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An infrared investigation in relation with chitin and chitosan characterization

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Abstract

The use of infrared spectroscopy for characterization of the composition of chitin and chitosan covering the entire range of degree of acetylation (DA) and a wide variety of raw materials is examined further. The ratio of absorbance bands selected was calibrated using ^{1}H liquid and ^{13}C CP-MAS solid-state NMR as absolute techniques. IR spectra of the structural units of these polymers validated the choice of baselines and characteristic bands. The bands at 1650 and 1320 cm^{-1} were chosen to measure the DA. As internal reference, the intensities at 3450 and 1420 cm^{-1} were evaluated. The absorption band ratios involving the reference at 3450 cm^{-1} had poorer fit.. The absorption ratio A_{1320}/A_{1420} shows superior agreement between the absolute and estimated DA-values ($DA\% = 31.92A_{1320}/A_{1420} - 12.20$; $r = 0.990$).

Keywords: Chitin; Chitosan; Degree of acetylation

1. Introduction

Chitin is the most important natural polysaccharide after cellulose found in crustaceous shells or in cell walls of fungi. However, it is not widely used for industrial applications up to now because it is insoluble in many solvents, relatively difficult to isolate from natural sources in pure form and to prepare in a reproducible way under good economic conditions. That is why it is also difficult to characterize this polysaccharide.

Its principal derivative is chitosan, obtained by deacetylation of chitin. It is soluble in aqueous acidic medium due to the presence of amino groups. The name chitosan is reserved to partially or fully deacetylated chitin soluble in acidic aqueous conditions, it usually also means that the average degree of acetylation (DA) is around or lower than 0.5; in addition, the solubility is also controlled by the distribution of the acetyl groups remaining along the chain.

For these different reasons, the characterization of chitin and chitosan is very delicate and has been largely discussed in the literature. Usually, a single technique cannot be adopted to cover the full range of DA, i.e. for chitin as well as for chitosan. For chitin, due to the lack of solubility, solid state NMR can be used [1–3], as well as infrared spectroscopy on film or powder [3–8]; for samples in the pure form, elemental analysis can also be used but with lower accuracy [9].

For chitosan, which is soluble in aqueous medium, more methods are available and they have been also often discussed in the literature. The main techniques suggested are potentiometry [10,11], ^{1}H NMR [12–14], UV spectroscopy [15–17] and infrared spectroscopy [3–8,18–26].

The most discussed technique is infrared spectroscopy because of its simplicity, but it needs a calibration versus an absolute technique. Many different calibration relations have been proposed, but they are still under discussion in the literature: some of the typical IR band ratios proposed will be discussed later. In this paper, we intend to improve the use of this technique as a way to characterize the degree of acetylation of chitin and/or chitosan.

The aim of this inter-laboratory study is to discuss the application of infrared spectroscopy for DA determination whatever the degree of acetylation, the source, the salt form, the purity and the solubility of the polymer. It will allow us

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Table 1
Identification of the samples used

Sample	Source	Method of purification (Ref.)	DA (%) and technique of determination
1	Crab shell	[24]	0.5[a]
2	Crab shell	[24]	0.5[a]
3	Crab shell	[24]	2[a]
4	Shrimp shell	[24]	3[a]
5	Shrimp shell	[24]	6[a]
6	Crab shell	[24]	7.8[a]
7	Shrimp shell	[24]	8.3[a]
8	Crab shell	[24]	8.5[a]
9	Shrimp shell	[27]	9.6[b]
10	Shrimp shell	[28]	10.1[b]
11	Crab shell	[27]	11.2[b]
12	Crab shell	[24]	12[a]
13	Shrimp shell	[24]	13.2[a]
14[c]	Squid pen	[29]	13.8[b]
15	Lobster shell	[30]	20.1[a]
16	Crab shell	[24]	21[a,b]
17	Crab shell	[d]	40[a]
18	Crab shell	[d]	44[a]
19	Shrimp shell	[31]	56.3[b]
20[c]	Squid pen	[31]	57.5[b]
21	Shrimp shell	[28]	86.9[b]
22	Shrimp shell	[32]	94.6[b]
23[c]	Squid pen	[e]	95.9[b]
24	Crab shell	[24]	97[b]
25[c]	Squid pen	[e]	97.9[b]

[a] Liquid ^{1}H NMR.
[b] Solid state CP/MAS ^{13}C NMR.
[c] Samples obtained from native β-chitin.
[d] Obtained by reacetylation of chitosan.
[e] Obtained by deproteinisation of squid pen meal with NaOH 1 M.

to propose a more reliable method for using the infrared spectral analysis to determine the composition of these biopolymers.

2. Experimental

The samples of chitin and chitosan in pure form were prepared in our laboratories as described previously [24]. They were selected from different sources as summarized in Table 1; some of them were commercial samples but purified by us and others were isolated from natural sources in our laboratories. Their DA were determined by NMR for calibration.

D-Glucosamine hydrochloride and *N*-acetylglucosamine — from Fluka and Janssen Chimica, respectively, — were used as model substances without further purification; D-glucosamine was prepared by neutralization of the hydrochloride form and freeze dried.

The FT-IR spectrophotometer used to record spectra was a FT-IR 1720 X from Perkin–Elmer. The samples were prepared in 0.25 mm thickness KBr pellets (1 mg in 100 mg of KBr) and stabilized under controlled relative humidity before acquiring the spectrum.

Samples 22 and 25 were analysed in a Nicolet Protégé (System 460 E.S.P) FT-IR spectrometer (Madison WI, USA) in pellet, powder or film. Transmission spectra were recorded either in KBr pellets or in dry films (casted from DMAc–LiCl 5% solutions) using a standard sample holder.

A Gemini sampling accessory was used to collect horizontal attenuated total reflectance (ATR) spectra using a standard ZnSe crystal (angle of incidence = 45°). The chitin films were pressed with a Minigrip device so as to assure uniform contact between the sample and the ATR crystal. These spectra were submitted to ATR correction to correct this kind of spectra for variation in the depth of penetration using the OMNIC software of the instrument.

Spectra of powder samples were collected directly using an accessory for diffuse reflectance IR-FT spectroscopy (DRIFTS) fitted with an aluminum sampling head attached to the Gemini accessory, against a background of KBr. DRIFTS spectra were transformed into Kulbeka-Munk units (similar to absorbance units of transmission spectra) in order to compensate for broader and decreased peak intensities at higher wavelengths using the same software as for the ATR spectra.

In all cases, IR spectra were recorded by accumulation of at least 64 scans, with a resolution of 2 cm^{-1}.

High resolution liquid ^{1}H NMR spectroscopy was carried out on a Bruker AC300 usually at 80°C; the solution of chitosan in D_2O was prepared at $C = 10$ mg/ml with HCl (pH ~ 4); the solution was freeze dried three times to exchange labile protons. Analysis of the spectra was performed as discussed previously [14].

^{13}C NMR solid-state spectrometry was conducted by single-contact 50.32 MHz ^{13}C CP-MAS (cross-polarization magic angle spinning) on a Bruker MSL CXP-200 spectrometer fitted with a Bruker-z32DR-MAS-DB probe. Samples in powder form were contained in a ceramic cylindrical rotor and spun at 4.5 KHz. Contact time for cross polarization was 2.5 ms and 1400–4000 scans accumulated. Spectra were referenced indirectly to a zero value for tetramethylsilane (TMS).

3. Results and discussion

3.1. Model analysis with the structural units

Figs. 1 and 2 give the IR spectra of the two molecules representing the repeating units in these polymers; many differences appear, especially when looking for a reference band. Comparing both spectra, it could be appreciated that a specific band appears at 1320 cm^{-1} for *N*-acetylglucosamine. The band located at 2900 cm^{-1}, often used in the literature as reference band to analyze chitin and chitosan, must be excluded as, for glucosamine, it may not be distinguished from the background. As reference peak, we

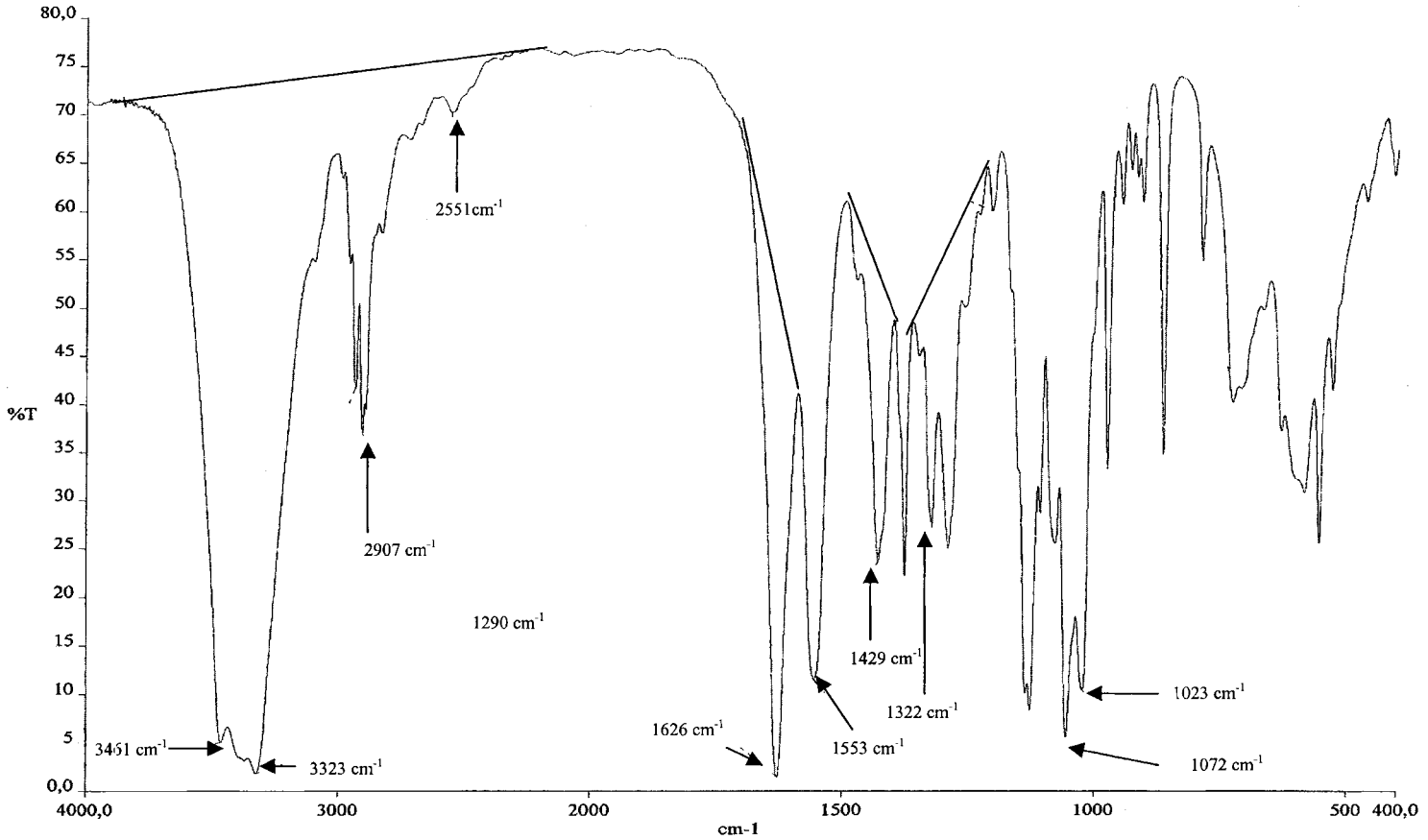

Fig. 1. IR spectrum obtained with *N*-acetyl D-glucosamine. Representation of the baselines adopted.

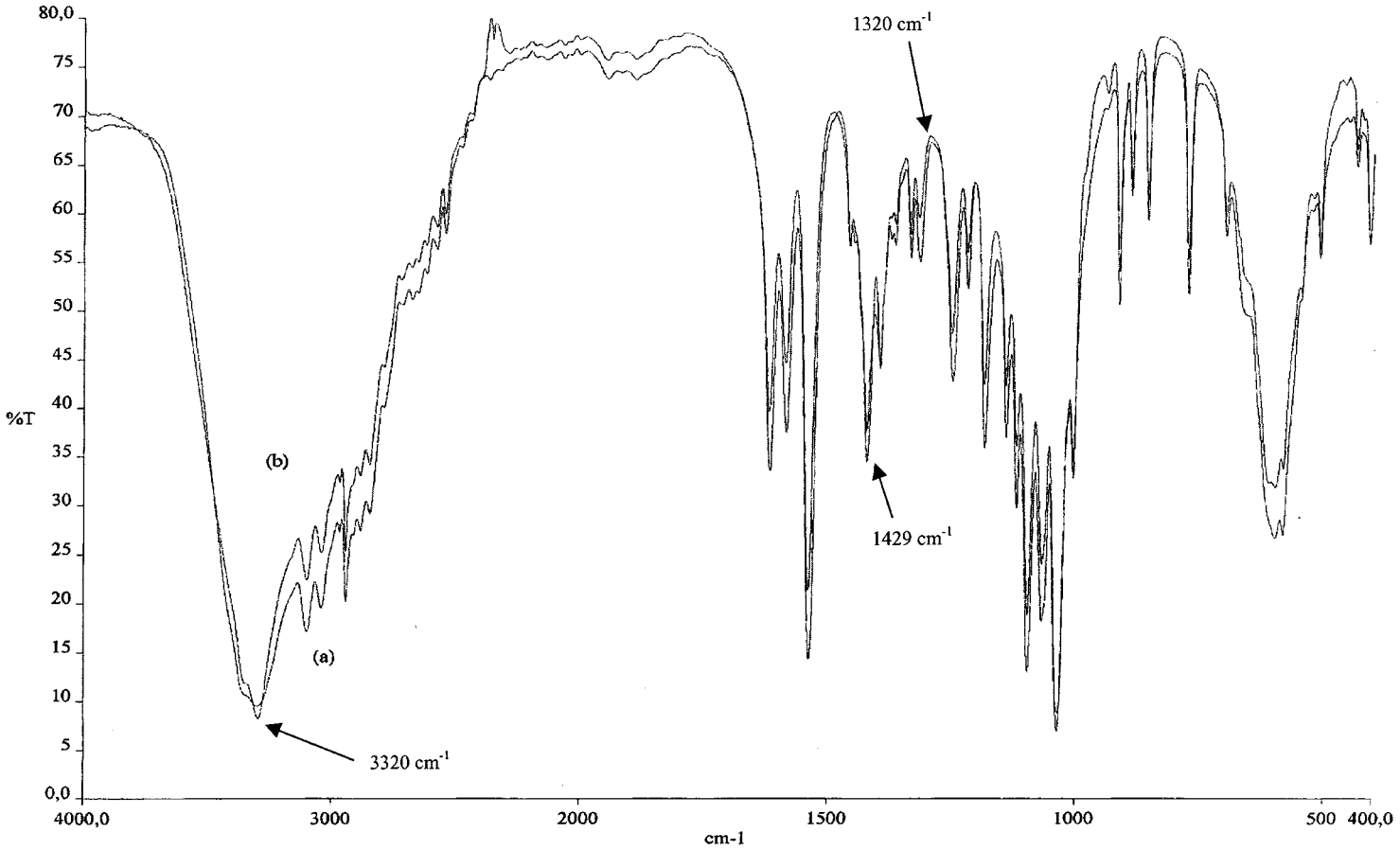

Fig. 2. IR spectra obtained for D-glucosamine: (a) in the chlorhydrate form; and (b) in the amine form.

evaluated two possibilities, either the large band centered at 3350 cm^{-1} (very near to that at 3450 cm^{-1} chosen for polymers) or the 1420 cm^{-1} band, which also seems to be suitable from the comparison of the two monomers.

When glucosamine is compared with its chlorhydrate form (Fig. 2), it can be seen that no modification is observed in the spectrum, which allows to conclude that the degree of protonation of the sample in the dried state will have no influence; this will be the situation when chitosan is isolated from solution without controlling the pH. We also tested the influence of water content in the sample (the spectrum is not shown) and it was observed no real influence in relation with the enlargement of the large –OH band at 3350 cm^{-1}.

Mixtures of glucosamine and *N*-acetyl glucosamine were prepared to consider the IR spectrum as a function of the composition of the mixture. The spectra obtained by computer-made linear addition of the spectra collected for the two structural units separately were calculated and compared with the experimental IR spectrum (Fig. 3). There is a very good agreement between the experimental and calculated spectra. A band at 2400 cm^{-1} appears in the experimental spectrum due to vibration band within CO_2 molecules. From this result, using the absorbance values of the bands at 3350 cm^{-1} (as the reference band) and 1320 cm^{-1} (as the measuring band) and the baselines indicated in Fig. 1, one gets a calibration curve (not shown) relating the absorbance ratio with the mixture composition (expressed as the monomeric unit fraction of *N*-acetyl-glucosamine).

3.2. Chitin and chitosan analysis

In Figs. 4 and 5 are given the IR spectra of the two polymers (samples 24 and 1, respectively) with the aim to show the role of the degree of acetylation in the shape of the spectra and to identify possible baselines (Fig. 4).

Many ways to analyze these spectra are proposed in the literature and recalled in Table 2. We have tested all these relationships as it has been also recently performed by other authors [8]. It must be mentioned that the validity of the calibration also depends on the absolute technique used to measure the DA. Titration is only valid for perfectly soluble materials as well as liquid NMR; solid state NMR has not been frequently used and is also delicate as discussed separately [33]. As already mentioned, elemental analysis is convenient but only in complete absence of residual proteins and generally is less precise.

In addition, in the literature, calibration covering all the values of DA has been obtained by mixing two samples (representative of chitin and chitosan) in different ratios [4]. This procedure should clearly give a linear relationship when choosing valuable base lines and a characteristic band for the *N*-acetyl substitution, but would not be appropriate as a general calibration to analyze samples regardless of their characteristics and nature.

Then, from Table 2, it can be appreciated that no relationship is available covering all the range of DA and different sources of materials.

In this work, for the first time, a large variety of samples prepared under purified form and characterized separately in our laboratories were examined using identical baselines and procedure. As it can be noted from Table 1, this set of samples covered all the range of DA values and comprised soluble and insoluble polymers obtained from a wide variety of sources.

During calibration, the DA was determined by solid-state ^{13}C NMR for samples with DA larger than 50%, while for those with lower DA, soluble in aqueous medium, liquid ^{1}H NMR was employed (except for samples 9, 10, 11 and 14; see Table 1). Sample 16 was analyzed using both solid-state and liquid NMR as a way to corroborate the reproducibility of these measurements [33].

In a separate experiment, the role of hydration on the IR spectrum of the polymers was tested. With this aim, a sample of chitosan was stabilized in 98% relative humidity and compared with another perfectly dried sample. No significant influence was observed as shown in Fig. 5. Similar results were reported previously by Domszy and Roberts [21].

Fig. 6 (a–d) illustrates the FT-IR spectra (shown in absorbance) collected for α-chitin (sample 22) analyzed using four different sampling techniques in powder, pellet or film state, namely ATR, DRIFTS and transmission. Fig. 6a shows an ATR spectrum recorded on a film. The ATR spectra reveals the very low resolution that can be achieved in this sampling technique even after collection of 64 scans typically recorded. Note that amide I band (doublet at 1655 and 1625 cm^{-1}) cannot be resolved as they appear fused into a single band when compared to a standard transmission spectrum of α-chitin collected in a KBr pellet (Fig. 6d). Also, the characteristic band at 1320 cm^{-1} has a very small intensity. The general poor quality observed in the ATR spectrum is likely to be the result of non-uniform contact between the dry film and the ZnSe crystal surface, hence perturbing the penetration of evanescent radiation into the sample. Therefore, this technique cannot be recommended as a standard procedure to characterize neither chitin nor chitosan films [34]. Whether films with greater uniform thickness and less imperfection will produce spectra of better quality remains to be tested experimentally, since in grafted chitosan films reproducible ATR spectra has been reported [35].

By contrast, DRIFTS analysis on powder of α-chitin (mixed with KBr) produced a spectrum (Fig. 6b) of much better resolution than those collected in the ATR mode. It is interesting to note that the amide I band in the DRIFTS spectrum of α-chitin is split into

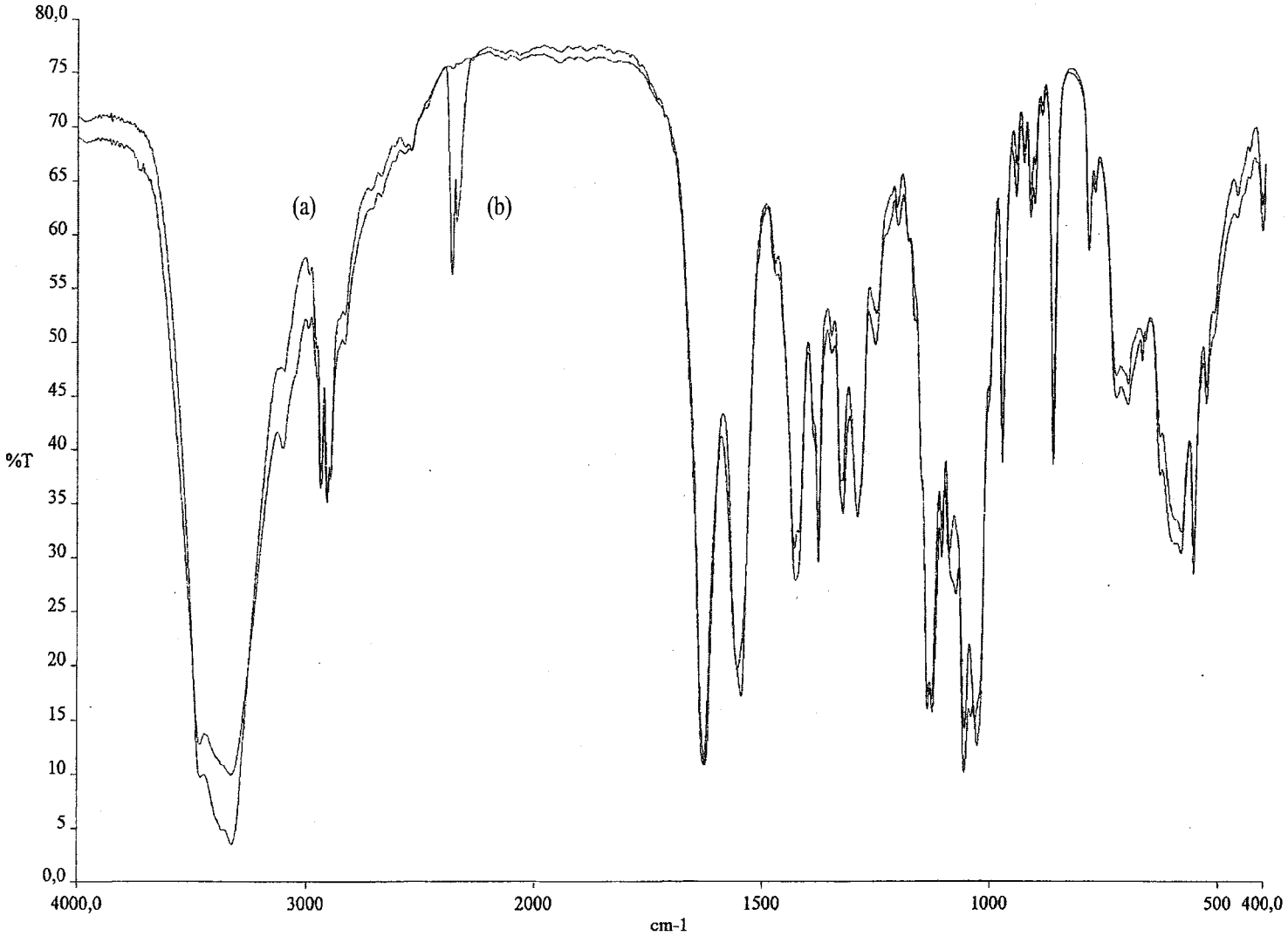

Fig. 3. IR spectrum for the mixture of *N*-acetyl glucosamine/glucosamine in a 80/20 weight ratio. Comparison between the calculated spectrum (a) and the experimental one (b).

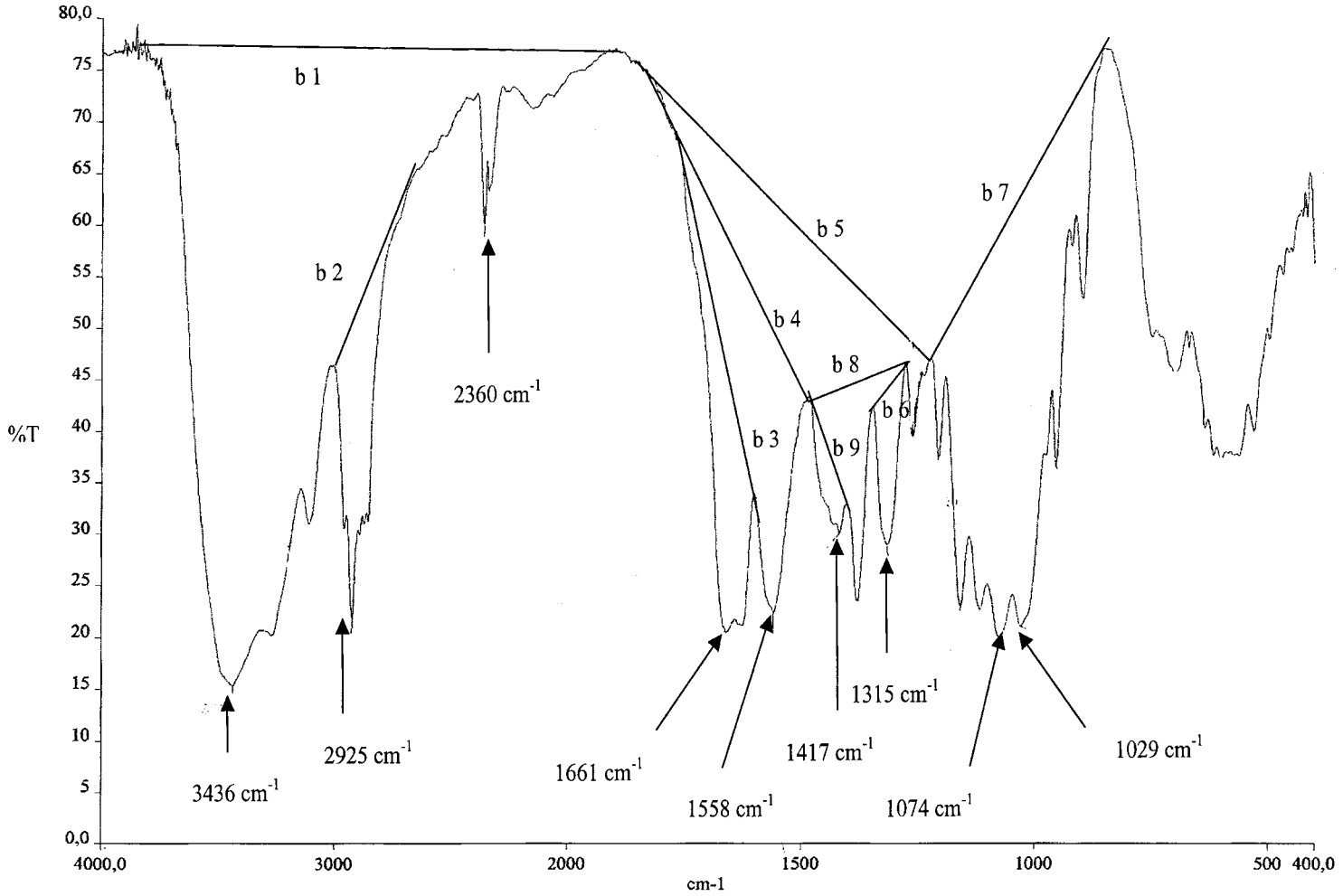

Fig. 4. IR spectrum for chitin (sample 24). Representation of the different baselines tested and mentioned in the literature.

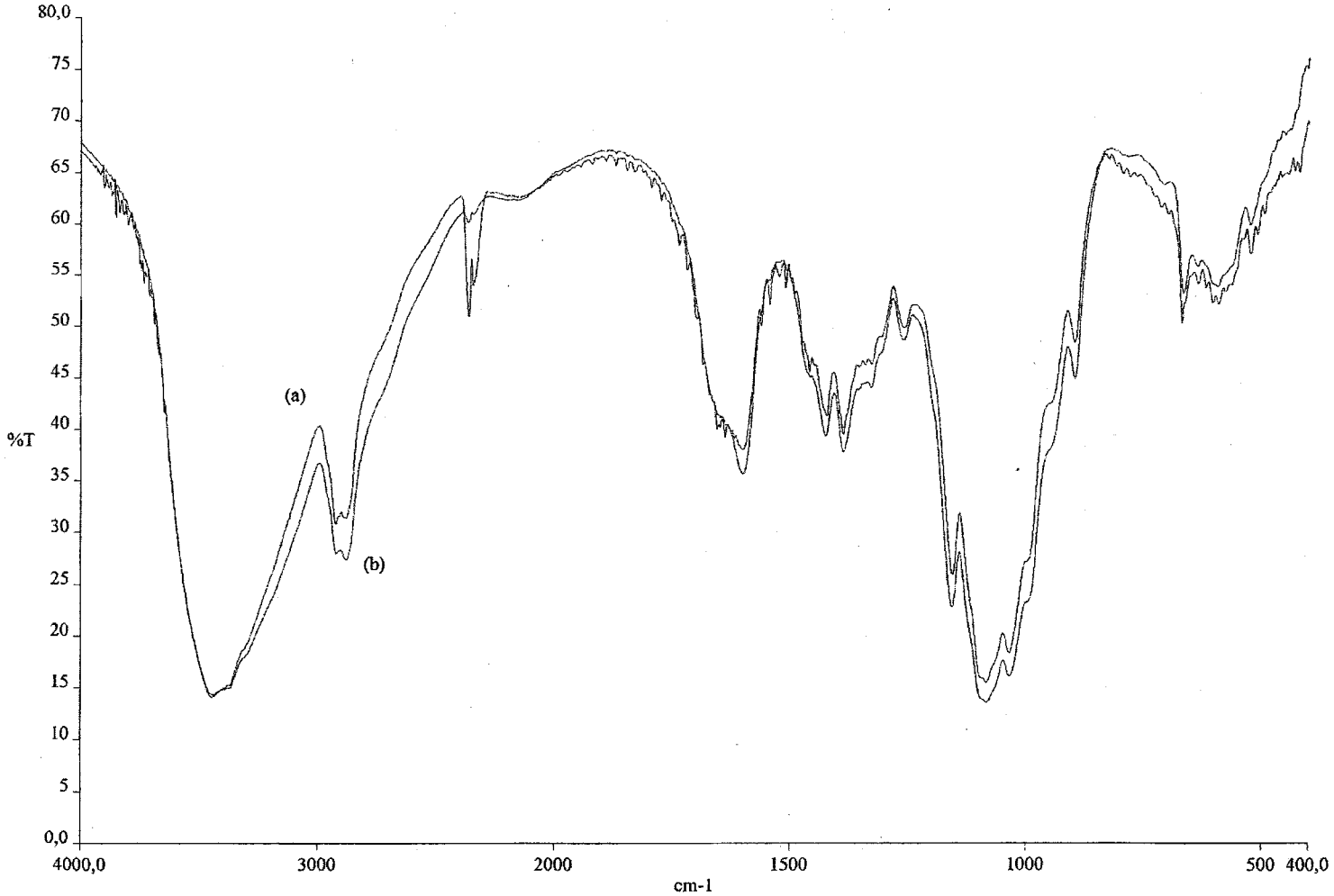

Fig. 5. IR spectra for fully deacetylated chitosan (sample 1): (a) dried state; and (b) hydrated sample.

Table 2
Characteristics of the main ways to analyze an infrared spectrum of chitin or chitosan (NR: Not reported)

Baselines		RB[a] (cm^{-1})	CB[b] (cm^{-1})	Range of DA (%) covered	Method of calibration	Natural Source	Ref.
For RB[a]	For CB[b]						
b 1	b 3	3450	1655	0–41	Potentiometry and ^{13}C CP/MAS solid state NMR	Shrimp and krill	[3]
b 7	b 4	1070	1560	Mixture of 0 and 100% acetylation	^{1}H NMR	Crab	[4,5]
b 7	b 4	1030	1560				
b 7	b 4	1070	1655 + 1630				
b 7	b 4	1030	1655 + 1630				
b 1	b 4	3450	1655 + 1630				
b 2	b 5	2878	1560	5–8	Elek and Harte method	Shrimp	[7]
b 2[c]	b 4	2877	1626	16–68	^{13}C CP/MAS solid state NMR	Crab	[8]
b 2	b 4	2877	1663 + 1626				
b 7	b 4	1074	1561				
b 7	b 4	1025	1561				
b 2	b 3	2867	1655	0–25	Colloidal titration	Crab	[19]
b 1	b 5	3450	1655	24–83	Periodate oxidation	NR	[20]
b 1	b 5	3450	1655	14–72	Hydrobromide salt titration, residual salicylaldhehyde determination	NR and prawn	[21]
b 1	b 3	3450	1655	0–55	Potentiometry and dye absorption	Scampi	[22]
b 1	b 5	3450	1655	10–30	Titrimetric method	NR	[23]
b 8	b 4	1430	1550	25–83	Chemical hydrolysis of acetyl groups [24]	Lobster	[26]

[a] RB corresponds to Reference Band.
[b] CB corresponds to Characteristic Band of the *N*-acetylation.
[c] b*x* corresponds to the baseline *x* represented in Fig. 4.

its two components. This is in close correspondence with experimental evidence obtained by transmission IR and FT-IR over the past decades [36]. This has been interpreted as a result of the two types of H-bonds formed by amide groups in the antiparallel alignment present in α-chitin crystalline regions [37]. Indeed, in β-chitin powder (sample 25), where a parallel chain alignment is present in the crystalline regions the DRIFT amide I band appears as a single peak (Fig. 6e). Also, in the transmission spectrum of α-chitin film (Fig. 6c), where the natural crystalline order should be expected to be lost, the amide I band shows a well-defined peak at 1650 cm^{-1} with a minor shoulder at 1625 cm^{-1}.

The different calibration curves were represented as usually proposed in the literature choosing different baselines and different characteristic bands for measuring the *N*-acetyl content. The best curves are given in this paper. All IR absorption bands were calculated from transmission spectra from either pellets or films.

In Fig. 7 is shown the calibration curve for the first band ratio considered and taking into consideration the information obtained on *N*-acetyl-glucosamine and glucosamine. The baselines were adopted as shown in Fig. 4 taking the large band centered at 3450 cm^{-1} (baseline 1) (corresponding to that located at 3350 cm^{-1} for the structural units) as the reference one and the band at 1320 cm^{-1} characteristic of –OH, $-NH_2$, –CO groups was chosen to measure the extent of *N*-acetylation (baseline 6); the correlation between the experimental DA values and the ratio of absorbance A_{1320}/A_{3450} is expressed by the relation

$$A_{1320}/A_{3450} = 0.03146 + 0.00226\mathrm{DA} \qquad \text{with } r = 0.97 \quad (1)$$

The values of absorbance ratios obtained for the mixtures of the structural units are in the same range of the values obtained for the polymers. In this work, we excluded definitively the ratio A_{1650}/A_{2900}, for the reasons given, from structural units investigation and especially the fact that an important absorbance at 1650 cm^{-1} exists in both glucosamine and *N*-acetylglucosamine spectra. This conclusion is in disagreement with the calibration relationship proposed recently [8].

Fig. 8 gives the calibration curve obtained taking the 1420 cm^{-1} band as reference with the baseline 9 (see Fig. 4) and the characteristic band located at 1320 cm^{-1} with the baseline 6. The linear correlation can be expressed by the

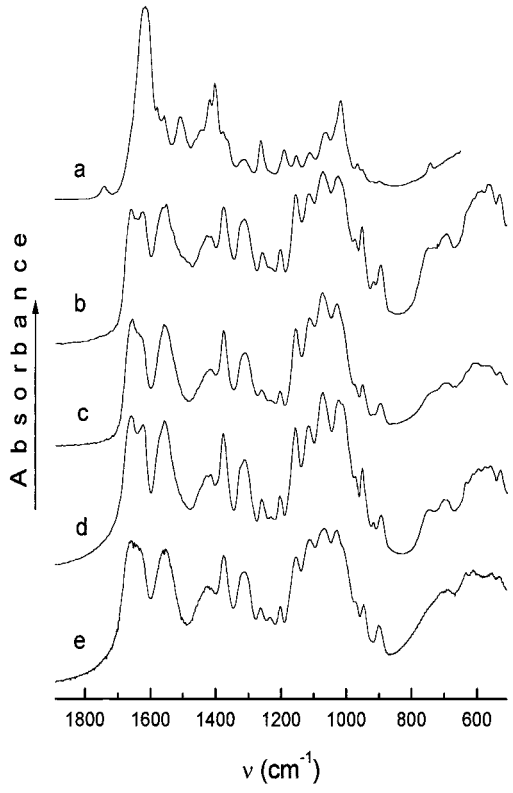

Fig. 6. Comparison of IR spectra (shown in absorbance) of α- and β-chitin (samples 22 and 25, respectively) recorded under different sampling techniques. For α-chitin: (a) ATR on film, (b) DRIFTS on powder, (c) Standard transmission on film, (d) Standard transmission on KBr pellet. For β-chitin: (e) Standard transmission on KBr pellet.

relation:

$$A_{1320}/A_{1420} = 0.3822 + 0.03133\ \mathrm{DA} \quad (2)$$

The agreement is surprisingly good in all the range of DA values ($r = 0.99$). This reference band at 1420 cm^{-1} was suggested first by Peniche et al. [26] and it is clearly identified in the comparison between both structural units (Figs. 1 and 2).

Only two points significantly lie out of the calibration curve and just correspond to β-chitin samples 23 (DA = 95.9%) and 25 (DA = 97.9%). This discrepancy may be related with the morphology of the native chitin and especially to the different H-bond network present in this polymorph as compared to the α-chitin. Moreover, it should be noted that samples 14 (DA = 13.8%) and 20 (DA = 57.5%) are also obtained from squid pen, but they show very good agreement with the linear correlation curve. This apparent contradiction reinforces the aforementioned explanation, since it should be expected that the strong alkaline treatment employed to deacetylate chitin destroys the native crystalline order of β-chitin. Then it can be concluded that the proposed calibration is not valid only for samples obtained from β-chitin with DA values greater than ~60%; in fact, this limit cannot be determined precisely because no sample is available to us in the range DA = 60 to 96%.

Analysis of the reproducibility of the two proposed absorbance ratios was also accomplished using four replicates of standard transmission spectra on both films and pellets. It revealed very good agreement between the values

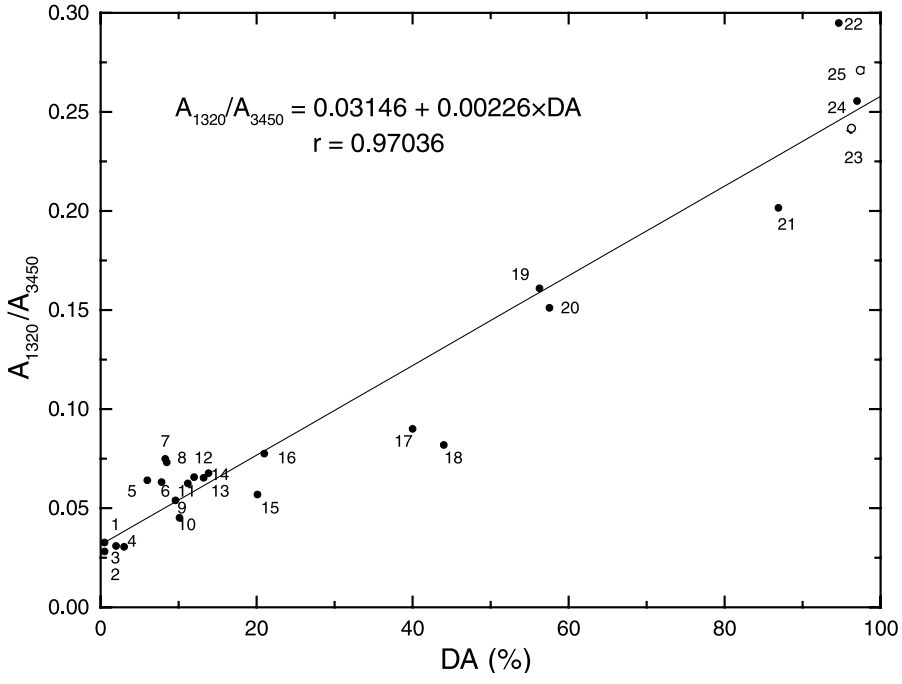

Fig. 7. Calibration curve giving A_{1320}/A_{3450} as a function of the degree of acetylation (DA). Baselines 1 and 6 (see Fig. 4). Open ○ is related to samples from β-chitin.

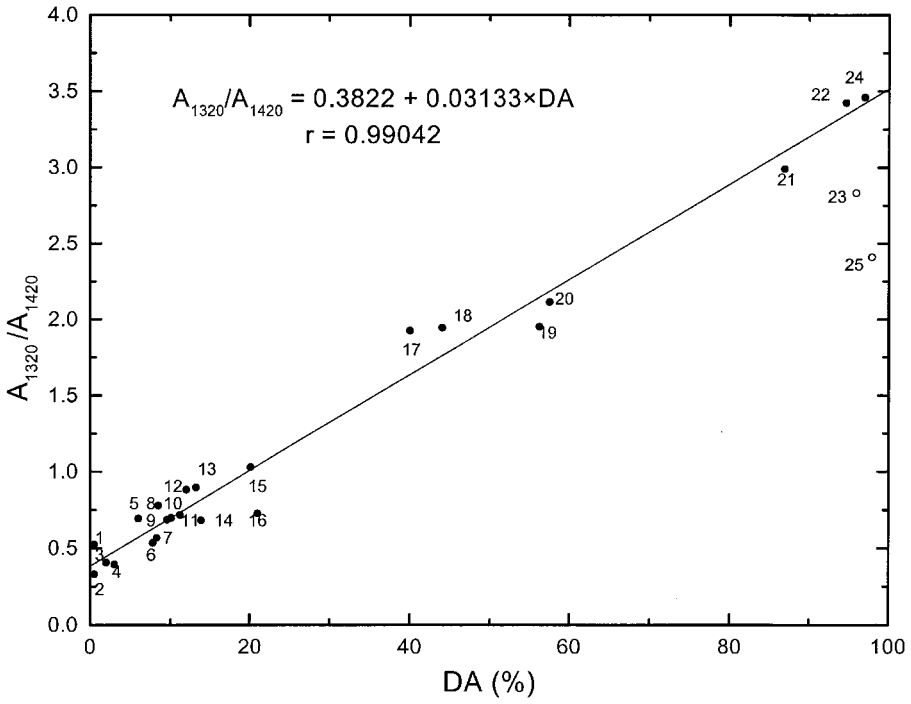

Fig. 8. Calibration curve giving A_{1320}/A_{1420} as a function of the degree of acetylation (DA). Baselines 6 and 9 (see Fig. 4). Open ○ is related to samples from β-chitin.

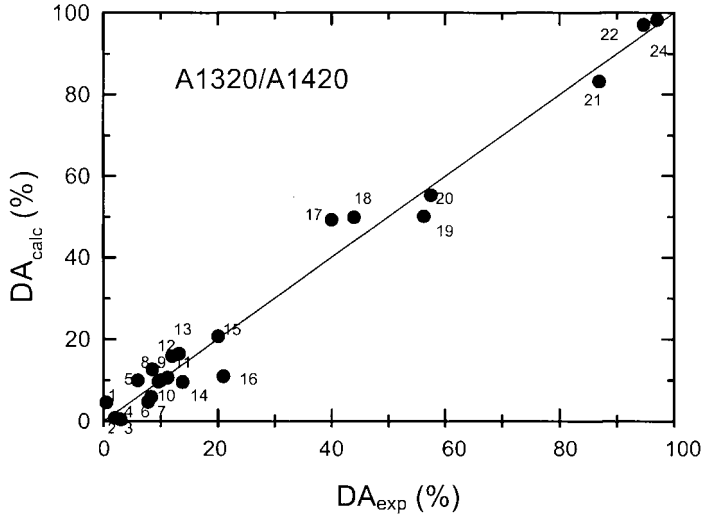

Fig. 9. DA(%) calculated from the ratios of absorbance A_{1320}/A_{1420} using the calibration relationship (2) as a function of the experimental values obtained from NMR.

irrespective of material state, with the A_{1320}/A_{1420} ratio better than A_{1320}/A_{3450}.

It can be appreciated clearly in Fig. 9 that the two band ratio A_{1320}/A_{1420} gives the narrower experimental error independent of the technique and state of the material. This evidence supports the use of A_{1320}/A_{1420} ratio, as being only sensitive to the chemical composition of chitin (or chitosan) irrespectively of technique, state and secondary structure.

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