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Methodological effects in Fourier transform infrared (FTIR) spectroscopy: Implications for structural analyses of biomacromolecular samples

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A B S T R A C T

A set of experimental data obtained by Fourier transform infrared (FTIR) spectroscopy (involving the use of samples ground and pressed with KBr, i.e. in a polar halide matrix) and by matrix-free transmission FTIR or diffuse reflectance infrared Fourier transform (DRIFT) spectroscopic methodologies (involving measurements of thin films or pure powdered samples, respectively) were compared for several different biomacromolecular substances. The samples under study included poly-3-hydroxybutyrate (PHB) isolated from cell biomass of the rhizobacterium *Azospirillum brasilense*; dry PHB-containing *A. brasilense* biomass; pectin (natural carboxylated heteropolysaccharide of plant origin; obtained from apple peel) as well as its chemically modified derivatives obtained by partial esterification of its galacturonide-chain hydroxyl moieties with palmitic, oleic and linoleic acids. Significant shifts of some FTIR vibrational bands related to polar functional groups of all the biomacromolecules under study, induced by the halide matrix used for preparing the samples for spectroscopic measurements, were shown and discussed. A polar halide matrix used for preparing samples for FTIR measurements was shown to be likely to affect band positions not only *per se*, by affecting band energies or via ion exchange (e.g., with carboxylate moieties), but also by inducing crystallisation of metastable amorphous biopolymers (e.g., PHB of microbial origin). The results obtained have important implications for correct structural analyses of polar, H-bonded and/or amphiphilic biomacromolecular systems using different methodologies of FTIR spectroscopy.
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1. Introduction

Fourier transform infrared (FTIR) spectroscopy in different methodological variants has been increasingly applied since its 'early days' [1–3] up to the last decades for both theoretical and experimental structural molecular-level characterisation of diverse biological materials, from simple organic and biological molecules to more sophisticated biomacromolecular substances, biomaterials, cells and tissues (see, e.g. [4–12] and references reported therein). Structural characteristics of samples under study in FTIR spectra are provided by various vibrational modes related to virtually all functional groups of molecules, as well as to all inter- and intramolecular interactions which noticeably modify bond geometries, lengths and/or energies. This is both an advantage (due to a rich informativity of a spectrum) and a drawback of the FTIR spectroscopy technique (due to its high sensitivity to slight variations of the bonding character, including those induced by external factors or methodological effects, resulting in spectroscopic artefacts in the latter cases).

One of the conventional methodologies in FTIR spectroscopy, which has been used since early days of the technique, involves grinding and/or pressing a small amount of a dry solid sample with powdered IR-transparent materials such as halide salts, with KBr being the most widely used (see, e.g [1,3,13–16].). However, it might be expected (as has been mentioned in some experimental reports [16–19]) that the highly polar halide-salt matrix, when used for pressing the powdered sample under study in a pellet for FTIR spectroscopic measurements, could affect the state of polar moieties and systems of H-bonds (which often determine the structure, properties and biological functions of native biomacromolecules), leading to a slight but noticeable FTIR band shift (even for intramolecular H-bonds; see, e.g., Table 1 in Ref. [16]).

The aim of this work was to experimentally assess, compare and discuss the halide-salt matrix effects on the results of FTIR spectroscopic measurements using different methodologies applied to a range of structurally complicated biological samples. The latter

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Table 1
Positions of the maxima of some representative vibrational bands in the samples under study: bacterial poly-3-hydroxybutyrate (PHB) isolated from cell biomass of *Azospirillum brasilense* Sp245; PHB-containing cell biomass of this bacterium, pectin from apple peel and its chemically modified fatty-acid esterified derivatives (see also Figs. 2–4).

Sample	Vibration modes	Wavenumbers (cm^{-1})	
		FTIR (thin film)* or DRIFT (powder)	FTIR (in KBr matrices)[a]
PHB	ν(C=O)[b]	1745*	1730
	ν(C—O—C/C—C—O)[b]	1292*	1279
Cell biomass	ν(C=O)[b]	1746	1728
	Amide I[c]	1661	1656
	ν(C—O—C/C—C—O)[b]	1300	1285
Pectin	ν(C=O)[b]	1756	1747
	ν(COOH)[d]	1627(broad)	1649
	ν(C—O—C)[e]	1158	1149
Pectin-palmitate	ν(C=O)[b]	1750	1738
	ν(COOH)[d]	1643	1634
	ν(C—O—C)[e]	1152	1133
Pectin-oleate	ν(C=O)[b]	1749	1742
	ν(COOH)[d]	1648	1634
	ν(C—O—C)[e]	1151	1146
Pectin-linoleate	ν(C=O)[b]	1750	1740
	ν(COOH)[d]	1642	1636
	ν(C—O—C)[e]	1152	1146

[a] Spectra measured in KBr pellets or, for cell biomass, as a powder ground with KBr.
[b] Ester moieties.
[c] Related to cellular proteins (ν(C=O) coupled to ν(C—N) of the peptide bonds).
[d] C=O of the non-ionised carboxylic moiety.
[e] Glycosidic bond.

included: (*i*) poly-3-hydroxybutyrate (PHB), biopolyester of the polyhydroxyalkanoate (PHA) family (biosynthesised and accumulated as intracellular nanosized granules by many microorganisms) [20,21] isolated from biomass of the rhizobacterium *Azospirillum brasilense* which is known to accumulate PHB (as a homopolymer, without other PHA copolymers [22,23]) up to high amounts under special environmental conditions [18,22]; (*ii*) PHB-containing bacterial biomass of this bacterium, (*iii*) pectin, natural carboxylated heteropolysaccharide of plant origin [24,25] (isolated from apple peel), and (*iv*) its chemically modified derivatives [19,26,27] obtained by partial esterification of its galacturonide-chain hydroxyl moieties with palmitic, oleic and linoleic acids [26].

The choice of the bacterium (*A. brasilense*) was determined by the fact that, under appropriate stress conditions, it is prone to accumulating homopolymeric PHB (up to over 80% of dry biomass) [22,23]. This facilitates FTIR spectroscopic analyses of methodological effects (which is the aim of this study), as the presence of various PHA copolymers (e.g., with longer aliphatic chains) in PHB, by modifying the physicochemical properties of the biopolymer [28–31], could *per se* induce noticeable band shifts for main vibration modes of functional groups of the copolymer [29,32]. Pectins (plant cell-wall carboxypolysaccharides) and their chemically modified derivatives are environmentally friendly substances of broad-range technological applications and potentials including food and package materials [7,12,14,15,17,24–27,33,34], so their structural and compositional analysis is also of great importance.

In order to compare the results of FTIR spectroscopic measurements involving a halide-salt matrix (KBr) powdered together with biological samples (used, e.g. for diffuse reflectance infrared Fourier transform (DRIFT) spectroscopic microanalyses [1,35]) and pressed in pellets (for conventional transmission FTIR measurements), the following matrix-free methodologies were used in this work: (*i*) DRIFT spectroscopy (for dry bacterial cell biomass), which had successfully been used earlier in our studies of complicated biological materials [18,19,36,37]; (*ii*) transmission FTIR measurements of thin sample films applied onto flat ZnSe discs (largely transparent for mid-IR radiation), which can be used both for biomacromolecular substances and for bacterial biomass or biofilm samples [13,38,39].

2. Materials and Methods

2.1. Materials

2.1.1. Bacterial Culture

In this study, the widely studied ubiquitous rhizobacterium *Azospirillum brasilense* [40–42] (wild-type strain Sp245 [43], taken from The Collection of Rhizosphere Microorganisms, [WDCM 1021] according to the World Federation of Culture Collections, http://www.wfcc.info/ccinfo/collection/by_id/1021, maintained at the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov, Russia: http://collection.ibppm.ru) was used for preparing PHB-containing biomass as well as for PHB extraction (vide infra). The strain was cultivated in a modified liquid malate salt medium (MSM; Day and Döbereiner, 1976 [44]), deficient in bound nitrogen (i.e. under a trophic stress which induces PHB biosynthesis and accumulation in *A. brasilense* cells [18,20–23,36]), containing the following components (grams per litre): K_2HPO_4, 3.0; KH_2PO_4, 2.0; NaCl, 0.1; $FeSO_4 \cdot 7H_2O$, 0.02 (added as chelate with nitrilotriacetic acid); $CaCl_2$, 0.02; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot 5H_2O$, 0.1; $Na_2MoO_4 \cdot 2H_2O$, 0.002; and sodium malate, 5.0 (obtained by mixing 3.76 g of malic acid with 2.24 g NaOH per litre); yeast extract, 0.1 (final pH 6.8–7.0). The cultures were grown for 9 d under aerobic conditions on a shaker (160–180 rpm) at 32 °C. Precultures were grown under aerobic conditions at 31–32 °C for 18–24 h in the MSM with added 1 g/l NH_4Cl. Cell growth was monitored by optical density OD_{600} (at $\lambda = 600$ nm; Specol 221, Germany). Other details of bacterial growth were reported elsewhere [18].

2.1.2. Preparation of Bacterial Biomass for Spectroscopic Measurements

Cells of *A. brasilense* Sp245 were collected by centrifugation (7000 *g*, 10 min, 10 °C) and washed three times with physiological saline solution (0.85% NaCl). The resulting finally centrifuged biomass was dried at ambient temperature up to a constant mass and powdered in an agate mortar prior to DRIFT spectroscopic measurements. Other details of preparation of bacterial cell biomass for spectroscopic measurements (vide infra) were reported elsewhere [18,36].

2.1.3. Extraction of Poly-3-hydroxybutyrate from Bacterial Biomass

For PHB isolation from bacterial cells, bacterial cell biomass grown as described above was collected by centrifugation (7000 *g*, 10 min, 10 °C), washed twice with physiological saline solution and, after centrifugation, frozen at −70 °C until further use. Frozen biomass was thawed and dried at 40–50 °C up to a constant mass.

PHB was extracted from the powdered dried biomass with hot chloroform on a water bath (at ~50 °C for 6 h per day, adding ~23 ml chloroform per 1 g of dried biomass) during 2 days. The PHB solution was separated from cell debris by filtering through a filter paper. The PHB extraction from the remaining biomass was repeated; both PHB extracts were combined and dried at ambient temperature (25 ± 3 °C). The resulting sample was a thin uniform flexible white film.

2.1.4. Pectin and its Chemically Modified Derivatives

Pectin (isolated from apple peel) as a powder sample with high molecular weight (30,000–100,000 g/mol) and a high degree of esterification (70–75%) was purchased from Fluka. Palmitic, oleic and linoleic acids and other chemicals for preparing chemically modified pectin derivatives (palmitate, oleate and linoleate esters synthesised as described earlier [26]) were purchased from Sigma–Aldrich.

2.2. Spectroscopic Measurements

For diffuse reflectance infrared Fourier transform (DRIFT) measurements, samples of bacterial cell biomass or pectin (chemically modified pectin derivatives) were used as dry finely ground powders (or, for the same bacterial biomass, as a finely ground mixture with spectroscopically pure KBr, "Merck") in a Micro sampling cup (Spectra-Tech Inc., USA), as reported earlier [18,19,36]. DRIFT studies were performed using a Nicolet spectrometer (model Magna-IR 560 E.S.P. for bacterial biomass or Nicolet 6700 FTIR spectrometer for pectin samples; vide infra; Thermo Electron Corporation, USA; DTGS detector; KBr beamsplitter) with a total of up to 100 scans (resolution 4 cm^{-1}).

For FTIR spectroscopic measurements in the transmission mode, the sample of PHB was analysed in two ways. For preparing KBr pellets, the sample was powdered and pressed in a pellet (200 mg; diameter 12 mm) at up to 7 MPa. For KBr-free transmission measurements, PHB sample was dissolved in a minimum volume of chloroform. The obtained PHB solution was applied onto a surface of a ZnSe glass disc, and dried at ambient temperature to form a film on the surface. The same spectrometer was used in this case as indicated above.

Transmission FTIR spectroscopic measurements of PHB film were performed on a Nicolet 6700 FTIR spectrometer. Spectra were collected with a total of 64 scans (resolution 2 cm^{-1}) against the ZnSe disc background. Spectroscopic data were manipulated using the OMNIC (version 8.2.0.387) software supplied by the manufacturer of the spectrometer (other details of this spectroscopic methodology were reported earlier [39]).

All spectroscopic measurements were repeated and appeared to be well reproducible. FTIR measurements of pectin and its chemically modified derivatives pressed in a halide-salt matrix (KBr pellets) were performed as described elsewhere [15].

3. Results and Discussion

It has to be mentioned that earlier, various effects have been discussed related to the use of different matrices (including finely ground halide salts) on DRIFT line intensities, which are important for (micro)analytical applications of the technique [35,45–48]. As for band positions, it has been demonstrated that for inorganic materials, where ion exchange could take place when the sample under study is ground and pressed in a halide-salt matrix, transmission FTIR spectra in the latter and in a non-polar matrix (such as Nujol, a mineral paraffin oil) can differ dramatically, as was found, e.g. for $CuSO_4 \cdot 5H_2O$ [47].

For biological samples, the use of halide matrices can mask some of the absorption bands related to polar functional groups, even those which are not involved in H-bonding. For instance, our earlier FTIR spectroscopic study of a commercial citrus pectin preparation (among other samples) [17], which contained ~30% of sucrose, showed (Fig. 1) that the non-hydrogen-bonded OH-group of sucrose [48], which for pure sucrose was clearly observed as a well-resolved ν(O—H) band at

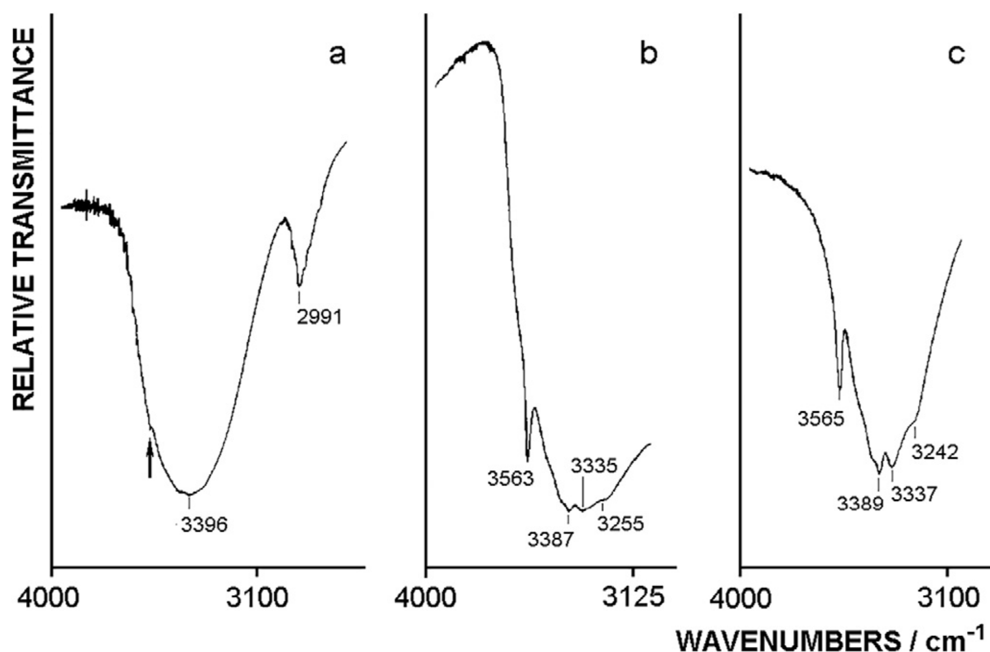

Fig. 1. FTIR spectra of a commercial citrus pectin preparation containing ~30% sucrose measured (a) in a KBr pellet and (b) in a Nujol mull, as well as (c) of pure sucrose (in a KBr pellet). The arrow in (a) shows the position of the non-H-bonded ν(O—H) band of sucrose (at ~3565 cm^{-1}) [48] largely masked by the KBr matrix [17]. In (b), the region of various ν(C—H) vibrations (3000–2800 cm^{-1}) obscured by Nujol is not shown.

~3565 cm^{-1} even in a KBr pellet (see Fig. 1c), was almost completely masked in a KBr pellet of the sucrose-containing pectin (see Fig. 1a). However, this band appeared to be well resolved (at 3563 cm^{-1}, together with other ν(O—H) bands of H-bonded hydroxo groups of sucrose in the region 3400–3200 cm^{-1}) in a Nujol mull of the sucrose-containing pectin (see Fig. 1b).

In this study, we consider in more detail the effects of a halide-salt matrix (in KBr pellets) on the positions of FTIR (DRIFT) absorption bands of polar functional groups in biomacromolecules and H-bonded biological systems.

3.1. Poly-3-hydroxybutyrate (PHB) Isolated from Bacterial Biomass

Transmission FTIR spectra in the mid-IR region of a thin PHB film and PHB in a polar matrix (KBr pellet) are shown in Fig. 2. All the main bands of PHB [18,30,32,36] are very well resolved facilitating a comparative analysis of the two images.

It has to be mentioned that the majority of the observed vibrational bands, e.g. those below 1230 cm^{-1}, at 1380, 1454 and over 2850 cm^{-1}, are evidently not affected by the KBr matrix, as their maxima in Fig. 1a and b either coincide or differ by only 1–2 cm^{-1} (which is within the spectroscopic resolution) and have similar relative intensities (cf. Fig. 1a and b). While many of them refer to non-polar functional groups (such as various ν(C—H) modes within 3000–2800 cm^{-1}, the stable scissoring $\delta(CH_2)$ band at 1454 cm^{-1}, the symmetric $\delta(CH_3)$ mode at 1380 cm^{-1}, skeletal C—C stretching modes contributing to ν(C–C–O) within 1250–1050 cm^{-1}, ν(C—C) at 979 cm^{-1} and different δ(C—H) modes at lower wavenumbers) [9,18,28,29,32,49,50], the band at 3435 cm^{-1} refers to ν(O—H) of H-bonded water impurities [9,29,51]. However, since these water molecules occur as relatively strongly bound H-bonded entities between biopolyester chains [9,29,51], they are evidently 'protected' from the polar KBr matrix.

From Fig. 2 it is clearly seen that broadened and evidently composite bands within 1750–1720 and 1300–1270 cm^{-1} are sensitive to the KBr matrix. The former region features the typical ester ν(C═O) mode which, in PHB, is sensitive to the degree of crystallinity of the biopolymer [9,50–52]. Thus, the band with the maximum at 1745 cm^{-1} refers to a more amorphous PHB fraction, whereas that at 1724 cm^{-1} (see

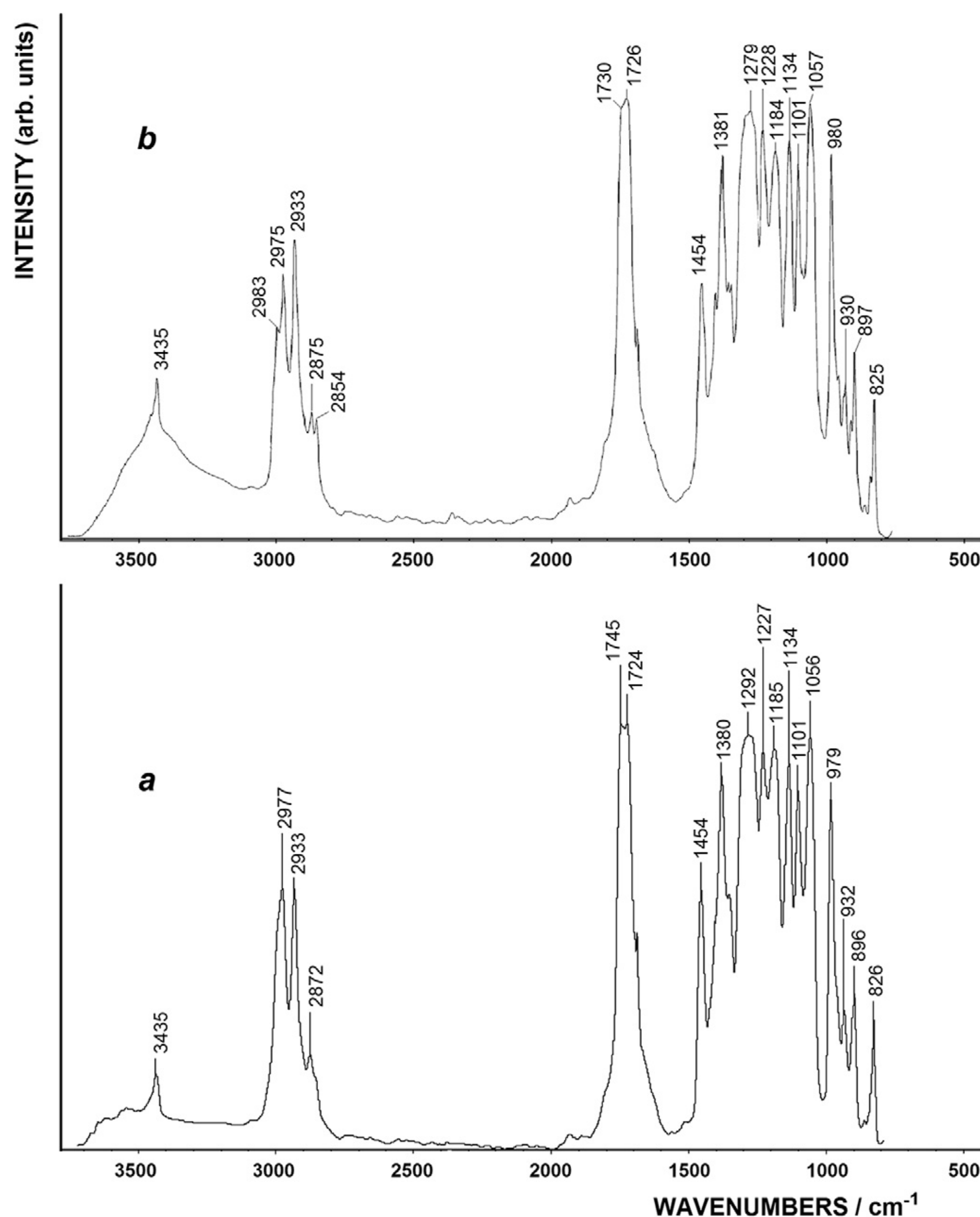

Fig. 2. Transmission FTIR spectra of (a) a thin film of poly-3-hydroxybutyrate (PHB; isolated from biomass of *A. brasilense* Sp245) and (b) of the PHB in a KBr pellet.

Fig. 2a) represents a more crystalline component. In the KBr matrix, however, the maximum of the amorphous part downshifted to 1730 cm^{-1} (i.e., by 15 cm^{-1}; see Fig. 2a). While this shift could be attributable to the influence of the polar matrix, however, its relatively large value (15 cm^{-1}) could as well be a result of partial crystallisation of the amorphous component induced by grinding and pressing the biopolymer sample with KBr. This assumption is corroborated by the concomitant downshift of the maximum of the broad ν(C—C—O/C—O—C) feature from 1292 to 1279 cm^{-1} (i.e., by 13 cm^{-1}; cf. Fig. 2a, b) which is also sensitive to the degree of PHB crystallinity [52] (Table 1). Note that similar relatively large downshifts of these two PHB-related bands sensitive to the degree of PHB crystallinity were observed also in DRIFT spectra of PHB-containing dry bacterial biomass in going from the biomass without KBr to that after grinding with KBr (see Subsection 3.2).

3.2. PHB-Containing Bacterial Biomass

In Fig. 3, DRIFT spectra are shown for dry biomass of PHB-containing *A. brasilense* Sp245 (grown under the conditions which induce PHB accumulation; see Subsection 2.1.1) which was ground prior to measurements without KBr (spectrum 1) and with KBr (spectrum 2).

Since the bacterium had been grown in nitrogen-deficient culture medium which is known to induce biosynthesis and accumulation of PHB in the form of granules in bacterial cells [18,20–22], besides the typical biomass bands (e.g. amide I at ~1660 cm^{-1} and amide II at ~1550 cm^{-1} related to cellular proteins [11,13,18]), a few PHB-related bands are clearly seen. Similar to the effects observed for PHB (see Subsection 3.1), the two PHB bands observed in pure dry biomass at 1746 and 1300 cm^{-1} (corresponding to amorphous PHB typical for intracellular granules [9,50,51]) significantly shifted to 1728 and 1285 cm^{-1} (i.e., by 18 and 15 cm^{-1}), respectively. This pronounced effect can also be attributed largely to crystallisation of metastable amorphous intracellular PHB induced by grinding with KBr. Note for comparison that a smaller shift (by 5 cm^{-1}) is also observed for the amide I band of cellular proteins (from 1661 to 1656 cm^{-1}; see Table 1).

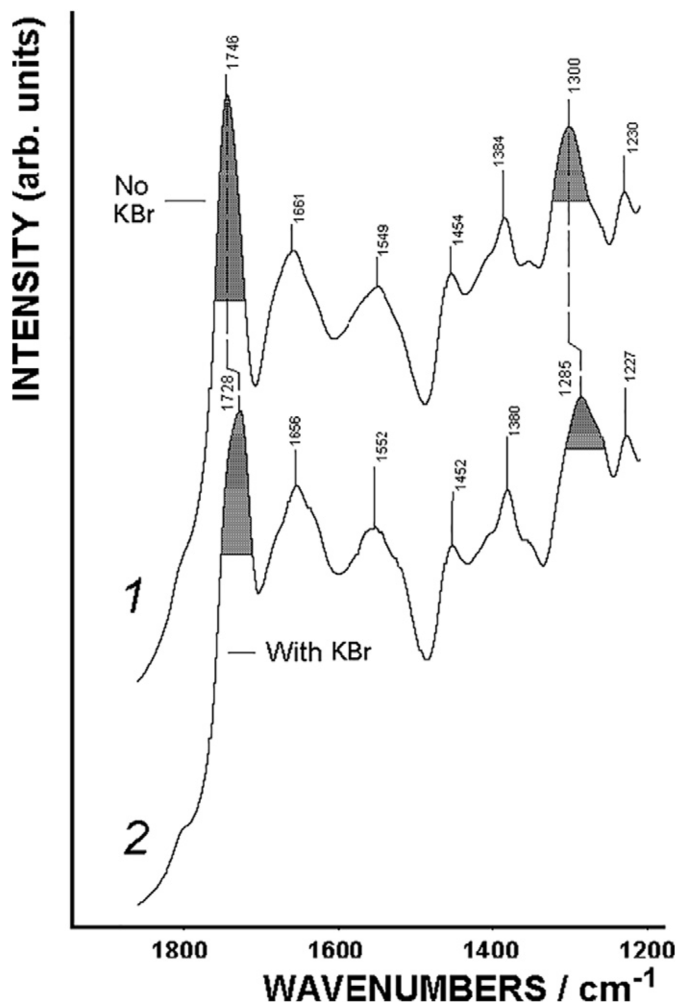

Fig. 3. Diffuse reflectance infrared Fourier transform (DRIFT) spectra of dry PHB-containing biomass of *Azospirillum brasilense* Sp245 (1) ground without KBr and (2) with KBr. The two typical PHB-related bands are highlighted with shading.

3.3. Pectin and its Chemically Modified Fatty Acid Derivatives

In Fig. 4, the carbonyl stretching region (1800–1500 cm^{-1}) is shown for pectin (obtained from apple peel) and its fatty-acid esterified derivatives measured in the DRIFT mode (without using KBr) or in KBr pellets.

The resulting spectra of the samples demonstrate that the KBr matrix induced a decrease in the ester carbonyl ν(C═O) band in all of the substances by 7–12 cm^{-1} (cf. panels A and B in Fig. 4 and Table 1). The non-ionised carboxylic ν(C═O) band in pure pectin (at 1627 cm^{-1}) was likely to undergo ion exchange upon grinding and pressing with KBr, appearing in a KBr pellet at 1649 cm^{-1} (cf. spectra A1 and B1 in Fig. 4). In fatty-acid esterified pectin derivatives, these bands shifted to lower wavenumbers by 6–14 cm^{-1} when measured in the KBr matrix, as compared to DRIFT spectra of their pure samples. In addition, in pectin and its derivatives, the ν(C—O—C) mode of the glycosidic bond (at ~1158–1151 cm^{-1}) showed a similar shift (see Table 1).

4. Conclusions

According to the experimental data obtained, for all the samples studied, several vibrational bands of polar and/or H-bonded functional groups related to polyester, polysaccharide or protein moieties were demonstrated to undergo significant downshifts of their maxima in KBr matrices. For partly amorphous biopolyester samples (PHB) or PHB-containing bacterial cell biomass, these shifts are likely to involve further PHB crystallisation induced by grinding with KBr (in the case of DRIFT spectra) and further pressing (when measured in KBr pellets). Therefore, the halide matrix-free DRIFT methodology or transmission measurements of thin films are preferable for such sensitive biomacromolecular samples to ensure their non-invasive analysis. The results obtained can have important implications for correct structural and compositional analyses of various biomacromolecular samples, especially those containing structurally relevant intra- or intermolecular H-bonding and/or a significant proportion of polar functional groups, by using different FTIR spectroscopic methodologies.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

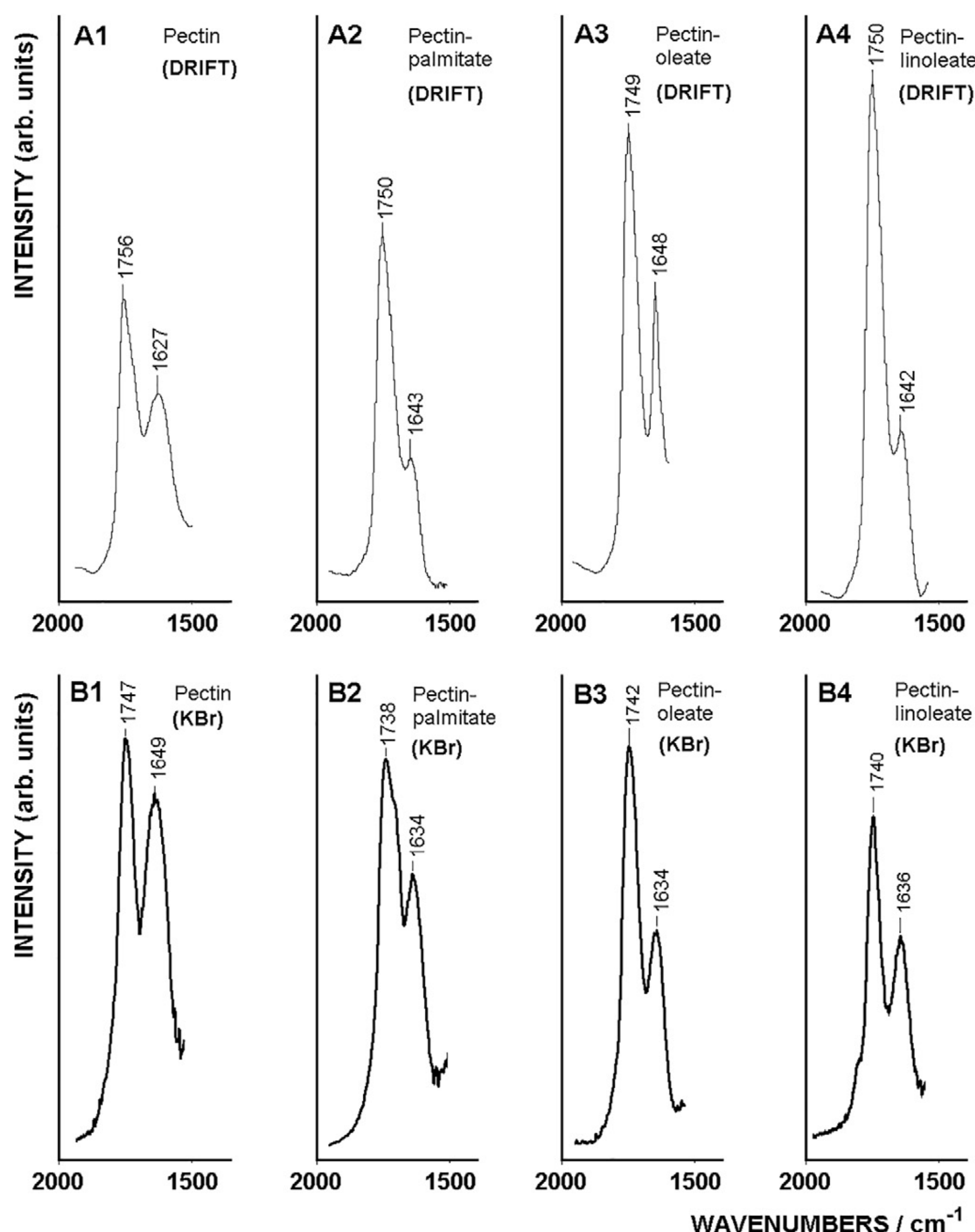

Fig. 4. FTIR spectra in the diffuse reflectance (DRIFT) mode (without KBr; A, upper panels) and in the transmission mode (in KBr pellets; B, lower panels) of pectin from apple peel (1) and its palmitate (2), oleate (3) and linoleate (4) chemically esterified derivatives in the region of ν(C═O) vibration modes of ester and carboxyl moieties.

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