



A new method for determining the relative crystallinity of chickpea starch by Fourier-transform infrared spectroscopy



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ABSTRACT

A new method for determining the relative crystallinity (RC) of chickpea starch was developed by using Fourier-transform infrared (FT-IR) spectroscopy, based on hypotheses as described as follows: there is a Gaussian holocrystalline-peak (HCP) in the 800–1300 cm⁻¹ region of FT-IR spectrum of starch which is divided into amorphous region and crystalline region; the crystalline region of HCP is the overlap of the HCP and the FT-IR spectrum of starch; the RC of starch is the ratio of the area of crystalline region to the area of HCP. It was found that there was no significant difference between the RC determined by FT-IR method and that determined by X-ray diffraction (XRD) method. The intra-class correlation coefficient was 0.998 ($p=0.000$, $n=9$) and the 95% confidence interval was 0.992–1.000 for the RC determined by XRD and FT-IR. Furthermore, the developed method showed good repeatability (coefficient of variation (CV), 1.1–2.9%) and good intermediate precision (CV, 2.8%).

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1. Introduction

Chickpea (*Cicer arietinum* L.) is the third important pulse crop in the world and rich in starch (37.5–50.8%) and protein (Chavan, Kadam, & Salunkhe, 1986; Jukanti, Gaur, Gowda, & Chibbar, 2012; Saini & Knights, 1984). Based on seed morphology and cultivation area, chickpea can be grouped into two cultivated varieties: Desi and Kabuli. The Desi chickpea has small and dark seeds and a thick coat, cultivated mostly in Asia and Africa; the Kabuli chickpea has large and light colour seeds and a thin and smooth coat, cultivated mostly in Europe, North America, West Asia and North Africa (Chavan et al., 1986; Jukanti et al., 2012).

The starch granules are semi-crystalline with various crystalline structures and degrees of crystallinity which have important impacts on the physical and functional properties of starch such as gelatinization and glycemic response (Cai & Wei, 2012; Parada & Aguilera, 2012; Tester, Karkalas, & Qi, 2004; Yang, Gu, & Hemar, 2013). Based on the difference of X-ray scattering in the crystalline and amorphous regions of starch granule, X-ray diffraction (XRD) is generally used to determine the starch crystallinity (Beninca et al.,

2013; Mutungi, Passauer, Onyango, Jaros, & Rohm, 2012; Primo-Martin, van Nieuwenhuijzen, Hamer, & van Vliet, 2007). According to the pattern, the crystalline structures of starches can be classified into A-, B- and C-type crystals (Tester et al., 2004). Nevertheless, it requires expensive equipment and under the best of circumstances. Despite differential scanning calorimetry (DSC) can also be used to estimate the starch crystallinity through measuring the enthalpy of starch in different states, it is not precise due to the crystallization effect during the heating and the variation of heat capacity with temperature (Kong & Hay, 2002; Primo-Martin et al., 2007; Vippagunta, Brittain, & Grant, 2001). Besides, solid-state ¹³C cross-polarization and magic angle spinning nuclear magnetic resonance (¹³C CP/MAS NMR) is alternative to determine starch crystallinity through the analysis of molecular (double-helical) order, but it is not widely used due to its expense and long analytic time (Cooke & Gidley, 1992; Mutungi et al., 2012).

Fourier-transform infrared (FT-IR) spectroscopy can provide useful information on crystallinity of various materials, such as poly(ethylene terephthalate) and cellulose (Chen, Hay, & Jenkins, 2012; Kljun et al., 2011; Nelson & O'Connor, 1964). This method has been advocated since it is simple, rapid, non-destructive and without complicated sample pretreatment before measurement. In theory, FT-IR can be applied to determine material crystallinity if there is a probe band (varying with material crystallinity) and a

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reference band (providing a kind of internal standard to eliminate the errors produced from sources such as the uncertainty in the sample dosage in KBr disc and the uncontrollable light scattering). Accordingly, starch crystallinity can also be determined by FT-IR if there are a suitable probe band and a suitable reference band in FT-IR spectra. At present, some absorption bands in the 1300–800 cm⁻¹ regions of FT-IR spectra, for example the 1047 cm⁻¹ and 1022 cm⁻¹ bands, have been reported to be related with the crystalline phase and amorphous phase within the starch granules (Ambigaipalan, Hoover, Donner, & Liu, 2014; Ispas-Szabo, Ravenelle, Hassan, Preda, & Mateescu, 2000; Sevenou, Hill, Farhat, & Mitchell, 2002; vanSoest, Tournois, deWit, & Vliegthart, 1995). However, to our knowledge, there is no independent method to determine starch crystallinity by FT-IR. Therefore, a new independent method to determine the relative crystallinity (RC) of starch by FT-IR was developed in the present study. In order to develop a novel method for the determination of RC of starch by FT-IR, native and resistant chickpea starch samples with different RC were prepared from Kabuli chickpea seeds and new FT-IR spectral bands were selected as a probe band and a reference band.

2. Material and methods

2.1. Materials and reagents

Chickpea seeds were provided by Xinjiang Agricultural University (Xinjiang, China). Sweet potato starch, corn starch and potato starch were purchased from Shandong Jincheng Co., Ltd. (Shandong, China). Wheat starch and tapioca starch were purchased from Shanghai Heyu Trade Co., Ltd. (Shanghai, China). Amyloglucosidase (100 U/mg) was purchased from Shanghai Kayon Biological Technology Co., Ltd. (Shanghai, China) and heat-stable-amyase (20000 U/mL) was purchased from Jiangsu Ruiyang Biotech Co., Ltd. (Jiangsu, China).

2.2. Preparation of chickpea starch samples

2.2.1. Preparation of native chickpea starch (NS)

Chickpea seeds were steeped in water containing 0.3% sodium sulfite for 24 h at room temperature. The grains were then ground by a laboratory blender, and the resulting ground slurry was screened through nylon cloth (100 mesh) and was allowed to stand for 5 h. The supernatant was removed by suction and the settled starch layer was resuspended in distilled water containing 0.04% sodium hydrate to remove protein and further washed by successive static precipitation in distilled water until neutrality. Most part of them was dried as the material for the production of resistant starch in an oven at 70 °C, and the rest was lyophilized as NS.

2.2.2. Preparation of resistant chickpea starch I (RSI)

The preparation of RSI was performed according to the method (Sievert & Pomeranz, 1989) with slightly modifications. Briefly, the chickpea starch was suspended in distilled water (starch/water ratio, 3:10), the resulting suspension was autoclaved at 121 °C for 30 min, cooled to 4 °C and stored at this temperature for 24 h. These autoclaving-cooling cycles were repeated up to three times. Then, the resulting sample was broken up and incubated with a heat-stable α -amylase (500 U/g starch) at 96 °C for 30 min (pH 6.0) and an amyloglucosidase (500 U/g starch) at 59 °C for 30 min (pH 4.5), respectively. The insoluble residues were washed with distilled water by centrifugation, and most part of them was used for further digestion with amylase in excess, the rest was lyophilized as RSI.

2.2.3. Preparation of resistant chickpea starch II (RSII)

RSI was resuspended in an equal volume of distilled water and then incubated with a heat-stable-amyase (5000 U/g wet basis) at

96 °C for 24 h (pH 6.0) and an amyloglucosidase (500 U/g wet basis) at 59 °C for 30 min (pH 4.5), respectively. The insoluble residues were washed with distilled water by centrifugation, and most part of them was used for further treatment in high concentration of K₂CO₃ solution, the rest was lyophilized as RSII.

2.2.4. Preparation of resistant chickpea starch III (RSIII)

RSII was suspended in high concentration of K₂CO₃ solution (ratio of K₂CO₃/water, 4:5). The floated matter was removed and the settled starch was washed by successive centrifugation in distilled water until neutrality and lyophilized as RSIII.

2.3. XRD analysis

XRD analysis was carried out on a Bruker D8 Advance Diffractometer. The radiation used was Cu-K α (wavelength of 0.15406 nm). The scan was performed at 40 kV and 40 mA for a 2 θ range of 5–37°, with a step size of 0.02° and a collection time of 6.7 s at each step. The RC of the starch was quantitatively estimated following the method (Nara & Komiya, 1983) by using MDI Jade 5.0 software (Materials Data Inc., Livermore, CA). All analyses were performed in triplicate.

2.4. FT-IR spectroscopic analysis

The FT-IR spectra of starch samples in KBr discs (2 mg starch/150 mg KBr disc) were recorded using a Nicolet IR200 spectrophotometer with a resolution of 4 cm⁻¹ in a spectral range of 4000–400 cm⁻¹. Omnic 8.0 software (Thermo Fisher Scientific Inc., WI, USA) was used for baseline-correction of the FT-IR spectra of starch samples, and Origin 8.0 (OriginLab Corp., MA, USA) was used for fitting the experimental curve. All analyses were performed in triplicate.

2.5. Method validation

The developed FT-IR method was validated following the assay procedures of the International Conference on Harmonisation (ICH) guidelines (ICH Harmonized Tripartite Guideline, 2005). Briefly, linearity, accuracy and specificity were determined by analyzing nine starch samples of NS, RSI, RSII, RSIII, sweet potato starch, corn starch, potato starch, wheat starch and tapioca starch with triplicate measurements. Repeatability was assessed from the results of triplicate determinations of NS, RSI and RSII. Intermediate precision was estimated by analyzing NS on six different days with triplicate measurements.

2.6. Data analysis

The data were expressed as means $\pm$ SD from three determinations. SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Significance of difference was tested at $P=0.05$ by using ANOVA and Post Hoc Test (LSD). A reliability analysis was carried out by using intra-class correlation coefficients with a two-way mixed effects model.

3. Results and discussion

3.1. XRD pattern of chickpea starch samples

The RC of NS, RSI, RSII and RSIII were determined by XRD. Fig. 1 shows the XRD patterns of chickpea starch samples, and the RC of NS, RSI, RSII and RSIII were calculated to be 31.0 ± 1.1 , 48.2 ± 0.9 , 56.2 ± 3.4 and $52.5 \pm 0.9\%$, respectively. Among them, the RC of NS was within the range in the literatures (27.6–34.4%) (Hughes et al.,

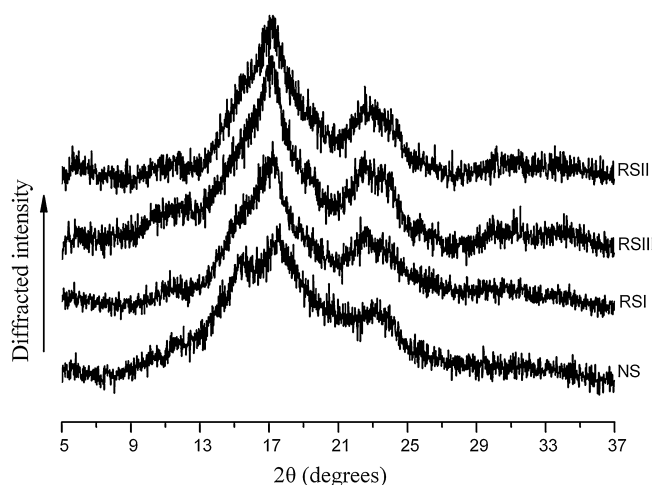


Fig. 1. XRD patterns of chickpea starch samples.

2009; Sandhu & Lim, 2008) and the RC of RSI was slightly higher than the reported value (45.6%) (Polesi & Sarmento, 2011).

3.2. FT-IR spectra of chickpea starch samples

In order to investigate whether there are a probe band and a reference band that can be used to determine the RC of chickpea starch, the FT-IR spectra of NS, RSI, RSII and RSIII as shown in Fig. 2a were baseline-corrected by drawing a tangent line in the 800–1300 cm^{-1} region (Fig. 2b). This region was mainly corresponded to vibration associated with C–C and C–O bonds and sensitive to change in starch polymer conformation (Bernazzani, Peyyavula, Agarwal, & Tatikonda, 2008). It was observed that the spectral areas in the 800–1300 cm^{-1} region of chickpea starch followed the order: RSII > RSIII > RSI > NS. Notably, they are in accordance with the results determined by XRD. Similar results are also reported for *Canna edulis* Ker starch and rice starch (Fan, Ma, Wang, Huang, Zhang, et al., 2012; Fan, Ma, Wang, Huang, Zhao, et al., 2012; Zhang, Chen, Liu, & Wang, 2010). Therefore, the band between 800 and 1300 cm^{-1} could be used as a probe band to determine the RC of chickpea starch.

Furthermore, it was found that the spectral area of NS increased with the sample dosage in KBr disc (Fig. 2c), indicating that the spectral area might be obviously affected by the sample dosage in KBr disc with FT-IR method. Therefore, it is need to find out a reference band to eliminate or weaken such an influence. In the present study, we tried to use Gaussian, Lorentz and Voigt functions to fit the data points composed of two tangency points of spectra and points of spectra at about 960 cm^{-1} and 1175 cm^{-1} . As shown in Fig. 3, it was found that the Gaussian fitting peaks were the best fits based on the adjusted *R*-square values. Thus, we considered a fitting Gaussian peak as a reference band. Taking RSI as an example, the steps of the Gaussian peak fitting procedure are followings: (1) the FT-IR spectrum of RSI is baseline-corrected by drawing a tangent line in the 800–1300 cm^{-1} region using Omnic 8.0 software; (2) the resulting two tangency points of spectra and the points of spectra in the 950–985 cm^{-1} and 1160–1190 cm^{-1} regions compose the fitting points; (3) the fitting Gaussian peak of RSI (Fig. 3) is plotted by using a Gaussian curve to fit the points selected from the above step of (2) with Origin 8.0 software.

To further determine whether the fitting Gaussian peak could be as a reference band and the ratios of different regions of probe band to reference band could be used to estimate the RC of the chickpea starch sample, the A_{SG}/A_G ratio (the ratio of the area of the spectra

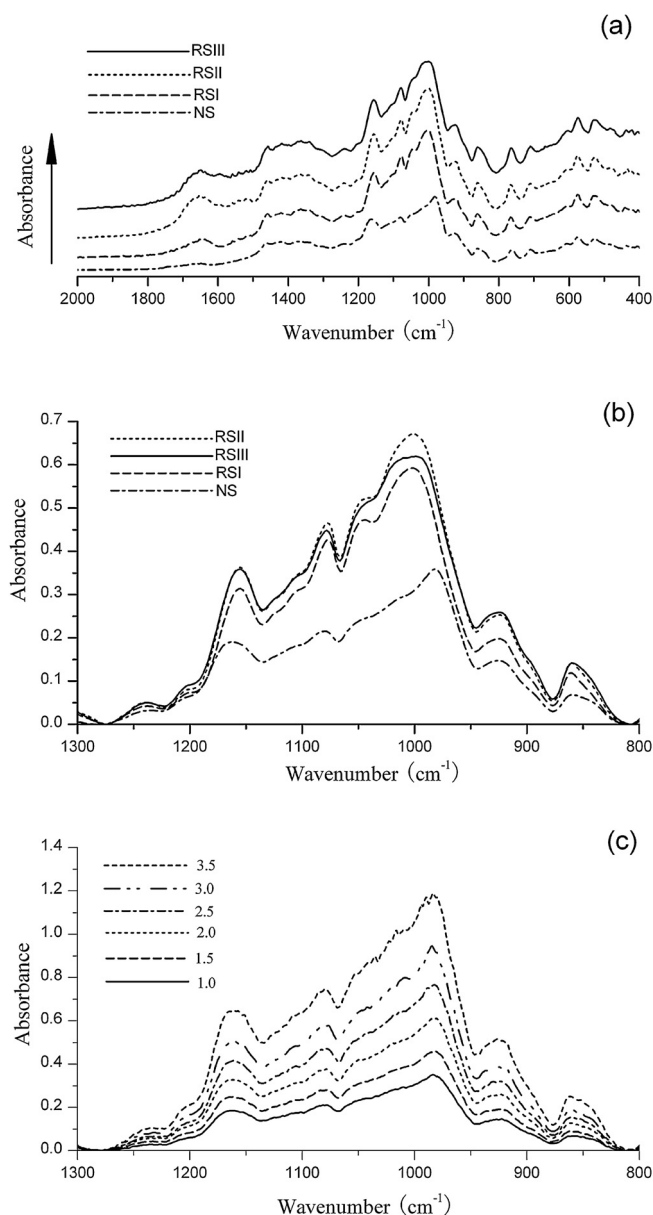


Fig. 2. FT-IR spectra of chickpea starch samples. (a) The FT-IR spectra of NS, RSI, RSII and RSIII in the 400–2000 cm^{-1} region. (b) The FT-IR spectra of NS, RSI, RSII and RSIII after baseline correcting by drawing a tangent line in the 800–1300 cm^{-1} region. (c) The FT-IR spectra of NS of different sample dosages in KBr disc (mg/150 mg KBr) after baseline correcting by drawing a tangent line in the 800–1300 cm^{-1} region.

in the fitting Gaussian peak to the area of the fitting Gaussian peak) and the A_S/A_G ratio (the ratio of the spectral area to the area of the fitting Gaussian peak) were calculated and are shown in Table 1 and Table 2.

It was observed that both the A_S/A_G ratio and the A_{SG}/A_G ratio of NS were not significantly affected by the sample dosage in KBr disc (Table 1). Therefore, the fitting Gaussian peak might be used as a reference band and the A_S/A_G ratio and A_{SG}/A_G ratio might be used to determine the RC of chickpea starch samples. Furthermore, it was found that the A_S/A_G ratio was significantly higher than the RC of chickpea starch measured by XRD, but there was no significant difference between the A_{SG}/A_G ratio and the RC (Table 2). Thus, the results suggest that the A_{SG}/A_G ratio is more suitable to be as the RC of chickpea starch.

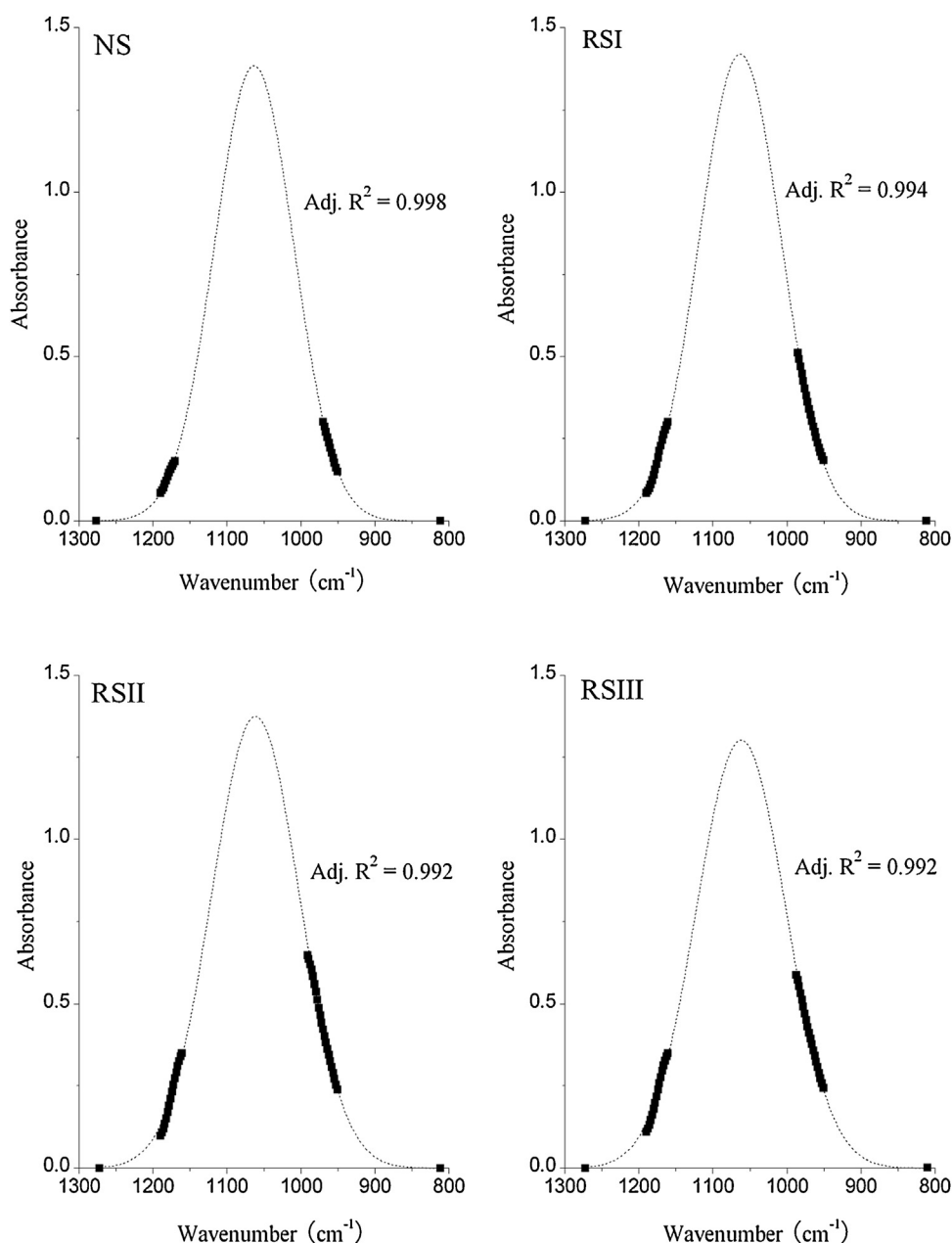


Fig. 3. The fitting points and fitting Gaussian peaks of FT-IR spectra of chickpea starch samples. The solid square dots represent the fitting points, the dot lines stand for the fitting Gaussian peak.

3.3. Hypotheses

Based on the above results, we propose hypotheses that can be illustrated in Fig. 4 and described as follows: (1) there is a Gaussian

Table 1
Effects of sample dosages on A_S/A_G ratio and A_{SG}/A_G ratio of NS.

Sample dosage (mg/150 mg KBr)	A_S/A_G ratio	A_{SG}/A_G ratio
1.0	$35.7 \pm 1.2\%^a$	$31.0 \pm 1.1\%^a$
1.5	$35.8 \pm 0.6\%^a$	$32.0 \pm 0.5\%^a$
2.0	$36.1 \pm 0.7\%^a$	$31.3 \pm 0.6\%^a$
2.5	$35.7 \pm 1.0\%^a$	$30.9 \pm 0.8\%^a$
3.0	$37.1 \pm 1.0\%^a$	$32.2 \pm 0.8\%^a$
3.5	$37.6 \pm 1.8\%^a$	$32.6 \pm 1.7\%^a$

Note: (1) The A_S/A_G ratio is the ratio of the spectral area to the area of the fitting Gaussian peak; (2) the A_{SG}/A_G ratio is the ratio of the area of the spectra in the fitting Gaussian peak to the area of the fitting Gaussian peak. Different small letters in the same column indicated significant difference at the 0.05 level.

holocrystalline-peak (HCP) in the 800–1300 cm^{-1} region of FT-IR spectrum of starch which is divided into amorphous region and crystalline region; (2) the crystalline region of HCP is the overlap of the HCP and the FT-IR spectrum of starch sample; and (3) the RC of starch sample is the ratio of the area of crystalline region to

Table 2
The A_S/A_G ratio, A_{SG}/A_G ratio and RC of chickpea starch samples.

Sample	A_S/A_G ratio	A_{SG}/A_G ratio	RC
NS	$35.9 \pm 0.5\%^b$	$31.3 \pm 0.3\%^a$	$31.0 \pm 1.1\%^a$
RSI	$55.0 \pm 1.8\%^b$	$48.3 \pm 1.2\%^a$	$48.2 \pm 0.9\%^a$
RSII	$63.2 \pm 1.3\%^b$	$56.2 \pm 1.6\%^a$	$56.2 \pm 3.4\%^a$
RSIII	$61.4 \pm 1.0\%^b$	$54.1 \pm 0.8\%^a$	$52.5 \pm 0.9\%^a$

Note: RC is the relative crystallinity of chickpea starch determined by XRD. Different small letters in the same line indicate significant difference at the 0.05 level.

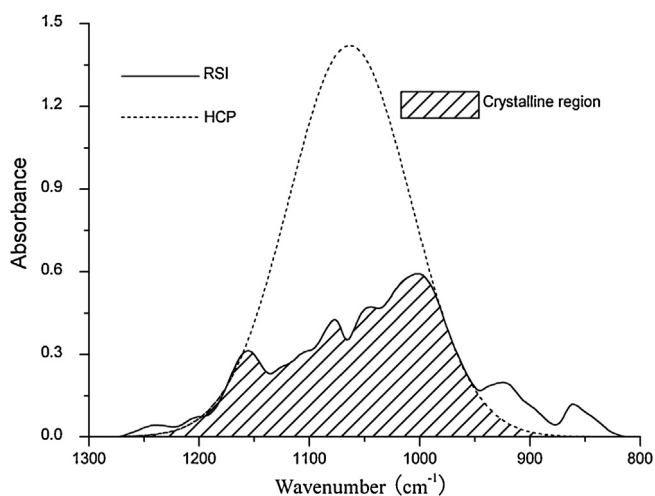


Fig. 4. The HCP and crystalline regions of RSI.

the area of HCP. With RSI as an example, the HCP and crystalline regions of starch are shown in Fig. 4.

3.4. Hypotheses tests and reliability analysis

To demonstrate whether the hypotheses are suitable for the determination of RC of other starches, the RC of sweet potato starch, corn starch, wheat starch, tapioca starch and potato starch were determined by their FT-IR spectra (Fig. 5a) and their XRD patterns (Fig. 5b). As shown in Table 3, the RC measured by FT-IR was not significantly different from that measured by XRD. In addition, it was found that the intra-class correlation coefficient was 0.998 ($p=0.000$, $n=9$) and the 95% confidence interval was 0.992–1.000

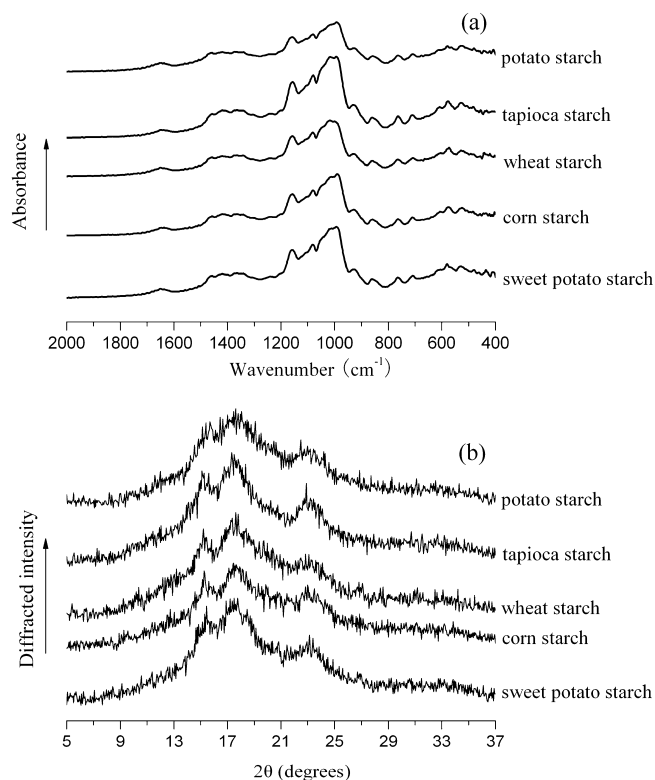


Fig. 5. FT-IR spectra (a) and XRD patterns (b) of sweet potato starch, corn starch, potato starch, wheat starch and tapioca starch.

Table 3

The RC of starch samples determined by FT-IR and XRD.

Starch sample	Calculated source	
	XRD	FT-IR
Sweet potato starch	$32.5 \pm 0.8\%^a$	$33.2 \pm 0.6\%^a$
Corn starch	$30.9 \pm 1.0\%^a$	$31.8 \pm 0.4\%^a$
Wheat starch	$35.5 \pm 2.1\%^a$	$35.4 \pm 1.1\%^a$
Tapioca starch	$34.7 \pm 1.8\%^a$	$33.2 \pm 1.1\%^a$
Potato starch	$36.8 \pm 0.5\%^a$	$36.6 \pm 1.8\%^a$

Note: Different small letters in the same line indicate significant difference at the 0.05 level.

for the RC of the nine starch samples between the two methods of XRD and FT-IR. All the results indicate that the RC determined by FT-IR is good in agreement with that determined by XRD.

3.5. Method validation

3.5.1. Linearity

A linear curve was established between the RC of starch determined by FT-IR (dependent variable) and by XRD (independent variable) with R^2 value of 0.993, fitted slope of 1.022 ± 0.032 and an intercept of -0.673 ± 1.303 . As can be seen, the FT-IR method can be deemed linear in the RC range of 31–56% for the slope and intercept (not significantly different from 1 and 0, respectively, given with their respective confidence intervals at a significance level of 95%).

3.5.2. Accuracy, specificity, repeatability and intermediate precision

Due to there was no significant difference between the RC determined by FT-IR method and XRD method (Tables 2 and 3), the developed FT-IR method was found with good accuracy and specificity, good repeatability (coefficient of variation (CV), 1.1–2.9%) and good intermediate precision (CV, 2.8%).

4. Conclusions

A new independent method to determine the RC of starch by FT-IR was developed in the present study. We observed that the spectral area in the 800–1300 cm^{-1} region increased with the RC of chickpea starch and could be as a probe band to determine the RC. Then, we found that a fitting Gaussian peak could be used as a reference band to eliminate the influence of the sample dosage in KBr disc on the spectral area and the A_{SG}/A_G ratio was more suitable to be as the RC of chickpea starch than the A_S/A_G ratio. Based on the above results, we proposed hypotheses that there is a HCP in FT-IR spectrum of starch which is divided into amorphous region and crystalline region and that RC of starch is the ratio of the area of crystalline region to the area of HCP. By applying these hypotheses, the RC of starch determined by FT-IR is in agreement with that determined by XRD. The developed FT-IR method was validated to have good linearity, accuracy, specificity, repeatability and intermediate precision. The results suggest that the developed FT-IR method in the present study can be used for the determination of starch RC in future.

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