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Determination of the polymorphic composition of smooth pea starch

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A method for estimating the proportions of 'A' and 'B' polymorphs comprising a sample of 'C' type starch is proposed which uses established experimental techniques with commercially available spreadsheet and X-ray analysis software. Waxy maize, potato and smooth pea starches were used to provide X-ray diffraction patterns characteristic of the 'A', 'B' and 'C' starch polymorphs. Samples of amorphous starches were also prepared. The method initially involved subtraction of the amorphous phase and instrumental background from the X-ray diffraction patterns of each starch sample using the spreadsheet program, Lotus 1-2-3. The remainder of the pattern, representing the crystalline portion of the starch sample, was then analysed by profile fitting to elucidate the positions and areas of individual diffraction peaks. The ratio of the total peak area to the areas under peaks characteristic of 'A' and 'B' type starches, respectively, were used to calculate the relative proportions of these polymorphs in smooth pea starch. These proportions were found to be 56±3% 'A' polymorph to 44±3% 'B' polymorph. A 'C' type pattern was constructed by using Lotus 1-2-3 to combine diffraction patterns from the crystalline portions of 'A' and 'B' type starches in the proportions given above. Polymorph patterns were obtained by manipulation of the diffraction patterns from the crystalline portions of starches using Lotus 1-2-3. An 'A' type pattern was obtained by subtraction of a 'B' type pattern from that of a 'C' type. Similarly, a 'B' type pattern was obtained by subtraction of an 'A' type pattern from that of a 'C' type. © 1997 Elsevier Science Ltd

INTRODUCTION

Native starch occurs as semi-crystalline granules with values of crystallinity ranging from 15 to 45% depending upon the source (Zobel, 1988). The crystalline structure of starches has been widely studied by wide angle X-ray diffractometry. The positions of the diffraction peaks obtained are used to classify each starch into one of three main polymorphs, 'A', 'B' or 'C' (Banks & Greenwood, 1975; Zobel, 1988). 'A' type patterns are generally characteristic of cereals (e.g. waxy maize), 'B' type patterns of tubers (e.g. potato) and 'C' type patterns of legumes (e.g. smooth peas). There is general agreement that the crystal structures of 'A' and 'B' type starches are based on parallel-stranded double helices, with the main difference between them lying in the packing of the helices and their water content. In 'A' type starch the helices are closely packed, whereas in 'B' type starch, there is a more loosely packed arrangement with a correspondingly greater amount of inter-helical water (Imberty *et al.*, 1988; Imberty & Perez, 1988; Sarko & Wu, 1978; Wu & Sarko, 1978). It has been proposed that the crystalline portion of 'C' type starches is simply a mixture of 'A' and 'B' type unit cells (Blanshard, 1987; Gernat *et al.*, 1990, 1993; Sarko & Wu, 1978; Wu & Sarko, 1978). A method for estimation of the relative proportions of the 'A' and 'B' polymorphs in samples of 'C' type starch was proposed by Gernat *et al.* (1990), (1993). These authors used mixtures of pure 'A' type (maize) and 'B' type (potato) starch standards in varying proportions to produce examples of 'C' type diffraction patterns. From this they concluded that semi-crystalline 'C' type diffraction patterns could be modelled mathematically as an appropriately proportioned combination of the semi-crystalline diffraction patterns from pure 'A' and 'B' polymorphs. They used their model to calculate the proportion of the 'A' and 'B' polymorphs in the 'C' type starch of smooth peas and broad beans by a linear regression method (Gernat, 1990). The stated assumptions of this method were that the amorphous/crystalline ratio and crystallite

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sizes of the 'A' and 'B' polymorphs in the 'C' type starch sample were identical to those of the pure 'A' and 'B' type starch standards, a situation which might not always occur in practice. Davydova *et al.* (1995) proposed an alternative method which partially overcame this problem by removing the influence of the amorphous portion of the starch on the calculations. A sample of amorphous starch was used to generate a background pattern which was then subtracted from the semi-crystalline diffraction patterns of all the starch samples analysed. The ratio of the area under the diffraction peak at 5.8° 2Θ (characteristic of 'B' type starch) to the summed areas of all the peaks, together with a calibration curve derived from mixtures of pure 'A' type (corn) and 'B' type (potato) starches, was used to calculate the proportion of the 'B' polymorph in five varieties of pea starch.

In the present paper a method is proposed involving the use of established X-ray diffractometry techniques with commercially available spreadsheet and X-ray analysis software. An amorphous background pattern is measured and then subtracted from a semi-crystalline 'C' type starch diffraction pattern. The resulting pattern is then resolved into the individual diffraction peaks corresponding to its constituent 'A' and 'B' polymorphs by the technique of profile fitting (Hindeleh *et al.*, 1980; Schreiner & Jenkins, 1983). No restrictions are placed on the resolved heights or widths of the fitted peaks. After profile fitting the areas under diffraction peaks characteristic of 'A' and 'B' type starch are used to estimate independently the proportions of the 'A' and 'B' polymorphs present. The advantages of the present method over the ones previously mentioned are that it does not require assumptions to be made about the crystallinity of the samples measured, it allows the proportion of each polymorph to be calculated independently and the sizes of the crystallites in the 'C' type sample and in the pure 'A' and 'B' polymorphs to be calculated and compared.

MATERIALS AND METHODS

Materials

Potato starch was obtained from Avebe UK Ltd, and waxy maize starch from Amioca. Smooth peas were obtained from the John Innes Centre and were of the BC/RR wild-type line. Starch from these peas was isolated according to the method of Davydova *et al.* (1995).

Methods

Preparation of amorphous starches

Amorphous samples were obtained by gelatinisation and freeze-drying of the pea, potato and waxy maize starches. The starch samples were gelatinised by heating a 2% aqueous suspension to 100°C with continuous stirring and then holding it at 100°C for 1 h. The samples were placed in sealed tubes and transferred to a block heater, preheated to 100°C. The block heater was then reset to 170°C, the samples remaining in it until this temperature was reached and a further 10 min thereafter.

The samples were cooled for a few minutes to about 100°C, removed from the tubes and immediately freeze-dried. Finally the freeze-dried samples were placed under vacuum at 70°C for 2 h.

X-ray measurements and subtraction of instrumental background

X-ray diffraction measurements were carried out using CuKα_1 radiation of wavelength 0.154 nm. The diffractometer was a Philips Scientific PW 1820 vertical goniometer with an Anton Paar TTK camera. The incident beam of X-rays was collimated using a Philips PW 1386/55 automatic divergence slit. With this device the divergence of the X-ray beam from the X-ray tube focus is continuously varied, so that at any angle of the goniometer the length of sample irradiated remains constant. This avoids the problem of variations in measured intensity, and hence diffraction peak area, with increasing angle encountered when using a fixed divergence slit with a constant width. Data were collected using a proportional detector, then stored and processed on a personal computer using Philips PC-APD (Version 3.6b) automated powder diffraction software. Samples were scanned over the range 4.0–30.0° 2Θ, at a speed of 0.005° 2Θ per second, with a step size of 0.15. All measurements were carried out at ambient temperature (24°C) and relative humidity (44%). As the water content of starch is known to have an influence on its crystallinity (Bear & French, 1941; Buléon *et al.*, 1982; Hartley *et al.*, 1995), both native and amorphous starch samples were equilibrated at a relative humidity of 44% before X-ray analysis. This was done by placing them in a desiccator over a saturated solution of K_2CO_3 for 30 d (Wolf *et al.*, 1984). Preliminary experiments involving dry weight measurements after equilibration established that the native and amorphous starch samples had water contents of 11–15% (w/w) and 2% (w/w), respectively. The higher water content in the semi-crystalline native starches probably arises as a result of water incorporated into the structure of their crystalline portions (Imberty *et al.*, 1988; Imberty & Perez, 1988; Sarko & Wu, 1978; Wu & Sarko, 1978). The loosely-packed helices of 'B' type starches can accommodate more water than the other polymorphs and potato starch was found to have the highest water content (15%; w/w). It is probably reasonable to assume that the water content after equilibration in the amorphous starch samples is an accurate representation of the water content in the amorphous portions of the native starch samples. It was established during preliminary experiments that no significant retrogradation occurred in either the amorphous or semi-crystalline samples during equilibration.

The empty sample holder was scanned under the same conditions as the samples so that the instrumental background could be determined. The diffraction patterns obtained from the samples and the empty sample holder were then converted to ASCII files and transferred to a spreadsheet program, Lotus 1-2-3 (Release 3). This program was used to subtract the measured intensities due to the empty sample holder from those of the native and amorphous starches. The resulting patterns consisting of native and amorphous starches without any instrumental background were converted to ASCII files and re-transferred to PC-APD for further analysis.

Starch crystallinity measurements

The crystallinity of the starches was calculated using the method of Hermans & Weidinger (1961) in which the semi-crystalline diffraction pattern is considered to be composed of crystalline diffraction peaks superimposed on an amorphous background. Melting of the crystallites, as in the method given above, allows the amorphous phase to be quantified which in turn allows the size of the crystalline phase to be deduced. The ratio of crystalline to amorphous material is taken to be a measure of the crystallinity of the sample. This measurement provides a reasonable working definition of crystallinity and has gained general acceptance amongst workers in this field (Blanshard, 1987).

No significant differences were found in the X-ray patterns from amorphous samples of waxy maize, potato and smooth pea starches under identical conditions of relative humidity, so the pattern from amorphous smooth pea starch was used in all the calculations to represent the amorphous portion of each semi-crystalline diffraction pattern. Fitting of the amorphous smooth pea X-ray pattern to those of native waxy maize (Fig. 1a), potato (Fig. 1b), and smooth pea starches (Fig. 1c) was done according to the method of Hermans & Weidinger (1961). The areas under the amorphous and native patterns were measured from 4.0 to 27.5° 2Θ using the Graphics facility of PC-APD. A scaling factor (f_{St}), deduced separately for each sample when fitting the amorphous to the semi-crystalline starch patterns, was applied to the measured area under the amorphous pattern. The crystallinity of the starch samples was calculated as follows:

$$\%\,\text{Crystallinity} = \frac{Q_{St} - (Q_{Am} \times f_{St})}{Q_{St}} \times 100\% \qquad (1)$$

where Q_{St}, Q_{Am} = the measured areas under the patterns from the semi-crystalline and amorphous starch samples respectively; f_{St} = scaling factor for a particular starch sample.

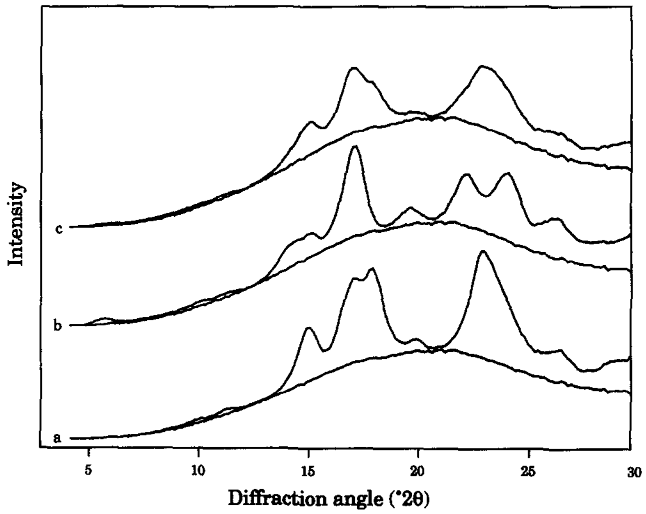

Fig. 1. Fitting of X-ray diffraction pattern from amorphous starch to diffraction patterns from: (a) waxy maize; (b) potato; and (c) smooth pea starches.

Lotus 1-2-3 was then used to subtract the amorphous pattern from smooth pea starch from the semi-crystalline diffraction patterns of native waxy maize, potato and smooth pea starches. The appropriate scaling factor (f_{St}) was applied to the amorphous pattern before subtraction from each starch pattern. The results (Fig. 2), representing the diffraction patterns from the crystalline portions of the starches without any contribution from amorphous starch or instrumental background, were used in all subsequent analyses.

The diffraction patterns from the crystalline portions of waxy maize and potato were used to simulate the changes in 'C' type patterns which occur as the proportions of their constituent 'A' and 'B' polymorphs change (Zobel, 1988; Gernat *et al.*, 1990). Lotus 1-2-3 was used to combine these diffraction patterns in different

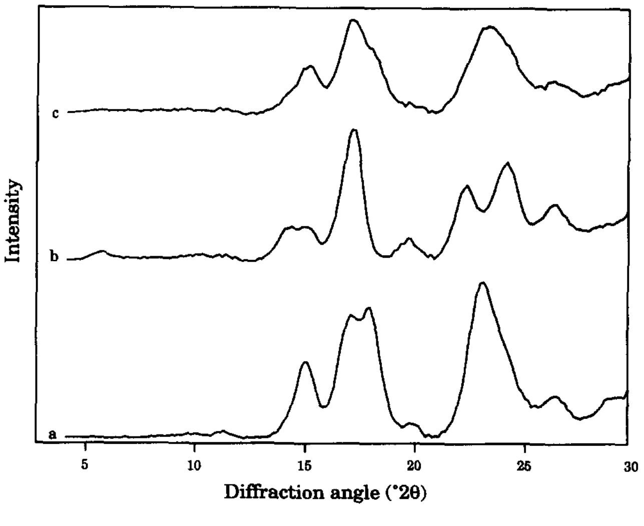

Fig. 2. X-ray diffraction patterns from the crystalline portions of: (a) waxy maize; (b) potato; and (c) smooth pea starches.

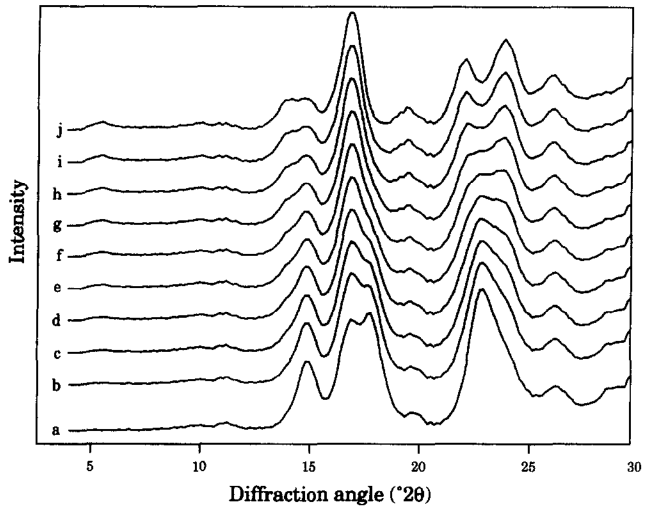

Fig. 3. Simulation of the crystalline transformations between 'A' and 'B' polymorphs by addition of the diffraction patterns from the crystalline portions of waxy maize and potato starches in the proportions (waxy maize:potato): (a) 1:0; (b) 0.8:0.2; (c) 0.7:0.3; (d) 0.6:0.4; (e) 0.5:0.5; (f) 0.4:0.6; (g) 0.3:0.7; (h) 0.2:0.9; (i) 0.1:0.9; and (j) 0:1.

proportions in order to produce a series of composite patterns (Fig. 3).

Profile fitting analysis

Despite the removal of the amorphous and instrumental backgrounds, the patterns obtained still consisted of overlapping peaks. Mathematical methods were used to separate these peaks and allow more detailed and accurate analysis of the pattern. The angular positions, full width at half maximum and areas of individual diffraction peaks contributing to the diffraction patterns of the crystalline portions of waxy maize, potato and smooth pea starches were determined using the Profile Fitting facility of PC-APD. This involved using a mathematical model to represent the expected diffraction profile shapes. The model employed 12 intrinsic parameters, which were independent of the sample, to describe the profile, geometrical aberrations and wavelength dependent contributions to the profile. These contributions were determined using an initial estimate of the Kα_1 peak position and intensity. The model also used three parameters to describe the sample dependent variables; angular position, intensity and line broadening (Schultz, 1982). These were fitted to the experimental diffraction profile using a Marquardt non-linear least squares algorithm (Schreiner & Jenkins, 1983). The initial estimated values of the Kα_1 peak positions for waxy maize and potato starches were taken from diffraction patterns of highly crystalline 'A' and 'B' type short chain amylose spherulites, obtained as described previously (Ring *et al.*, 1987), from which the instrumental and amorphous backgrounds had been subtracted (Fig. 4). The positions of the diffraction peaks from waxy maize and potato starch determined by profile fitting (Table 1), which were taken to be representative of the pure 'A' and 'B' polymorphs, were then combined

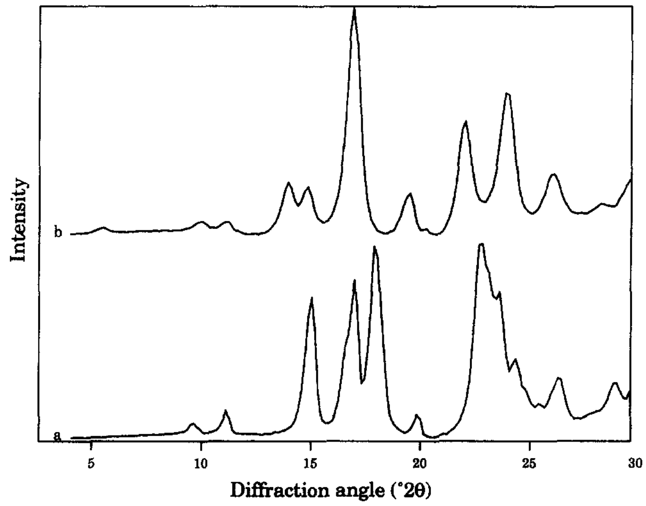

Fig. 4. X-Ray diffraction patterns from the crystalline portions of: (a) 'A' type spherulites; and (b) 'B' type spherulites.

Table 1. Positions of diffraction peaks from profile fitting analysis of X-ray diffraction patterns from starches and calculated diffraction patterns produced using a spreadsheet

Waxy maize (° 2Θ)[a]	Potato (° 2Θ)[a]	Smooth pea (° 2Θ)[a]	'C' type–'B' type (° 2Θ)[a]	'C' type–'A' type(° 2Θ)[a]
	5.8	6.1		5.8
9.8	9.8	9.4	8.4	8.6
11.2	11.2	11.2	11.2	11.2
	14.0	14.1		14.0
15.0	14.9	15.0	15.0	15.0
16.9	17.0	16.9	16.8	17.0
17.9		18.0	18.0	
19.9	19.6	19.7	20.2	19.2
	22.2	22.1		22.2
22.9		22.9	23.0	
23.9	24.0	23.9	24.1	23.7
26.4	26.4	26.4	26.2	26.4

[a] Standard deviation±0.1° 2Θ.

and used as the initial estimated Kα_1 peak position values for profile fitting of the diffraction pattern from the crystalline portion of smooth pea starch.

Line-broadening analysis

The technique of X-ray line broadening (Schultz, 1982) was used to approximate the average size (D) in nm of the crystallites in the samples, by means of the Profile Widths facility of PC-APD, using the Scherrer formula:

$$D = \frac{0.9\lambda}{\beta \cos\Theta} \tag{2}$$

where $\lambda = 0.154$ nm; β = peak broadening (given by full width at half maximum [in radians] of Kα_1 diffraction peak minus full width at half maximum [in radians] of a peak from a standard sample containing crystallites > 1 μm; $\Theta = 0.5 \times$ angular position of peak (in radians).

Peak broadening also occurs as a result of variations in the interplanar spacing of crystallites which arise due to distortions in the crystalline lattice. The PC-APD software was able to distinguish these effects from those related to crystallite size and quantified them as mean lattice distortion, or lattice strain (e) using the following formula:

$$e = \frac{\beta}{4\tan\Theta} \tag{3}$$

where e = lattice strain (or mean lattice distortion); β = peak broadening (given by full width at half maximum [in radians] of Kα_1 diffraction peak minus full width at half maximum [in radians] of a peak from a standard sample containing crystallites > 1 μm); $\Theta = 0.5 \times$ angular position of peak (in radians).

RESULTS AND DISCUSSION

Experimental error

Values of experimental error quoted are based on observed variations in values obtained from a number of different aliquots taken from each starch sample.

Starch crystallinity

The percentage crystallinity values calculated for the three samples are shown in Table 2. The values obtained for waxy maize (33±2%) and smooth pea starches (21±2%) are similar to those of 31 and 19% reported by Gernat *et al.* (1993), whereas Davydova *et al.* (1995) reported slightly higher values in the range 26–31% for five varieties of smooth pea starches.

Profile fitting analysis

The positions of the diffraction peaks found in the patterns from waxy maize and potato starches (Table 1) were typical of those reported for 'A' and 'B' type starches, respectively (Wu & Sarko, 1978; Buléon *et al.*, 1987). These patterns had most of their diffraction peak positions in common, except for the peaks at 17.9±0.1° and 22.9±0.1° 2Θ in waxy maize starch, and 5.8±0.1° and 14.0±0.1° 2Θ in potato starch. The diffraction peak positions found in the pattern from the crystalline portion of smooth pea starch (Table 1) closely approximated to a combination of those found in the patterns from the crystalline portions of waxy maize and potato starches. These results tend to confirm the proposition that the crystalline portion of 'C' type starch is composed of a mixture of 'A' and 'B' type unit cells (Blanshard, 1987; Gernat *et al.*, 1990, 1993; Sarko & Wu, 1978; Wu & Sarko, 1978).

Table 2. Crystallinity and polymorphic composition of waxy maize, potato and smooth pea starches

Starch	% crystallinity[a]	% polymorphic composition[b] 'A' type	'B' type
Waxy maize	33	100	
Potato	25		100
Smooth pea	21	56	44

[a]Standard deviation ±2%.
[b]Standard deviation ±3%.

Proportions of 'A' and 'B' polymorphs in smooth pea starch

It has been shown previously that the relationship between the area under the peak at 5.8±0.1° 2Θ and the total peak area can be used to calculate the proportion of the 'B' polymorph present in 'C' type starch (Davydova *et al.*, 1995). A modification of this method was used to calculate the proportions of both polymorphs present in smooth pea starch. The total peak area was defined as being only the sum of the areas under the diffraction peaks whose maxima lay in the range 13–25° 2Θ. This was the region containing the largest peaks which could be most clearly defined by profile fitting. The diffraction patterns from the crystalline portions of waxy maize and potato starches were used as standards, representing the pure 'A' and 'B' polymorphs respectively. The peaks at 17.9±0.1° 2Θ (Fig. 5b.1) and 14.0±0.1° 2Θ (Fig. 5b.2) in the 'C' type diffraction pattern from the crystalline portion of smooth pea starch were assigned to the 'A' and 'B' polymorphs, respectively. The proportion of the 'A' polymorph present in smooth pea starch was calculated as follows:

$$\text{Proportion of A polymorph} = \frac{a_{\text{pea}}}{a_{\text{maize}}} \times 100\% \tag{4}$$

where a_{maize} and a_{pea} are given by:

$$a_{\text{pea}}; a_{\text{maize}} = \frac{\text{Peakarea}_{17.9^\circ}}{\text{Combined peak areas}_{13-25^\circ}} \tag{5}$$

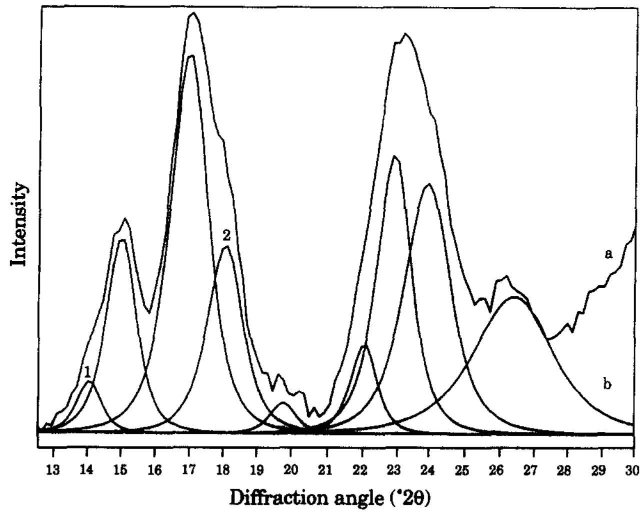

Fig. 5. Partial plot of the X-ray diffraction pattern from the crystalline portion of smooth pea starch illustrating: (a) raw data; and (b) constituent diffraction peaks elucidated by profile fitting analysis of the pattern.

in the diffraction patterns from the crystalline portions of waxy maize and smooth pea, respectively.

The proportion of the 'B' polymorph present was calculated in a similar manner:

$$\text{Proportion of B polymorph} = \frac{b_{\text{pea}}}{b_{\text{potato}}} \times 100\% \qquad (6)$$

where b_{potato} and b_{pea} are given by:

$$b_{\text{pea}}; b_{\text{potato}} = \frac{\text{Peak area}_{14.0^\circ}}{\text{Combined peak areas}_{13-25^\circ}} \qquad (7)$$

in the diffraction patterns from the crystalline portions of potato and smooth pea, respectively.

Using this method the proportions of 'A' and 'B' polymorphs in smooth pea starch were calculated to be 56±3 and 44±3%, respectively (Table 2). These results are similar to the proportions reported by Gernat *et al.* (1990, 1993) for smooth pea starch. The diffraction pattern from the crystalline portion of smooth pea starch (Fig. 2c) was compared to a composite pattern produced by using Lotus 1-2-3 to combine the diffraction patterns from the crystalline portions of waxy maize and potato starches in the ratio 0.6:0.4 (Fig. 3d). Figure 6 shows that these two patterns are very similar in appearance which suggests that the polymorphic proportions calculated above may be of the correct order.

Line-broadening analysis

Table 3 shows the results from line-broadening analysis of the three samples. The average sizes (*D*) and mean lattice distortion (*e*) of 'A' type crystallites were approximated using the broadening of the peaks at 17.9±0.1° 2Θ in the diffraction patterns from waxy maize and smooth pea starches. The average sizes and mean lattice distortion of 'B' type crystallites were approximated using the peaks at 14.0±0.1° 2Θ in the diffraction patterns from potato and smooth pea starches. The average size of the 'A' type crystallites in waxy maize starch was found to be 10±1 nm and that of the 'B' type crystallites in potato starch was found to be 14±1 nm. Values obtained for the 'A' and 'B' type crystallites in smooth pea starch were also 10±1 and 14±1 nm, respectively.

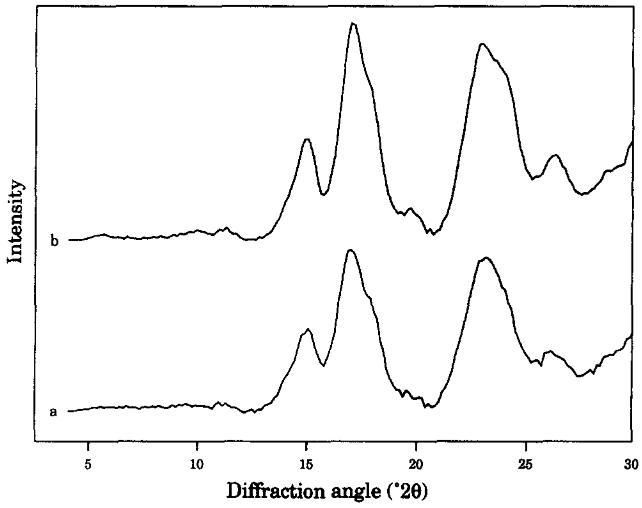

Fig. 6. Comparison between (a) the 'C' type X-ray diffraction pattern from the crystalline portion of smooth pea starch and (b) a composite 'C' type pattern formed by a combination of the diffraction patterns from the crystalline portions of waxy maize ('A' type) and potato ('B' type) in the ratio 0.6:0.4.

Table 3. Average sizes of polymorph crystallites (*D*) and mean lattice distortion (*e*) from line broadening analysis of X-ray diffraction patterns from starches and calculated diffraction patterns produced using a spreadsheet

	'A' type		'B' type	
	D (nm)[a]	*e* (%)[b]	*D* (nm)[a]	*e*(%)[b]
Waxy maize	10	2.9		
Potato			14	2.7
Smooth pea	10	2.9	14	2.9
'C' type–'B' type	10	3.0		
'C' type–'A' type			14	2.7

[a] Standard deviation ±1 nm.
[b] Standard deviation ±0.4%.

The mean lattice distortion of the 'A' type crystallites in waxy maize starch was found to be 2.9±0.4% and that of the 'B' type crystallites in potato starch was found to be 2.7±0.4%. Values obtained for the 'A' and 'B' type crystallites in smooth pea starch were 2.9±0.4 and 2.9±0.4%, respectively. The values for average size and lattice disorder of the 'A' and 'B' type crystallites in the pure 'A' and 'B' type starch standards and the 'C' type starch are very similar. Variations in the effects of these parameters on diffraction peak width between the standard samples and the 'C' type starch will not, therefore, constitute a major source of error in the calculation of the relative proportions of the 'A' and 'B' polymorphs in smooth pea starch.

Separation of 'C' type pattern into 'A' and 'B' type patterns

'A' and 'B' polymorph patterns (Fig. 7a, b) were obtained from the 'C' type diffraction pattern of the crystalline portion of smooth pea starch by subtraction of the appropriately proportioned diffraction patterns from the crystalline portions of potato and waxy maize starch respectively. Profile fitting (Table 1) and line-broadening analysis (Table 3) of the resulting patterns gave results which were in close agreement with those from 'A' type (waxy maize) and 'B' type (potato) starches.

CONCLUSIONS

Using samples of native and amorphous starches, together with a combination of PC-APD and Lotus 1-2-3 software, the crystalline portion of 'C' type starch has been shown to be comprised of a mixture of 'A' and 'B' polymorphs. The proportions of these polymorphs in smooth pea starch were calculated to be 56±3% 'A' type to 44±3% 'B' type, respectively. It has been demonstrated that a diffraction pattern similar to that from the crystalline portion of a 'C' type starch can be constructed by combining the diffraction patterns from the crystalline portions of 'A' and 'B' type starches in the correct proportions. It has also been shown that the pattern from the crystalline portion of a 'C' type starch can be broken down into its constituent 'A' and 'B' polymorphs. The methods outlined in this present work will be used to study the different crystalline structures found in the starches from wrinkled peas, which arise as a result of a number of different genetic mutations in smooth peas.

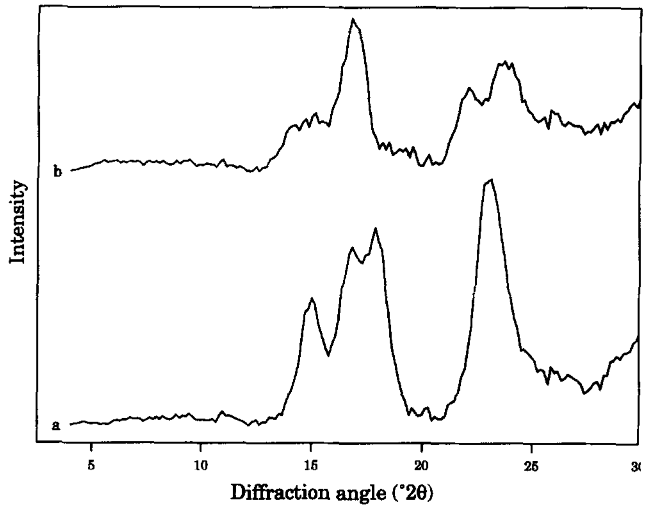

Fig. 7. Polymorph patterns produced by manipulation of the diffraction patterns from the crystalline portions of starches using Lotus 1-2-3: (a) an 'A' type pattern produced by subtraction of a 'B' type pattern from a 'C' type pattern; and (b) a 'B' type pattern produced by subtraction of an 'A' type pattern from a 'C' type pattern.

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