L^{-1} were added to Fig. 7A, named "theoretical BHI". They are very close to the actual values from HA in BHI. This indicates that the main effect of BHI complex environment is coming from its salt content. Other biomolecules composing this medium probably have no or minor influence, confirming previous observations (Song et al., 2009).

The spectrophotometric method commonly used to quantify HA in solution is the so-called carbazole method. The standard curves of 2 MDa HA in phosphate buffer and BHI obtained with the carbazole method are presented in Fig. 7B. As expected, the carbazole method is perfectly efficient to quantify HA when using phosphate buffer. Its sensitivity is even higher than that of the CTM. However, in BHI, a saturation of the spectrophotometer occurs in every HA concentration preventing any use of the method in such media. The carbazole method is based on (i) a full hydrolysis of HA into free carbohydrates (glucuronic acid and N-acetyl glucosamine) under a strong acidic condition and high temperature, (ii) a conversion of these free carbohydrates into hydroxymethylfurfural compounds and (iii) a combination of the compound yielded from glucuronic acid to produce a chromophore quantified by spectrophotometry. However, the last step is poorly specific and many hydroxymethyl furfural obtained from other carbohydrates (with 6 carbons) may interfere. The high content in free sugars (and particularly glucose) in BHI or other culture media prevent the use of the carbazole method for HA quantification in complex environment.

3.2. Assessment of the CTM accuracy for the quantification of BDDE-cross-linked HA

The cross-linking of HA by BDDE helps to tailor the mechanical properties and the degradation rates (Jeon et al., 2007; Schanté, Zuber, Herlin, & Vandamme, 2011). This specificity is mostly used in the treatment of osteoarthritis of the knee or for wrinkle fillers. The following study focuses on the quantification of cross linked HA. This last HA form leads to aggregates in solution. And then, it

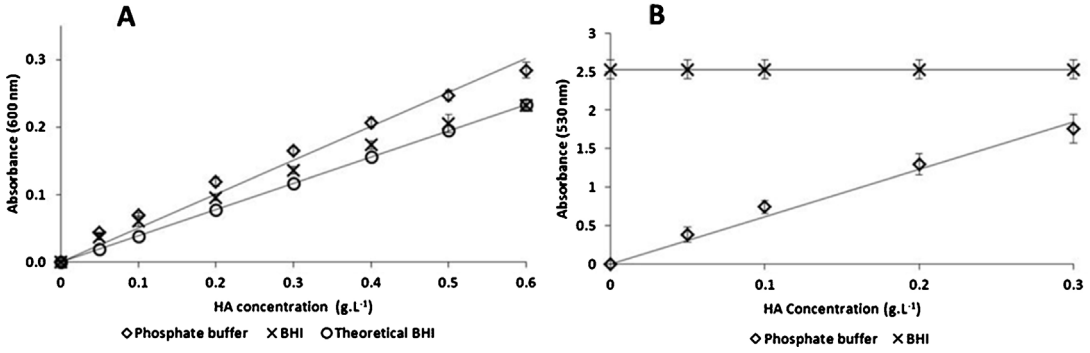

Fig. 7. CTM (A) and carbazole method (B) for assaying HA in phosphate buffer (◊) and BHI (×). Standard curve obtained from Eq. (1) using 0.125 M NaCl is also displayed (○).

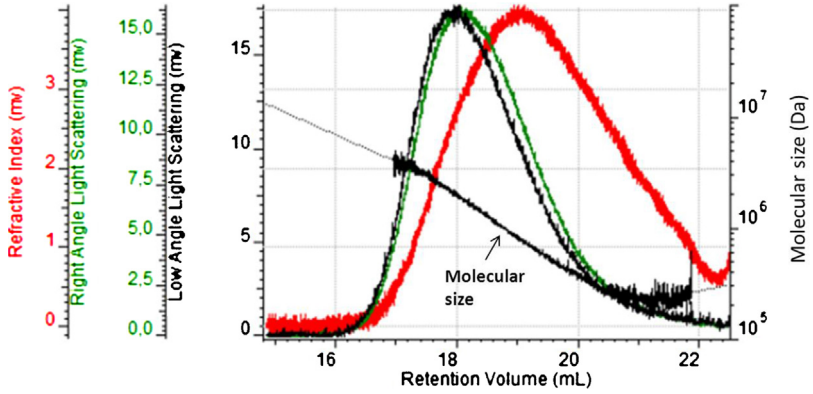

Fig. 8. GPC–LS–RI chromatogram of cross-linked HA degraded by sonication. Elution was performed by phosphate buffered saline at a flow rate of 0.35 mL min^{-1}. Dn/Dc used to calculate the HA concentration was 0.147. PEO 19k universal calibration was used to determine the HA size. Average HA size calculated was 980 kDa.

has to be partially degraded by sonication to obtain a homogeneous solution. Cross-linked HA treated by sonication was quantified simultaneously by the CTM, the GPC–LS–RI and the carbazole methods. The chromatogram shown in Fig. 8 was obtained by GPC–LS–RI. The right angle light scattering (green curve) and the low angle light scattering (black curve) were used to calculate the size of the HA for each elution volume by the Zimm equation (black "linear" curve). The mean value of sizes was 980 kDa. Thus, a sonication treatment degraded the reticulated HA but its final size remained high enough (included into 70 kDa and 4 MDa) to be assayed by the CTM. The cross-linked HA concentration determined by the refractive index (red curve; Dn/Dc = 0.147) was 25 $mg{\cdot}g^{-1}$(Fig. 8). When assessed by the carbazole method and the CTM, the cross-linked HA concentration was 26 $mg{\cdot}g^{-1}$. The three methods gave similar results. Then, the sonication of HA did not hinder its quantification. The CTM was thus efficient for the quantification of cross-linked HA.

4. Conclusion

The HA quantification is classically performed by using the carbazole method to determine HA concentrations. But this method presents many drawbacks, particularly a low specificity and, a time consuming experimental procedure. Moreover, its set up is dangerous because of the use of sulfuric acid. Based on the formation of a complex between HA and CTAB, the CTM is an alternative method that proved to be highly specific and safer. Previous studies on the CTM were rather limited, therefore the purpose of this study was to complete them and particularly to determine the limits of its application in terms of incubation duration, linearity range, HA size and form (natural linear HA or BDDE-cross-linked HA), and environment. The turbidity evolution with time was noticed and a compromise between sensitivity and handling issues led to set the incubation duration to 10 min. The CTM led to a linear curve in the HA concentration range of 0 $g{\cdot}L^{-1}$ to 0.6 $g{\cdot}L^{-1}$. It was not dependent on the HA size from 70 kDa to 4 MDa. Chloride ions interfered with the CTM reagent but when the chloride ion concentration was known, the slope of the standard curve could be determined and used to quantify HA concentrations. The CTM proved to be very robust in a fermentation medium and with the modifications of solution pH and/or ionic strength. All the results showed that the CTM was stable in a wide range of conditions. As a consequence, the CTM could be used to follow the HA concentration during a microbial process but also in all downstream processes and after BDDE-cross-linking. In view of these results, the CTM constitutes an alternative to the conventional carbazole method. In addition, CTAB may react with other capsular polysaccharides. Anderson and Smith (1977) and Bartoloni et al. (1995) purified a bacterial capsular polysaccharide from *Haemophilus influenzae* and *Neisseria meningitidis* by CTAB. Accordingly, the CTM should be a simple method to determine the concentration of various bacterial capsular polysaccharides.

Acknowledgement

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