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Characterization of insoluble calcium alginates by solid-state NMR

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ABSTRACT

Three series of 9 insoluble calcium alginate powders with different average calcium contents (1.5, 3.5 and 8%, w/w) are investigated by means of ^{13}C solid-state NMR spectroscopy. The effect of the increased calcium content on the determination of the mannuronate (M) to guluronate (G) ratio from spectral deconvolution of the ^{13}C CP/MAS spectra is discussed, and the variations observed are commented in function of possible structural modifications related to the interaction with the divalent cations. The possibility of using solid-state NMR spectroscopy for the quantification of the calcium content in unknown alginate samples is explored performing principal component analysis (PCA) of the spectra. The results obtained show that a clear separation of alginates with slightly different calcium content is possible. The proposed method relies on the sole use of the chemical shifts of the signals corresponding to pyranose carbons, suggesting that PCA of solid-state NMR data holds promises as a rapid and undestructive method for screening the calcium content of alginate-based materials with biomedical uses.

1. Introduction

Alginates (Draget, Moe, Skjåk-Bræk, & Smidsrød, 2006) are natural polysaccharides extracted from marine brown algae (*Phaeophyceae*). They are binary, linear copolymers of (1 → 4) linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) (Scheme 1). Alginates occur naturally as mixed salts of alginic acid, the sodium form being the predominant one. Both the chemical composition (indicated as M/G ratio) and monomer sequence can vary significantly depending on the seaweed species and growing conditions (Indergaard, Skjåk-Bræk, & Jensen, 1990). It is now well-established that the monomers do not occur randomly on the chain but preferentially form block-like sequences with a smaller contribution from alternating structures (Haug, Larsen, & Smidsrød, 1967). One of the most important properties of alginates is their ability to selectively bind divalent cations (e.g. Ca). Notably, the presence of G-rich regions of the chain increases the ability of alginates to selectively bind Ca ions, while it has been shown that alternating and M-block structures do not have any selectivity (Smidsrød, 1973). This phenomenon has been explained taking into account the formation of an "egg box" structure (Grant, Morris, Rees, Smith, & Thom, 1973) and possible lateral association of G blocks with increasing the G content and the calcium concentration (Stokke, Draget, Yuguchi, Urakawa, & Kajiwara, 2000). Such ability allows alginates to form gels, whose properties vary in function of the content of calcium ions, the monomer sequence and the M/G ratio (Grant et al., 1973). In fact, the selective binding of calcium ions is an essential prerequisite for the gel formation (Haug, Myklesta, Larsen, & Smidsrød, 1967; Steginsky, Beale, Floss, & Mayer, 1992).

Because of their ability to form stable gels (de Nooy, Capitani, Masci, & Crescenzi, 2000), alginates have been largely used in many biomedical and pharmaceutical applications, going from delivery vehicles for drugs (George & Abraham, 2006; Laurienzo et al., 2006; Oddo et al., 2010), wound dressings materials (Ågren, 1996; Segal, Hunt, & Gilding, 1998; Thomas, 2000), dental impression materials (Hanks, 1993), or biomaterials for tissue engineering (Smidsrød & Skjåk-Bræk, 1990). It is then clear that the knowledge of the primary structure and the relative amount of M and G monomers, as well as of the calcium content of the alginate, can determine its final application. For this reason, efforts have been devoted in the last decade to find an effective way of determining the M/G ratio in alginates. For sodium alginates, methods based on vibrational spectroscopy (infrared and Raman) and solution-state ^{1}H nuclear magnetic resonance (NMR) spectroscopy have been reported (Grasdalen, Larsen, & Smidsrød, 1979; Salomonsen, Jensen, Stenbæk, & Engelsen, 2008a). The method based on vibrational spectroscopy has the advantage that it does not require any significant sample preparation, but needs a reliable reference method for the measurement of the M/G ratio. On the contrary, the method based on liquid-state NMR does not require a reference calibration, but is only applicable to soluble alginates. In fact, the

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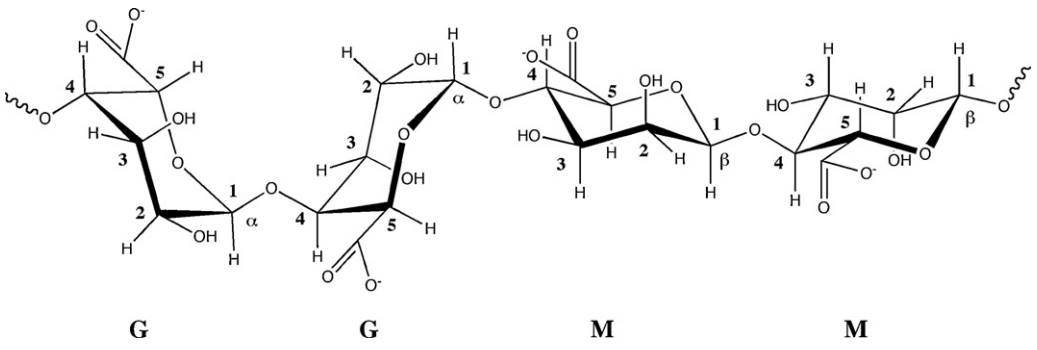

Scheme 1. Chemical structure of alginate at the junction between poly-α-L-guluronate (G) and poly-β-D-mannuronate (M) blocks.

presence of divalent cations increases the viscosity of the solution, causing line broadening of the signals corresponding to G moieties. This constitutes a limitation in its use for the determination of the M/G ratio, which results often overestimated. In these cases, a laborious enzymatic degradation of the alginate is necessary to reduce the viscosity. It is also worth noting that discrepancies between the M/G ratios determined through liquid-state NMR and vibrational spectroscopy were observed for samples with high calcium contents (Salomonsen, Jensen, Stenbæk, & Engelsen, 2008b). As an alternative, high resolution magic angle spinning (HRMAS) NMR was recently used to improve spectral resolution of alginate viscous solutions (Salomonsen, Jensen, Larsen, Steuernagel, & Engelsen, 2009a). However, as for liquid-state NMR, the results obtained revealed a general overestimation of the M/G ratio. Despite the lower spectral resolution showed by ^{13}C CP/MAS spectra of solid sodium alginates, in the same work Salomonsen and co-workers showed that the determination of the M/G ratio by means of solid-state NMR resulted more reliable with respect to liquid-state or HRMAS NMR methods. Moreover, this method showed the advantage of requiring a minimal sample preparation. In a different paper, the same authors reported different possible protocols for analyzing the ^{13}C CP/MAS spectra to find the best method to determine alginate monomer composition, and compared the M/G results obtained with those estimated from solution NMR methods (Salomonsen, Jensen, Larsen, Steuernagel, & Engelsen, 2009b). The results were collected on more than 40 sodium alginates with low calcium content (between 0.01 and 0.2%, w/w, with only three samples having calcium amounts between 1.1 and 2.4%). The large number of samples available allowed the authors to perform statistical analysis of the spectra, which revealed that only a slight separation of the samples could be obtained on the basis of their M/G ratios in liquid-state NMR and HRMAS experiments, while the separation became more evident when analyzing ^{13}C CP/MAS results. *A priori* determination of M/G ratio of powder alginates with low calcium content was also demonstrated using the multivariate curve resolution method (Salomonsen et al., 2009b).

Typically, alginates with higher calcium contents (between 3 and 10%, w/w) are used in most of the previously described biomedical applications (Illarionova et al., 2002). These samples are nearly or totally insoluble, making their characterization by means of solid-state NMR techniques mandatory. However, important spectral changes can be induced by the presence of an increased amount of calcium ions, which can potentially affect the use of solid-state NMR in the structural investigation of insoluble alginates.

In this work, we discuss the potential of solid-state NMR for the characterization of insoluble alginates with calcium contents of interest for biomedical applications. In particular, 9 samples of sodium alginates with very low sodium contents were treated following two different specific protocols for the production of calcium alginates, leading to three series of 9 calcium alginates with average calcium contents ranging between 1.5 and 8% (w/w). The ^{13}C CP/MAS spectral modifications induced by the presence of large calcium ions concentrations are discussed in terms of their effects on the estimation of the M/G ratio. The use of unsupervised statistical analysis of the ^{13}C spectra to separate the alginate samples on the basis of their calcium content and the potential applications of this method in the determination of calcium concentration of unknown alginate-based materials of biomedical interest are also discussed.

2. Experimental

2.1. Samples

A total of 9 different commercial sodium alginate samples were purchased from FMC Biopolymer (Philadelphia, USA), Kimika Corporation (Tokyo, Japan) and Danisco A/S (Brabrand, Denmark). The samples were chosen so as to cover a range of M/G ratios between 0.3 and 2. The calcium content of 8 of the 9 sodium alginates was found to lie between 0.05 and 0.2% (w/w): only in one case the calcium content was 1% (w/w). The M/G ratio of the commercial samples was determined using ^{1}H liquid-state NMR following the procedure described by Grasdalen et al. (1979). The commercial references of the 9 sodium alginates as well as their respective M/G ratios are reported in Table 1. The following 3 distinct procedures were applied to all of the 9 sodium alginates (referred to as "Na" in the following) to increase their calcium content:

(a) 20 g of sodium alginate powder was dissolved in 50 ml of $CaCl_2$. The solution was agitated vigorously for 30 min, then filtered and dried using a vacuum pump. The filtrate was then washed using deionized water to eliminate the unreacted chloride. The absence of residual chloride was tested by means of the silver nitrate method. The resulting powder was then dried using a laminar flow. The obtained material was finally lyophilized and finely ground in a mortar. The average calcium content of

Table 1
Commercial references of the 9 sodium alginates investigated and corresponding M/G ratios.

Sample n.	Producer	M/G ratio[a]
1	FMC	0.3
2	FMC	0.3
3	Kimika	0.3
4	FMC	0.5
5	Danisco	0.6
6	Danisco	0.8
7	Danisco	1
8	Kimika	1.2
9	Kimika	2

[a] The M/G ratio has been determined by means of ^{1}H liquid-state NMR according to the procedure described by Grasdalen et al. (1979). The estimated error is ±5%.

the alginates obtained through this procedure was 8% (w/w). The 9 alginate samples prepared through this procedure will be referred to as "Ca" in the following. These samples are nearly insoluble in aqueous solvents.

(b) The "FIRA-gel" protocol (Payet, Ponton, Agnely, Colinart, & Grossiord, 2002) was applied to the 9 "Na" alginates. This protocol makes use of 4 starting solutions: solution 1 is composed by EDTA 36 g/l; solution 2 is $CaCl_2$ 14.7 g/l; solution 3 is obtained adjusting the pH of 60 g of solution 1 and 60 g of solution 2 between 7 and 7.5; solution 4 was obtained diluting 120 g of solution 3 into 280 g of deionized water. 0.35 g of sodium alginate was dissolved into 37.5 g of solution 4 and stirred until complete dissolution of the powder. 3.8 ml of freshly prepared glucono-delta lactone (GDL) were added to this solution and stirred for about 20 min. The obtained solution was diluted adding 50 ml of deionized water, then left untouched for one night to allow the formation of the gel phase. The formed gel was then left in atmosphere of 70% EtOH for 4 days, and then in atmosphere of 100% acetone for 3 days. The obtained gel was lyophilized and finely ground in a mortar to obtain the final powder. This procedure let to an average calcium content of 3.5% (w/w). The 9 alginate samples prepared through this procedure will be referred to as "F20" in the following. These samples are almost insoluble in aqueous solvents.

(c) The same procedure was repeated using exactly the same conditions except for the concentration of solution 2, which was 7.35 g/l. This procedure led to an average calcium content of 1.5% (w/w). The 9 alginate samples prepared through this procedure will be referred to as "F10" in the following. These samples are almost insoluble in aqueous solvents.

2.2. Ca determination

The calcium content of the 36 alginate powders was determined by inductively coupled plasma emission spectroscopy using a Hitachi Z5000 instrument equipped with ten lamps. About 30 mg of alginate powder was calcinated for 45 min at 800 °C, dissolved into 2 ml of concentrated HCl and then diluted with 1998 ml of water. 10 ml of this solution was poured in a 100 ml flask with 200 μl of concentrated HCl, 200 μl of lanthanum chloride and water (QS 100). 50 ml of this solution was used for the analysis. The calibration standards were prepared from single-elemental standard solutions from VWR. The wavelength used for the measurement of Ca was 422.7 nm, the flame type was air-acetylene, the fuel flow was 2.4 l/min, the oxidant flow was 15 l/min, the oxidant pressure was 160 kPa and the burner height was fixed at 9 mm.

2.3. Solid state NMR

^{13}C solid-state NMR spectra of the 36 alginate samples were recorded using a Bruker Avance III spectrometer (Bruker Biospin GmbH, Germany) operating at the Larmor frequencies of 400.43 MHz and 100.70 MHz on ^{1}H and ^{13}C nuclei, respectively, by using a 4 mm cross-polarization/magic angle spinning (CP/MAS) probehead. The zirconia rotors were filled using approximately 80 mg of alginate powder and spun at 10 kHz. 2048 scans were used to acquire the ^{13}C spectra by means of CP/MAS (Pines, Waugh, & Gibby, 1972; Pines, Gibby, & Waugh, 1973). The ^{1}H 90° pulse length and power level were 3.8 μs and 80 kHz, respectively. The spectral width was 35.7 kHz and 2048 points were acquired to describe the free induction decay. TPPM decoupling (75 kHz) was used during ^{13}C acquisition. The acquisition time was 28.7 ms. All the spectra were externally referenced to the carbonyl peak of glycine at 176.03 ppm downfield of TMS. No linebroadening was applied. All the spectra were acquired using the same receiver gain.

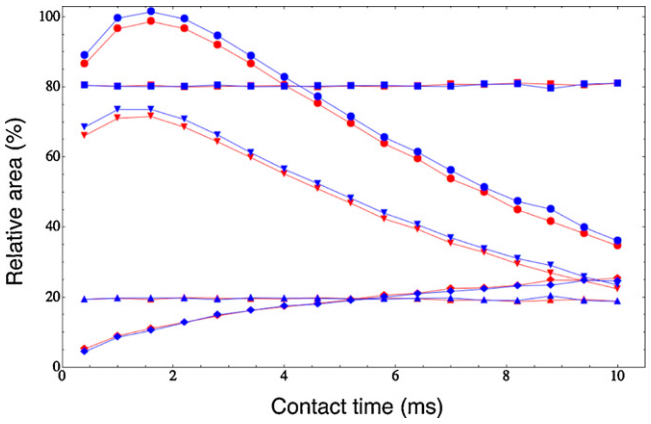

Fig. 1. Overall integrated area of all the signals (circles), integrated area of carboxylic (diamonds), anomeric (upper triangles) and pyranose (squares) carbon signals relative to the integrated area of the anomeric and pyranose signals, and absolute integrated area of the pyranose signals (lower triangles) in the ^{13}C CP/MAS spectrum of sample n.1 as a function of contact time. Blue and red curves/symbols indicate sample n.1 in the "Na" and "Ca" series, respectively. Lines are drawn to guide the eyes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2.4. Quantitativity of ^{13}C CP/MAS spectra

Cross polarization build-up curves, showing the trend of the integrated area of the ^{13}C signals in function of the contact time, were recorded for sample n.1 (see Table 1) in both the "Na" and "Ca" series to check for possible variations caused by the different calcium content. These curves are shown in Fig. 1. These samples were chosen since they were representative of the lowest and highest calcium content available, respectively. The curves shown in Fig. 1 confirm that the same cross-polarization trend is observed for the integrated area of the total spectrum and the pyranose region of sample 1 in the "Na" and "Ca" series. We can safely infer that the same contact time can be used for all the samples, independently of the calcium content. A contact time of 2 ms was chosen in all the cases. As previously discussed by Salomonsen et al. (2009b), cross-polarization curves can be used to find the experimental conditions in which the spectra can be considered "internally quantitative" (in the present case, this must be intended as "within each alginate series"). For consistency with the discussion reported by Salomonsen et al. (2009b) about quantitativity of CP/MAS spectra of powdered alginates, Fig. 1 also shows the trend of the integrated area of the carbonyl, anomeric and pyranose signals relative to the integrated area of the anomeric + pyranose signals for both the "Na" and "Ca" samples. The constant trend of the anomeric and pyranose signals indicates that their relative intensities do not depend on the chosen contact time in the range investigated, while contact times longer than 6 ms are necessary for carbonyl carbons to reach optimal cross-polarization conditions. The behavior observed for both the "Na" and "Ca" samples reflects exactly the ones reported by Salomonsen and co-workers for low-calcium content alginates, which were compared to the results obtained through quantitative single pulse ^{13}C spectra. Therefore, these results confirm that choosing a contact time of 2 ms for all the samples guarantees internal quantitativity between the spectra. CP experiments using different relaxation delays (3 and 10 s) were also performed to find the conditions guaranteeing full recovery of the proton longitudinal magnetization. Using a relaxation delay of 3 s revealed to be sufficient for quantitativity purposes.

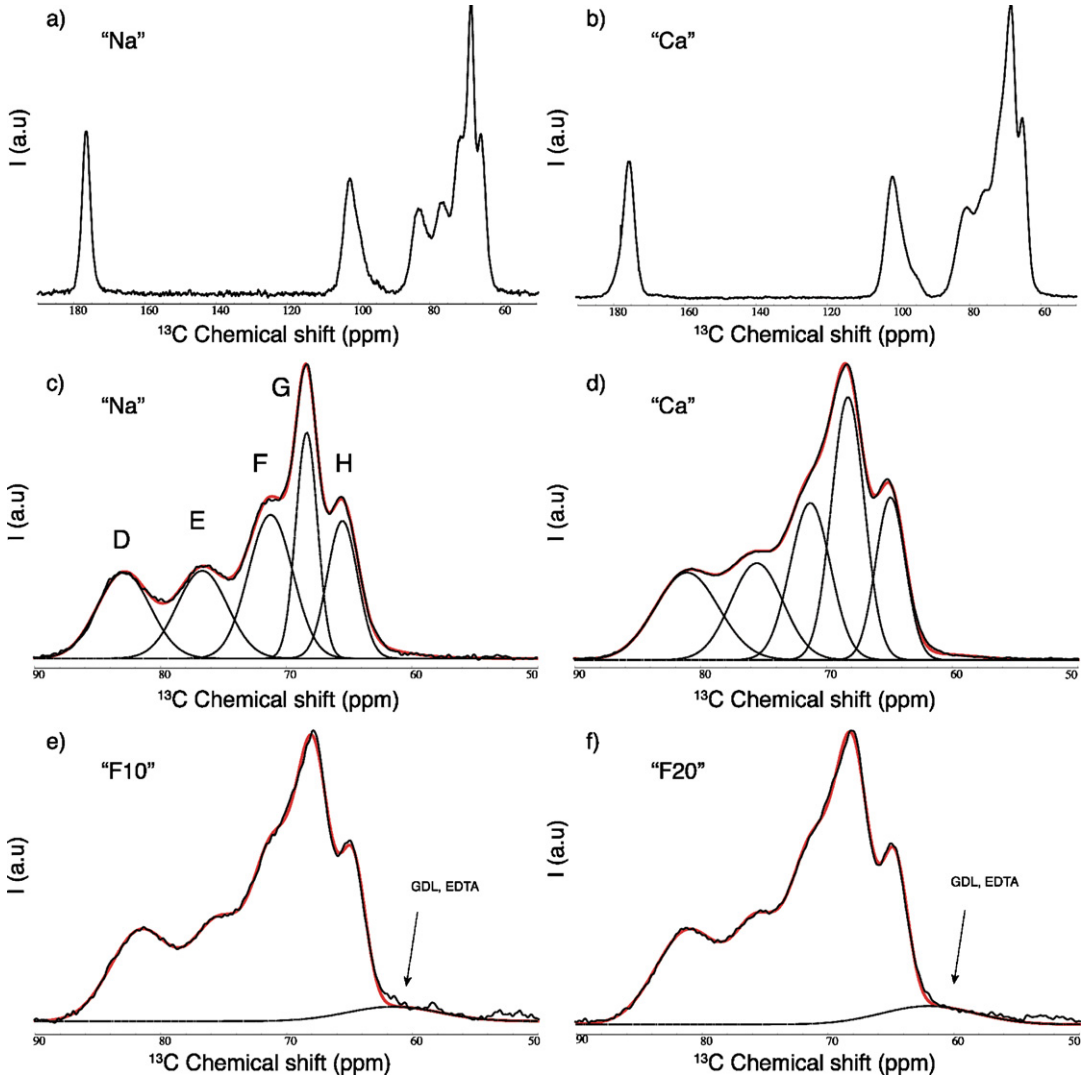

Fig. 2. ^{13}C CP/MAS spectra of sample n.1 (see Table 1) in the "Na" (a) and "Ca" series and expansions of the 50–90 ppm (pyranose) region of the ^{13}C CP/MAS spectra of sample n.1 in the series "Na" (c), "Ca" (d), "F10" (e) and "F20" (f) (black solid curves). The individual 5 Gaussian functions used in the deconvolution procedure of the pyranose region are shown as black dotted curves for the "Na" (c) and "Ca" (d) sample, while the best-fitted function is shown as a red solid curve for all the expanded spectra. An additional Gaussian peak has been used to fit the spectra of "F10" (e) and "F20" (f) samples (black dotted curve), only, to account for the residual presence of GDL and EDTA (see text for details). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2.5. ^{13}C CP/MAS spectra deconvolution

A deconvolution procedure was applied to all of the spectra in order to extract the exact values of chemical shift, line width and intensities of the signals in the spectral region between 50 and 90 ppm (pyranose region). A total number of 5 peaks were used to reproduce the pyranose region of the experimental spectra, as previously reported by Llanes, Sauriol, Morin, and Perlin (1997) and Salomonsen et al. (2009a, 2009b). The presence of residual GDL and EDTA in alginate samples prepared through the FIRA method was evident from the lower chemical shift region of the pyranose signals. As shown in Fig. 2c and d, this was taken into account in the analysis through the use of an additional peak centered at about 62 ppm in the deconvolution procedure of the corresponding spectra. The best fitting results were obtained using Gaussian shapes for all the peaks. The spectral fitting procedure (deconvolution) was performed using the *NonlinearModelFit* function implemented within Mathematica 8.0 (Wolfram Research Inc., 2010). No restraints were used for peak line widths and intensities. However, the observation of a general line broadening with increasing the calcium content of the samples, together with the variation of the relative peak intensities due to differences in the M/G ratio of the samples, made the identification of the isotropic chemical shift of peak F (see Fig. 2) ambiguous in some cases, where it appeared as a shoulder overlapped with the peak G of higher intensity. To keep a systematic approach in the spectral deconvolution, the chemical shift of this peak was fixed in all the deconvolutions to a value optimized case by case on the basis of the R^2 (correlation coefficient) value of the fitting.

2.6. Principal component analysis

Principal component analysis (PCA) (Hotelling, 1933) was performed on 36 ^{13}C solid-state NMR spectra of powdered alginates using Mathematica 8.0 (Wolfram, USA). PCA is an advanced statistical method for finding underlying patterns in data of high dimensions and expressing the data so as to highlight their similarities and differences. Essentially, it allows grouping of data after a suitable combination of a set of variables. A big advantage of this technique is that, once the patterns have been found, the transformed data set can be compressed in a reduced number of dimensions without any significant loss of information. The

two-dimensional data matrix (samples × variables, $m \times n$ dimensions) is decomposed into the outer product of two matrices (scores and loadings) plus some residuals. The scores ($m \times n$) and loadings ($n \times n$) matrices describe the actual systematic data variation: the scores contain the information about the samples while the loadings contain the information about the variables. This multidimensional information is usually extracted by plotting the PCA scores and loadings on the first two principal components, indicated as PC1 and PC2, which account almost entirely for the systematic variation.

3. Results and discussion

3.1. Determination of M/G ratio for different calcium contents

3.1.1. Quantitative analysis of ^{13}C solid-state NMR spectra

The ^{13}C CP/MAS spectra of the "Na" alginate sample n.1 (see Table 1) before and after three treatments leading to different calcium contents are shown in Fig. 2. The ^{13}C spectra of all the powder alginate samples investigated in this study can be separated into three spectral regions: the pyranose region between 50 and 90 ppm, the anomeric region between 90 and 110 ppm and the carboxylic region between 170 and 180 ppm (Salomonsen et al., 2009b). In the following discussion, only the peaks appearing in the pyranose region will be taken into account (indicated with the letters D–H in Fig. 2) since, as previously explained by Salomonsen et al., they can be suitably combined to determine the M/G ratio of the corresponding alginate sample. The assignment of this spectral region has been already reported for sodium alginates in the solid state (Llanes et al., 1997). In the present study this assignment was verified by comparing the reported spectra with the spectrum of an alginate sample containing 100% of mannuronate (results not shown, and is reported in Table 2). As shown in Fig. 2, the ^{13}C signals look generally broader after increasing the calcium content of the original "Na" samples, somehow limiting an accurate determination of the chemical shift of the signals in this region. For this reason, to evaluate the effect of a higher calcium content on the quantification of the M/G ratio through the methods proposed by Salomonsen et al. (2009b), a deconvolution of the ^{13}C spectra of all of the investigated samples was performed (see Section 2) to obtain the exact chemical shifts, line widths and intensities of the peaks corresponding to the pyranose region. An example of the deconvolution procedure, showing both the individual peaks and the total fitted spectrum (50–90 ppm region), is shown in Fig. 2a and 2b for the sample n.1 of the "Na" and "Ca" serie, respectively. The resulting chemical shift and line width values of each signal for the 4 series of alginate samples with different calcium content are shown in Table 2 together with the spectral assignment suggested by Grasdalen et al. (1979) and Llanes et al. (1997).

As shown by the standard deviations reported in Table 2, either the chemical shifts or the line widths of the ^{13}C signals falling in the pyranose region do not vary significantly within the same series of alginate samples (different M/G ratios but same calcium content). The main chemical shift variation is observed for signals D and E, corresponding to G4 and M4–M5 carbons, respectively. On one hand, with varying the M/G ratio, the signal corresponding to the D peak (G4) shows the most significant chemical shift variations in the "F10", "Na", and "Ca" series ($\Delta\delta$ = 0.7, 0.6 and 0.5 ppm, respectively). On the other hand, the largest chemical shift variations of peak E (M4–M5) are observed for the "Na" and "Ca" series ($\Delta\delta$ = 0.6 and 0.5 ppm, respectively) with increasing the M/G ratio. It is worth remembering that these effects concern carbon 4 of both M and G units, which is involved in the 1–4 glycosidic linkage. It is well known that, especially in the solid state, the chemical shift of such carbons is very sensitive to changes in the neighboring residues and in the geometrical properties of the chain ("primary" and "secondary" structures, respectively). The former effect can be related to changes in the triads distribution and/or ratio with increasing the M/G ratio of the alginate, as discussed by Grasdalen et al. in the liquid state (Grasdalen et al., 1979). However, effects related to the supramolecular structure of the polysaccharide are generally very relevant in the solid state and could explain the behavior observed for the signals D and E in the present study. This hypothesis is consistent with the line width data reported in Table 2, where the largest values are observed for the signals D and E in all the samples. This suggests that G4 and M4–M5 carbons experience a large chemical shift distribution that reflects the presence of heterogeneous chain geometries at these sites.

For what concerns the other pyranose signals (G and H), the chemical shift variation within the different series appears to be

Table 2
^{13}C chemical shifts and line widths of the four alginate series with different calcium content as obtained from spectral deconvolution of the pyranose region (50–90 ppm). Data are reported as average values ± standard deviation; the data range are also reported.

Nomenclature[a]	D	E	F[b]	G	H
Assignment[c]	G4	M4, M5	M3, M2	G3, G5	G2
Average chemical shift of the pyranose region (ppm)[d]					
"Na"	82.6 ± 0.2	76.5 ± 0.3	71.5 ± 0.1	68.4 ± 0.1	65.6 ± 0.1
Range	82.2–82.8	75.9–76.8	71.3–71.6	68.3–68.5	65.5–65.8
"Ca"	81.0 ± 0.2	75.8 ± 0.3	71.3 ± 0.0	68.2 ± 0.1	64.9 ± 0.1
Range	80.7–81.2	75.5–76.1	71.3–71.3	68.0–68.3	64.9–65.1
"F20"	81.2 ± 0.1	75.6 ± 0.1	71.2 ± 0.0	67.9 ± 0.1	64.7 ± 0.1
Range	81.0–81.3	75.5–75.7	71.1–71.2	67.8–68.0	64.6–65.0
"F10"	81.4 ± 0.2	75.3 ± 0.1	71.1 ± 0.1	67.8 ± 0.1	64.9 ± 0.1
Range	80.9–81.6	75.3–75.5	71.0–71.2	67.8–67.9	64.7–64.9
Average signal line width of the pyranose region (ppm)					
"Na"	5.5 ± 0.3	4.5 ± 0.7	4.0 ± 0.5	2.3 ± 0.1	2.9 ± 0.1
Range	5.1–6.1	3.7–6.1	3.7–4.7	2.0–2.5	2.7–3.1
"Ca"	5.8 ± 0.1	4.2 ± 0.6	4.1 ± 0.5	2.7 ± 0.3	2.7 ± 0.1
Range	5.5–5.9	3.4–4.8	3.7–4.7	2.1–3.0	2.6–2.9
"F20"	5.8 ± 0.2	4.4 ± 0.3	4.1 ± 0.0	2.9 ± 0.3	2.4 ± 0.1
Range	5.6–6.1	4.1–4.9	4.1–4.1	2.4–3.1	2.2–2.6
"F10"	5.8 ± 0.2	4.9 ± 0.3	3.7 ± 0.1	2.9 ± 0.2	2.5 ± 0.1
Range	5.5–6.1	4.6–5.4	3.7–3.9	2.6–3.1	2.3–2.8

[a] As reported in Fig. 2.
[b] The chemical shift of peak F was kept fixed in all the fittings (see text for details).
[c] According to Grasdalen et al. (1979).
[d] Downfield of TMS used as an external reference.

less important. This effect is particularly evident for peak G, for which a maximum chemical shift change of 0.3 ppm is measured for the "Ca" series. The observed behavior suggests that the corresponding pyranose carbons (G3, G5 and G2) are not significantly affected by a different M/G ratio, which agrees with the fact that these signals are not in strategic positions with respect to possible chemical environment changes related to the primary structure of the polysaccharide (sequence of M and G monomers).

3.1.2. Determination of the M/G ratio of high-calcium content alginates from ^{13}C solid-state NMR spectra

The results of the spectral analysis previously discussed were used to evaluate the effect of a different calcium content on the determination of the M/G ratio from the ^{13}C solid-state NMR spectra of the 9 investigated alginate samples.

In a recent work, Salomonsen et al. (2009b) compared several methods for the determination of the M/G ratio from solid-state NMR spectra of powdered alginates based on spectral deconvolution of the pyranose region. In fact, as in the present case, the occurrence of strongly overlapping ^{13}C signals required to find a method providing an effective separation of the contributions arising from M and G carbon signals. Two of the best methods for the determination of the M/G ratio combined different sets of spectral parameters obtained from the deconvolution of the ^{13}C alginate spectra, which could be unambiguously ascribed to G or M signals: (a) the (E + F)/(D + G + H) ratio, which accounts for the total (fitted) area of the M signals over the total (fitted) area of G signals in the pyranose region; (b) the ratio of the heights of the Gaussian deconvoluted peaks F and G, assigned to M3–M2 and G3–G5 carbons, respectively. These methods were compared with (c) the ratio of the absolute ("spectral") intensities of peaks F and G, which could be easily obtained from the intensity of the ^{13}C spectra at the chemical shift of the F and G peaks without any deconvolution procedure. The proposed methods were tested on a large number of sodium alginates with calcium content comparable to that of the "Na" samples investigated in the present study. The authors analyzed the correlation coefficient obtained comparing the ^{1}H liquid state NMR data and the data obtained from solid-state NMR measurement using the methods (a) to (c) and concluded that the fitted intensity ratio (method b) gave a good estimate of the M/G ratio in case only a restricted number of samples is available. They also demonstrated that multivariate curve resolution (MCR-ALS) is the best method to use in case a statistically relevant number of samples is available, since it allows the precise determination of the M/G ratio without any *a priori* knowledge by means of other methods. However, the application of the MCR-ALS method was not suitable for the present case for two reasons. On one hand, the limited number of alginate samples with different M/G ratio available in our study (9) did not constitute a statistically relevant data set. On the other hand, the significant spectral changes induced by the increased calcium contents of the 9 alginate samples when passing from the "Na" series to the "Ca", "F10" and "F20" series (see below) did not allow the entire set of 36 alginate samples to be treated as a statistical data set for this kind of analysis. In fact, the spectra of the pure chemical components into which the experimental alginate spectra should be decomposed are also expected to be modified by a different calcium content. Therefore, the M/G ratios of the 9 powder alginate samples studied were determined by means of the methods (a) to (c). This analysis was repeated for each series of samples ("Na", "Ca", "F10", and "F20") in order to evaluate the biasing effect of the calcium concentration on the different methods used for its determination. It is worth noting that the procedures used to increase the calcium content of the original series of 9 sodium alginate samples ("Na") did not include any step that could modify the M/G ratio of the samples. Therefore, comparable M/G ratios are expected for corresponding samples with different calcium content using the methods (a) to (c). However, as the results in Fig. 3 show, a significant variation in the value of the M/G ratio was observed using these methods in the present case, which was possibly induced by the changes in the spectral features connected to a different calcium content.

The M/G values calculated for each of the 9 alginate samples in the 4 series with different calcium content using methods (a) to (c) are compared in Fig. 3a–c with the M/G values obtained from ^{1}H liquid-state NMR data. As shown in Fig. 3a, the M/G ratios determined using the (E + F)/(D + G + H) ratio of the fitted peak areas display a certain degree of variation through the 4 series of alginate samples with different calcium content. In particular, the data show an increased discrepancy through the series with increasing the M/G content. The results for the "Na" series reflect those obtained previously (Salomonsen et al., 2009b), with the solid-state NMR data overestimating and underestimating the M/G ratio obtained from liquid-state NMR for low and high M/G values (M/G > 1.2), respectively, and a sum of squares of the residuals (SSR) of 0.87. The SSRs of the other series vary between 0.59 ("F20") and 0.81 ("F10"), indicating a good match with the M/G values obtained from solution state NMR. The M/G values obtained using the ratio of the intensities of the Gaussian deconvoluted F and G peaks are shown in Fig. 3b. In this case, the increase in calcium content produces an increased discrepancy through the series with increasing the M/G ratio. In this case, the SSRs vary between 0.57 ("Na") and 1.70 ("Ca") through the series, and the data result generally overestimated with

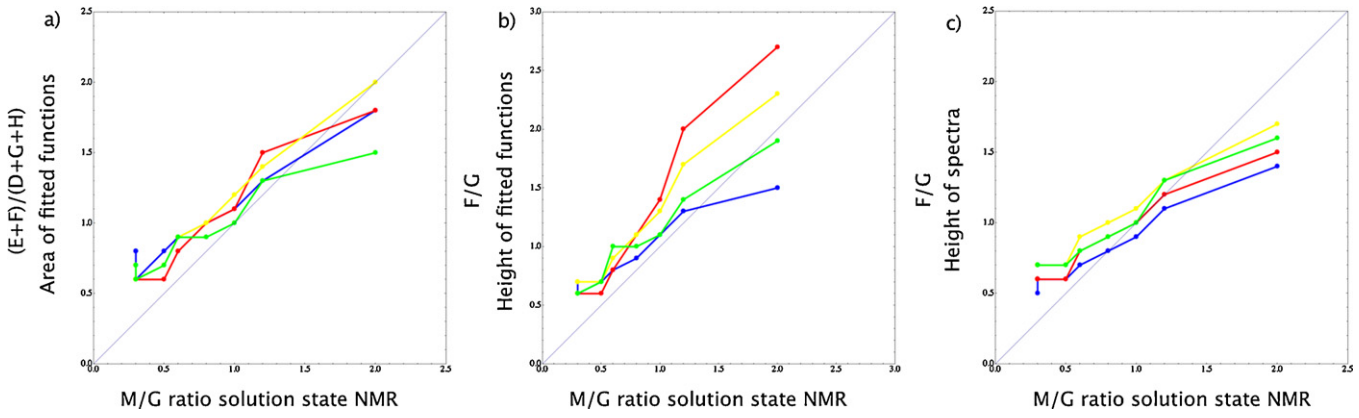

Fig. 3. M/G ratios of the 9 alginate samples in the "Na", "Ca", "F20" and "F10" series (indicated by blue, red, yellow and green filled circles, respectively) estimated from: (a) the ratio of the fitted Gaussian areas of the (E + F) and (D + G + H) signals; (b) the ratio of the height of the fitted Gaussian peaks corresponding to F and G signals; (c) the ratio of the absolute intensities (height of spectra) of signals F and G. The values are compared with the M/G ratios of the 9 "Na" samples obtained through liquid-state NMR. Lines are drawn to guide the eyes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

respect to the M/G ratios obtained from solution state NMR in the whole M/G range. On the contrary, the data in Fig. 3c, showing the M/G ratios obtained using the ratio of the absolute spectral intensities of the F and G signals appear to be the less biased by the presence of a different calcium content. This method provides the lowest SSRs for the different series, which vary between 0.62 ("Na") and 0.76 ("F20"). Moreover, differently from the other methods, a systematic deviation of the "Ca", "F10" and "F20" series from the behavior of the "Na" series is observed in this case, which does not change with the M/G ratio. This systematic deviation is positive throughout the whole investigated M/G range and increases with increasing the calcium content.

Method (c) is based on the sole absolute spectral intensity and does not therefore take into account the highly overlapping nature of the ^{13}C signals. This means that contributions to the observed intensity of a given peak could arise from other signals, whose area reaches the chemical shift value at which the intensity is measured. F and G peaks are expected to be the most affected from these effects. In the worst perspective, mannuronate and guluronate signals could contribute to G and F signals, respectively, hence giving biased results. At the same time, as previously discussed by Salomonsen et al. (2009b), the spectral deconvolution procedure is intrinsically subject to a certain degree of initial guessing about the number and the position of the peaks to be used in the analysis. In fact, a total of 8 signals are expected to contribute to the pyranose spectral region on the basis of the molecular structure of the alginate polymer (4 from each type of monomer). While the 5 signals used in the present deconvolution procedure are fully reliable because of evident spectral features, it is not sure to what extent the three left expected carbons could be contributing to the total spectrum: these peaks could be easily "hidden" under the fitted peaks at very similar or equivalent chemical shift values. It is clear that the methods for the determination of the M/G ratio based on the results of the deconvolution procedure are more sensitive to the modification of the spectral features due to a different calcium content. However, if we consider that corresponding samples with different calcium content "must have" the same M/G ratio on the basis of the preparation procedure used, method (c) seems to be the most effective in compensating effects of line width and chemical shift variations related to a significant increase in calcium content.

3.2. Separation of alginates with different calcium content by means of principal component analysis of ^{13}C solid-state NMR spectra

In view of possible applications of alginate powders with high calcium content in the medical and pharmaceutical field, it would be desirable to have access to the calcium percentage of the alginate powder without necessarily resorting to destructive methods like absorption spectroscopy. In the present study, we investigated the possibility of using the results of the deconvolution of solid-state NMR spectra to find an accessible way of separating alginate samples with different calcium contents. Before describing the method used in the present study to achieve this goal, the most remarkable spectral changes induced by the presence of a significant amount of calcium in the powdered alginate samples are discussed.

3.2.1. ^{13}C spectra of alginates with different calcium content: discussion of spectral features

The data reported in Table 2 show significant variations of chemical shifts and line widths with varying the calcium content of the samples. In particular, the average line width of signals D and G, assigned to G4 and G3–G5 signals, respectively, tends to significantly increase with increasing the calcium content of the samples. This could reflect the well-known tendency of G-rich chain sequences to coordinate calcium ions, generating very stiff structures. Notably, calcium ions are believed to be coordinated through oxygen ligands from the 2-OH and 3-OH, (1 → 4)-O-linkage and carboxylate and ring oxygen of the subsequent residue of the G-units. Lateral association of multiple poly-guluronate blocks can then contribute to the calcium ions coordination to form the so-called "egg-box" structure (Grant et al., 1973). The carboxylate group is directly attached to C-5 of the pyranose ring, 3-OH is adjacent to the C3 carbon, and the (1 → 4) linking oxygen is adjacent to the C4 carbon of G units. It is then reasonable that the ^{13}C signals corresponding to G5, G3 and G4 carbons are those mostly affected by the presence of an increased calcium content. A deconvolution of the line shape of the carbonyl signals present in the 170–180 ppm region of the alginate spectra revealed the presence, in all the cases, of two overlapped peaks centered between 175 and 177 ppm and characterized by line widths of about 1 and 3 ppm, respectively. In particular, the line width of the narrower peak regularly increased from about 1 to about 1.6 ppm with increasing the calcium content of the alginate through the series, hence confirming the effects discussed.

Moreover, Table 2 shows that an increase in the calcium content leads to a significant shift of the carbon signals of the pyranose region towards lower chemical shift values with respect to the "Na" samples. In particular, signals D, E and H show the largest variation, suggesting that the electron density distribution of the corresponding carbons is perturbed by the increasing amount of calcium. Those variations are more evident for signal D (≥1.2 ppm) than for signals E (≥0.7 ppm) or H (≥0.6 ppm). In fact, although signals D and E signals both refer to carbons that are involved in the formation of the glycosidic linkage between different monomeric units of the polysaccharide, carbons G4 (signal D) are expected to be directly influenced by the active involvement of G monomers in calcium coordination, hence justifying the larger chemical shift variations observed. On the contrary, the chemical shift of signal G varies only slightly, which agrees the indirect involvement of G5 carbons in the calcium coordination.

3.2.2. Principal component analysis

The ^{13}C CP/MAS spectra of the 36 alginate powders were analyzed by means of principal component analysis (PCA) in order to explore the potential of this methodology for discriminating the samples according to their calcium content. On the basis of the previous discussion (Section 3.2.1) the PCA statistical analysis primarily focused on the isotropic chemical shifts and line widths of the 4 D, E, G and H peaks falling in the pyranose region. Peak F was not included in the analysis for the reasons discussed in Section 2. The results of the PCA performed using two different sets of variables are shown in Fig. 4, where they are reported as the PCA scores and loadings plots.

In a first attempt to find the best set of variables for discriminating alginate powders according to their calcium contents, the chemical shifts and line widths of all the peaks were included in the analysis. As shown by the PCA scores plot in Fig. 4a, this approach provides a good separation between the different series of alginate samples on the basis of their calcium content. The PCA score plot shown describes 78.3% of the total spectral variation, distributed as 61.9% along the first principal component (PC1) and 16.4% along the second (PC2). This plot shows a very good level of separation of the 4 datasets along PC1, while the samples appear to be less separated along PC2. Notably, PC1 allows a neat separation between samples with low calcium content (all "Na" samples showing positive PC1 scores) from the rest of the samples. The "Na" and "Ca" series result to be the more distributed along PC2. Looking at the loadings plot of the 8 variables (Fig. 4b), it is clear that the chemical shifts of the signals D, E, G and H contribute to a similar extent to the separation observed along PC1. In particular, "Na"

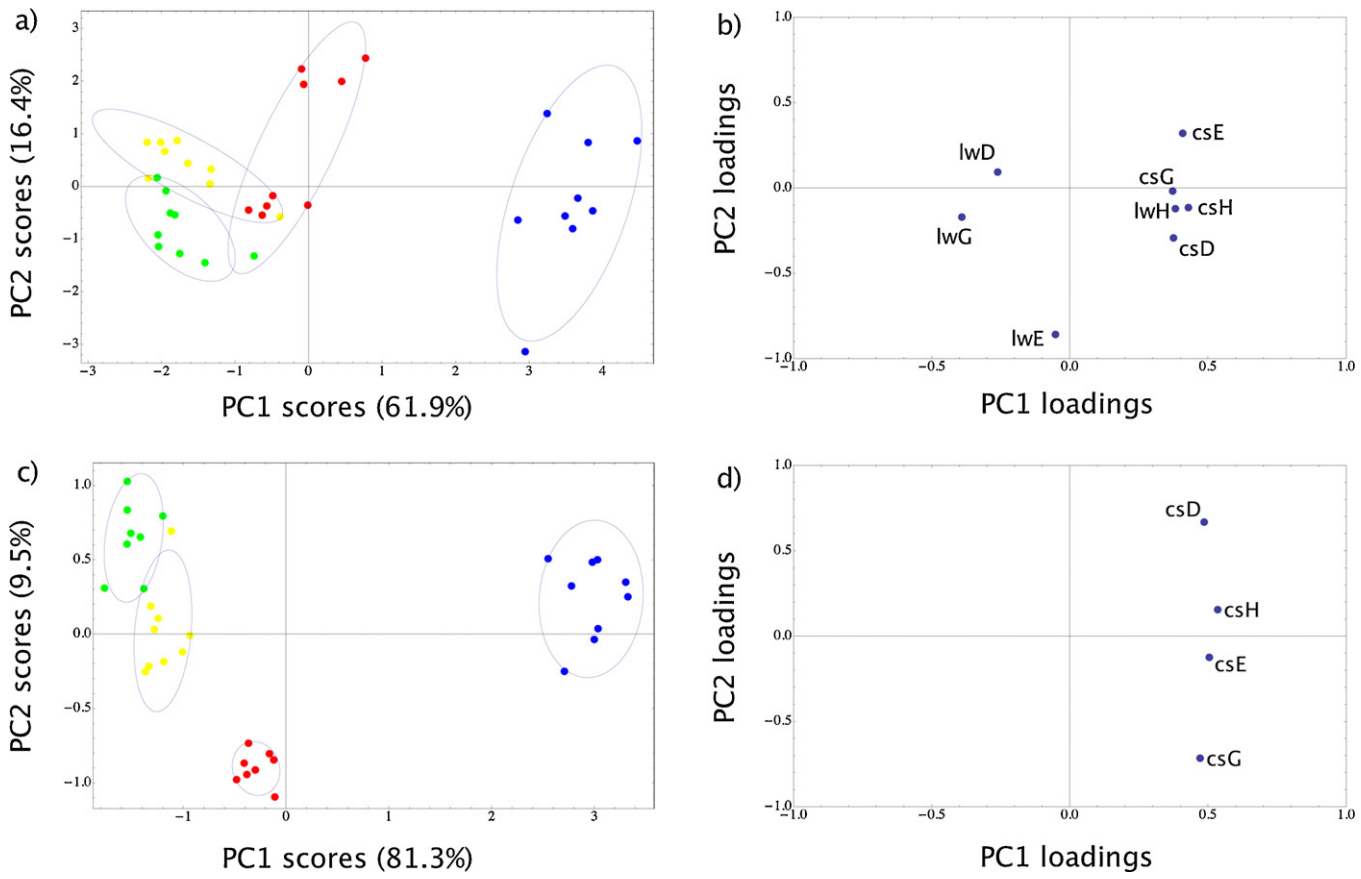

Fig. 4. PCA scores (a and c) and loadings (b and d) plots of the ^{13}C CP/MAS spectra of the 9 alginate samples in the "Na", "Ca", "F20" and "F10" series (indicated by blue, red, yellow and green filled circles, respectively). The results shown have been obtained using: (i) both the isotropic chemical shifts and the line widths of D, E, G and H signals (a and b plots); (ii) only the isotropic chemical shifts of D, E, G and H signals (c and d plots). The percentile confidence ellipsoids shown in (a) and (c) account for 90% of the data in each of the series. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

samples are all associated with higher values of the chemical shift of the 4 signals, while samples with larger calcium contents have lower chemical shift values. Therefore, the chemical shift of signals D, E, G and H seems to be directly correlated with the PCA scores and loadings along PC1. A similar contribution to this separation is given by the line width of signal H, which, as shown in Table 2, is the only one decreasing with increasing the calcium content of the samples. Again, H signals in "Na" samples are characterized by the largest line width, which is associated with larger PC1 loadings. On the contrary, the line widths of signals D and G, characterized by negative PC1 loadings, seem to be the most relevant in separating along PC1 the ensemble of samples with higher calcium content from the "Na" series, and the "Ca", "F10" and "F20" series, reciprocally. This can be explained considering the very large line width of these signals for the calcium-enriched alginates and their variations through the series with different calcium content: large line widths are again associated with high loadings (in absolute value) along PC1. All these variables lie at PC2 loadings values near zero, indicating that they do not significantly contribute to the separation of the samples along this direction. The highest absolute values of the loadings along PC2 are observed for the chemical shift of peak E (csE, positive), the chemical shift of peak D (csD, negative) and the line width of peak E (lwE, negative). While lwE seems to contribute to the separation of the "Ca" and "F10" series from the other samples along PC2 (the E signal shows the smallest and largest average line width in "Ca" and "F10", respectively), csE is probably the most effective in discriminating "Ca" from "F10" and "F20". All together, these observations are in agreement with the previous discussion about the changes in spectral features observed with increasing the alginate calcium content.

Although the results obtained using these 8 variables are very encouraging, especially considering that no *a priori* assumption or constraint was applied to the data, the ideal case would be to achieve the best separation with the smallest set of variables. Therefore, we performed a second analysis including only the isotropic chemical shifts of the 4 pyranose signals D, E, G and H. Fig. 4c and d shows the so-obtained PCA scores and loadings plots, respectively. The former plot shows that eliminating the contribution of signal line width from the analysis significantly reduces the data dispersions, generating very well-separated sets of samples corresponding exactly to the 4 alginate series with different calcium content. Most importantly, this plot constitutes a very clear evidence of the fact that the variation of the M/G ratios within each series of alginates does not influence the separation of the samples based on the calcium content. The PCA scores plot describes 90.8% of the total variation between the spectra, 81.3% along PC1 and 9.5% along PC2. PC1 shows very positive scores for the "Na" series and negative scores for the other series: in particular, the PC1 scores of the "F10", "F20" and "Ca" (high-calcium content) series decrease with increasing the calcium content. A better separation with respect to the previous analysis is also obtained along PC2. As discussed for the previous analysis, all the chemical shifts of the pyranose signals give a strong contribution to the separation of "Na" samples from the samples with higher calcium content along PC1 (see Fig. 4d). PC1 discriminates series of alginate samples according to their calcium content, while PC2 seems to be more efficient in giving a further level of separation between series

of calcium-enriched alginates characterized by slightly different calcium contents.

4. Conclusions

In this study, the potential of solid-state NMR in the investigation of insoluble calcium alginates for biomedical applications is evaluated for the first time. ^{13}C CP/MAS spectra deconvolution allowed the effects induced by the presence of increasing calcium contents on the alginate spectra to be assessed. These effects were tentatively discussed in terms of changes in the supramolecular structure of the polysaccharide chains, which leads to a progressive coordination of the calcium ions by means of the G-rich blocks of the copolymer. Moreover, three methods previously proposed for the estimation of the M/G ratio from solid-state NMR spectra of sodium alginates (soluble) were tested on the alginates with higher calcium content (insoluble). The spectral changes induced by the higher calcium content revealed to affect the value of the M/G ratio determined through the three methods to a different extent, suggesting that the presence of geometrical effects dependent on the calcium content must be accounted for if one wants to determine the M/G ratio properly. Taking the ratio of the absolute (non-fitted) intensities of the peaks corresponding to F and G signals appeared to be the most robust method with respect to variations in the calcium content of the alginate. In a second part of the study, the possibility of using solid-state NMR to separate sets of insoluble alginates on the basis of their calcium content was considered. Unsupervised statistical analysis (principal component analysis) of the ^{13}C spectra based on the chemical shifts of the signals falling in the pyranose region allowed a clear discrimination of the samples according to their calcium content, suggesting that this methodology could be straightforwardly used for the screening of the calcium content of large sets of insoluble alginate samples in a non-destructive way. At the current stage of development, the proposed method does not allow the calcium content of the samples to be directly obtained since no evident correlation between the PC1 or PC2 scores and the calcium content can be established. A possible future development of the method includes the use of a statistically relevant set of samples with known, sufficiently different, calcium contents to serve as a "basis set": the calcium content of an unknown alginate sample added to this basis set could be inferred from the match with one of the basis set samples. This study is currently in progress and will be the object of a dedicated paper.

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