Reprinted from: Issues in new crops and new uses. 2007. J. Janick
and A. Whipkey (eds.). ASHS Press, Alexandria, VA.

Scale-Up of Sicklepod Processing

Rogers E. Harry-O'kuru*

Sicklepod (*Senna obtusifolia* L., Fabaceae) is a leguminous plant that thrives unwanted in crop fields such as soybean farms and is therefore described as a weed especially in many Southeastern States in the US. Although the recent introduction of "round-up-ready" soybeans mitigates infestation of the beans by sicklepod, the latter is so prolific that even volunteer stands yield over a 1120 kg/ha. The composition of sicklepod seed has been reported to include mainly: anthraquinones 1%–2%, fats 5%–7%, proteins 14%–19%, and carbohydrates 66%–69% (Khanna and Gupta 1967; Varshney et al. 1973; Crawford et al. 1990; Abbott et al. 1998). Sicklepod flour from Indian seed is in the market as a hydrocolloid additive in pet foods because of its unique galactomannan ratio which enhances flexibility in the range of formulation compositions (Bayerlein et al. 1989). In a previous report we have shown a laboratory-scale process for partitioning the crushed seed components in order to separate the three classes of compounds in the seed (Harry-O'kuru et al. 2005). To effect a scale-up of that process beyond a hundred gram level, cost as well as technical considerations have resulted in the development of a two-pronged approach for enhanced isolation of the different seed components into their corresponding classes. In both approaches, water is the principal solvent used except for defatting the meal. To isolate the various proteins, we have used solubility as a guiding principle (Osborne 1895; Csonka and Jones 1927). And traditional saline treatment has been applied to sicklepod defatted seed meal to allow for solubilization of water soluble proteins. For non water-soluble proteins, alternative aqueous based techniques were used to effect complete extraction. This paper reports scaled-up procedures for the extraction of sicklepod seed components mainly proteins and carbohydrates.

MATERIALS AND METHODS

Materials and Reagents

Sicklepod seed were obtained from the Wilder Farm, Raleigh, via Department of Agriculture, North Carolina State University. Petroleum ether was purchased from Sigma-Aldrich Co. (St. Louis, Missouri); amberlite XAD-4 was obtained from SUPELCO, Bellefonte, Pennsylvania; dialysis tubing (regenerated cellulose, MWCO 3,500), sodium chloride and sodium hydroxide were obtained from Fisher Scientific (Chicago, Illinois). Sample centrifugations were achieved using a Beckman J2-HS centrifuge (Fullerton, California for smaller scale) and a Clinton Separators Inc. centrifuge (Warminster, Pennsylvania for kilogram scale). Volume reduction of dilute solutions was attained using Reverse Osmosis Concentrator, Seprotech Systems, Inc., Ottawa, Canada.

Instrumentation

The ^{13}C NMR spectra were obtained on a Bruker ARX-500 with a 5-mm dual proton/carbon probe (Bruker Spectrospin, Ballerica, Massachusetts); the internal standard was tetramethylsilane. Protein analysis was performed using a LECO model CHN 2000, St. Joseph, Michigan.

Fourier-Transform Infrared (FTIR) Spectrometry

FTIR **s**pectra were measured on a Bomem Arid Zone FTIR spectrometer (Bomem MBSeries, Bomem, Quebec) equipped with a DTGS detector. Dried test samples of isolated solids were pulverized with KBr (1/300 mg) and pressed at 24,000 psi) to generate transparent discs for FTIR analysis. Liquid derivatives were pressed between two NaCl discs (25 × 5 mm) to give thin transparent oil films for analysis by FTIR spectrometry. Absorbance spectra were acquired at 4 cm^{-1} resolution and signal-averaged over 32 scans. Interferograms were

*Names are necessary to report factually on available data. The USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

The author owes a depth of gratitude to Dr. Karl Vermillion for the ^{13}C NMR spectrum, to Kathleen Payne-Wahl, Mark Klokkenga, Jo Dean Sarins, Jeffrey Forester, Billy Deadmond and Corey Shannon for technical assistance. ARS CRIS Project 3620-41000-092 is hereby acknowledged for support of this study.

Fourier transformed using cosine apodization for optimum linear response. Spectra were baseline corrected, adjusted for mass differences and normalized to the methylene peak at 2927 cm^{-1}.

Isolation of Proteins

Albumins and Globulins. Defatted sicklepod meal (100 g) was stirred into 0.5 M sodium chloride solution (1.0 L) at room temperature (25 min). The resulting mixture was centrifuged (12,000 × g) for 20 min and the centrifugate saved. The resulting pellet was resuspended and stirred in a fresh equivalent volume of sodium chloride solution for same time interval followed by centrifugation. This procedure was repeated a third time so as to exhaustively extract the albumin and globulin components of the sample. The pooled supernatants (centrifugates) were then dialyzed against deionized water using regenerated cellulose tubing, molecular weight cut off (3,500). The dialysis was carried out at 5°C. The dialysis retentate comprised the soluble albumins and precipitated globulins which were separated and freeze dried to give 5.10 g, 5.1% and 11.40 g, 11.4% respectively. The FT-IR spectra of these isolates are: albumin ν_{KBr} cm^{-1}: 3399 bs, 3073 bm, 2960 m, 2928 m, 2873 w-m, 1656 vs, 1534 s, 1452 w-m, 1397 w-m, 1316 w, 1233 w-m, 1079 bm-s, 619 w; the globulin cm^{-1}: 3338 bs, 3068 bm, 2961 m, 2933 m, 2875 w-m, 1657 vs, 1518 s, 1451 m, 1392 m, 1235 w-m, 1155w-m, 618 w.

Prolamines. The solids (pellet) leftover from the sodium chloride treatment were then triturated with 80% ethanol (1.0 L) for 30 min at room temperature and centrifuged (12,000 × g) for 20 min. The supernatant was saved while the pellet was treated two more times with same volumes of fresh 80% ethanol. The combined ethanol extracts were dialyzed against deionized water at 5°C and freeze dried to give the prolamine component (5.88 g, 5.9%). FT-IR ν_{KBr} cm^{-1}: 3411 bvs, 3082 m, 2929 m, 1654 vs, 1527 m-s, 1447 m, 1397 m, 1316 w-m, 1237 w-m, 1159 m-s, 1071 bs, 614 w.

Glutelins Fraction. The glutelin component was extracted from the lyophilized pellet (61.2 g) left over from the prolamine extraction. This solid was stirred into 0.1 M sodium hydroxide solution (1.0 L × 3) for 30 min and centrifuged for 20 min at 12,000 g. Each 1,000 mL batch was centrifuged as above and the supernatant separated and saved whereas the pellet was again triturated with the alkali. The combined NaOH extracts were treated with HCl to pH 6.5–6.9, and then dialyzed against deionized water at 5°C. The dialysis retentate comprised a supernatant and a precipitate; the mixture was centrifuged as above for 20 min. The supernatants were filtered and the filtrate lyophilized separately from the combined filter cake. Thus two glutelin components were obtained from alkali extraction, namely, a water-soluble fraction, β-glutelin (7.4 g, 7.4% overall or 12% of the residue) and a water-insoluble fraction, α-glutelin (12.2 g, 12.2% overall or 20% of the residue), respectively. FT-IR spectrum of the freeze dried β-glutelins ν_{KBr} cm^{-1}: 3413 bvs, 2930 m, 1630 s, 1540 m, 1414 m, 1390 w-m, 1239 w, 1093 s, 1046 s, 634 w; and the water-insoluble α-glutelins cm^{-1}: 3407 bs, 3077 m, 2961 m, 2933 m, 2875 w-m, 1653 vs, 1525 s, 1451 m, 1392 m, 1235m, 1077w-m, 617 w.

Sodium Hydroxide-Insoluble Residue. There was material that was recalcitrant to NaOH treatment. A suspension of this component in DI water had a pH 11.5; on acidulation (HCl) to pH 6.5–7.0 followed by dialysis against DI water. The resulting retentate contained a mother liquor and a precipitate. These were separated and freeze dried to give a minor component from the supernatant (1.0 g, 1.0%) and a major component from the precipitate (36.0 g, 36%) overall or 58.8% based on the residue after prolamine removal. The light-brown major product has an IR ν_{KBr} cm^{-1}: 3389 vs, 2928 w-m, 1653s, 1529m-s, 1438m, 1419m, 1318w-m, 1152m, 1067s, 894 w, 813 w, 612 w; and the minor supernatant fraction (1.0 g) with an IR cm^{-1}: 3413 vs, 2930 m, 1630 s, 1540 m, 1414 m, 1093 s-vs, 1045 s-vs, 634 w.

Isolation of Carbohydrates and Anthraquinones

Defatted sicklepod meal (2.40 kg) was stirred into 70 L of deionized water (ca. 1 hr) then pumped into the centrifuge (Clinton Separators, Inc.) running at 5,000–6,000 rpm. The centrifugates were recycled until clear supernatants were obtained and saved. The resulting pellet was removed from the bowl and stirred with a fresh volume of deionized water (ca. 70 L) for same amount of time as before and centrifuged to a clear supernatant

that was again saved. The resulting cake comprising water-insoluble proteins was removed from the centrifuge drum and freeze dried. Meanwhile portions of the combined supernatants were heated to 92–93°C for 40 min and filtered through a pad of celite in a medium porosity sintered glass funnel; portions of the cooled filtrate were passed through a column bed of amberlite XAD-4 (3180.9 cm^3) at a flow rate of about 15 mL/min. The column was rinsed with deionized water and the rinse volume combined with the earlier carbohydrate eluate. This dilute (0.3–0.7% solids) eluate was concentrated to 3.0% solids for lyophilization or spray drying. The lyophilized portion of the extract gave a colorless powder with IR spectrum ν_{KBr} cm^{-1}: 3418 vsb, 2923 m, 1624 m, 1413 m, 1074 vs, 1034 vs, 812 w. ^{13}C-NMR (DMSO-d_6) δ (ppm): [104.5, 103.6, 99.54, 97.26, 92.21, 92.08] anomerics; [83.00, 82.83, 77.56, 77.50, 77.31, 77.06,] C-4; [74.78, 74.72, 73.38, 73.33, 73.28, 72.95, 72.10, 71.94, 71.69, 71.38, 70.74, 70.32, 69.93, 69.25, 69.02, 67.16] C2, C3, C5; [63.53, 62.66, 62.61, 62.55, 60.99, 60.96] C-6. Acetylation of this fraction with neat acetic anhydride and Et_3N as catalyst gave an IR ν_{film} on NaCl disc cm^{-1}: 3482 vw, 2960 m (CH_3 asym.), 2944 m (CH_2 asym.), 2855 w (CH_3 sym.), 1750 vs (C=O), 1434 m (CH_2 deform), 1370 s (CH_3 deform), 1224 vs (C-CO), 1040 s-vs (C-CH_2-O), 907 m, 601 m. ^{13}C-NMR $CDCl_3$ δ (ppm): [170.5, 170.1, 170.0, 169.7, 169.6, 169.5, 169.3, 169.27, 169.2, 169.11, 169.1, 169.06, 168.9, 168.55] C=Os; [100.27, 100.20, 95.95, 95.34, 92.78, 91.79, 91.35, 91.28, 89.87, 89.78, 88.72] anomerics; [80.64, 79.85, 78.96, 78.81, 78.20, 78.08] C-4; [72.39, 72.28, 70.07, 69.56, 69.33, 68.26, 68.09, 67.64] C-2, C-3, C-5; [63.38, 63.27, 63.13, 62.58, 61.96, 61.83, 61.62, 61.38, 61.31, 60.96, 59.93] C-6s; [20.54, 20.38, 20.33, 20.30, 20.27, 20.25, 20.22, 20.18, 20.12, 20.10, 20.07, 20.02, 19.99] -CH_3 of the acetoxy groups. The column bed was further rinsed with deionized water, and this rinse discarded. The resin bed was then eluted with 0.1 M ammonium hydroxide in ethanol to recover the anthraquinones, as a reddish solution. The anthraquinone component recovered will be described and characterized elsewhere. The column bed was conditioned for further use by several washes with aqueous ethanol and finally with dilute ammoniacal ethanol until the light pink color faded at ca. pH 10.

RESULTS AND DISCUSSION

In contrast to the use of extracting solvents such as acetone-water, ethanol-water, 2-propanol-water, to isolate sicklepod flour from the endosperm (Bayerlein et al. 1998), the use of 0.5 M sodium chloride solution as a triturating solvent for the defatted endosperm allowed removal of albumins and globulins from the meal (Osborne 1895; Csonka and Jones 1927) without the filtration difficulties usually encountered in the former process. The flow charts (Fig. 1) summarize the extraction regimen for the various classes of proteins. This was intended to obviate the filtration difficulties brought about by fouling of filter systems by watersoluble proteins in the extraction and decolorization steps of carbohydrate isolation from the meal. In processing small quantities of sicklepod meal for carbohydrates, soluble proteins were a manageable problem, however, the problem becomes intolerable at the kilogram scale. An additional advantage of the current process is provision of a means of separating the biologically active proteins (albumins and globulins) from the purely storage types in the meal. The many steps involved in the overall process allowed separation of the various classes of proteins

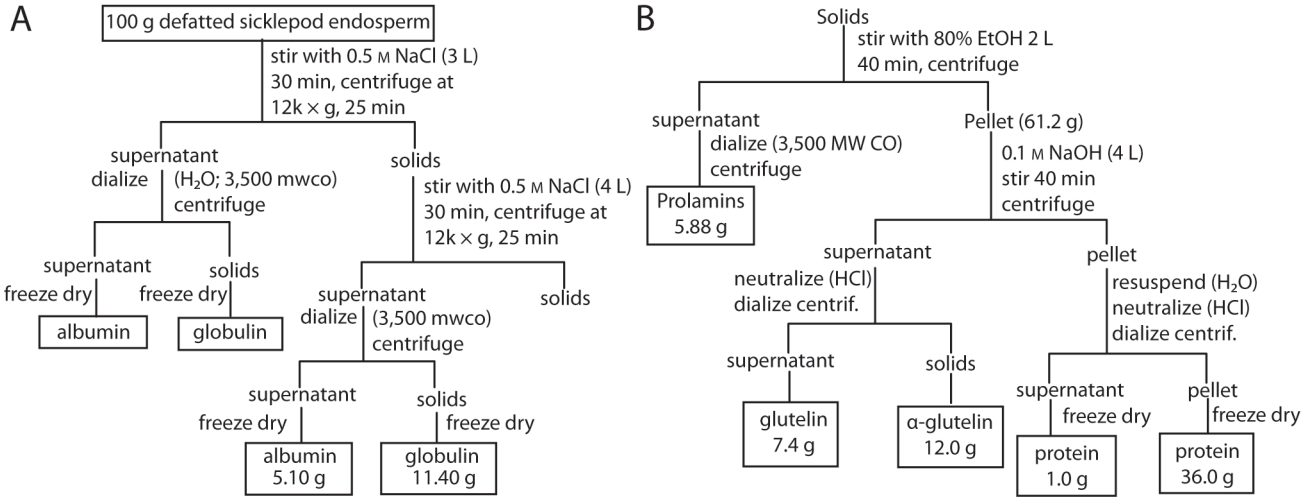

Fig. 1. A and B. Flow chart of protein extraction regimen to improve the process.

in reasonable purity as shown in Table 1. Analysis of the albumin and globulin isolates by IR spectrometry show characteristic amide A and B peptide absorption bands—N-H stretching modes or Fermi resonance usually seen around 3280 cm^{-1}, 3090 cm^{-1}, but observed here at 3399 cm^{-1}, 3077 cm^{-1} for albumins (Fig. 2A) and 3338 cm^{-1} and 3068 cm^{-1} for globulins (Fig. 2B). The main protein characteristic in IR spectroscopy, i.e., the strong amide I (C=O stretch) and amide II (N-H in-plane bending) bands are observed at 1656 cm^{-1} and 1534 cm^{-1}, respectively, for the albumins fraction which also shows a broad medium to strong band at 1079 cm^{-1} (C-C-O stretch). The globulins fraction gives strong amide I and II absorption bands at 1657 cm^{-1} and 1518 cm^{-1}, respectively. Analysis of the percent N of the globulins component gave a value corresponding to 92.2% dry protein content compared to 64.6% dry protein for the albumins. The spectrum of the aqueous ethanol-soluble prolamines fraction (Fig. 3A) shows a very strong, broad band centered at 3418 cm^{-1} which overlaps the amide A absorption. The frequency and strength of this band is reminiscent of a hydrogen bonded OH stretch. The remaining spectral features are a strong amide I and II absorption bands at 1653 cm^{-1} and 1534 cm^{-1}, respectively. In addition the spectrum exhibits a reasonably strong (C-C-O) band at 1069 cm^{-1}. The combination of the high frequency, high intensity band (3418 cm^{-1}) overlapping the amide A absorption, with the strong amide I and II (1653, 1534 cm^{-1}) absorbances together with the strong 1069 cm^{-1} band imply presence of a glycosyl component as part of this seed protein. The water-insoluble α-glutelins fraction from NaOH treatment readily precipitated out of the dialysis retentate leaving the more soluble β-glutelins in the supernatant solution as had been observed by earlier workers in wheat, barley, and rye flours (Csonka and Jones 1927, 1929). The IR spectrum (Fig. 3B) of the freeze dried α-glutelins (12.2%) overall yield from the defatted meal or 20% from the residue left over after prolamines extraction gave a typical protein absorption spectrum. A broad amide A and B absorbance centered at 3407 cm^{-1} and 3077 cm^{-1}, a very strong amide I band at 1653 cm^{-1}, and a strong amide II at 1525 cm^{-1}. The analysis for percent N in this isolate gave a value equivalent to 99.5% dry protein content.

The material that was recalcitrant to sodium hydroxide treatment turned out to be complex. Its IR spectrum (Fig. 3C) gave very strong absorbances at 3389 cm^{-1} (Fermi resonance), a strong amide I band at 1653 cm^{-1}; a medium to strong amide II band at 1529 cm^{-1} and a strong band at 1067 cm^{-1}. The intensities and frequencies of these IR bands indicate a material with both protein and carbohydrate characteristics and therefore could best be described as a glycoprotein. It comprises 36% of the defatted starting meal or 58.8% of the solid residue after ethanol extraction. Interestingly, the IR spectral characteristics of this isolate are exactly identical to that of an earlier water-insoluble residue obtained from exhaustively extracting defatted sicklepod meal with warm water. The scale-up of sicklepod meal extrac-

Table 1. Analysis of % dry protein content of sicklepod protein isolates.

Sample	Dry protein (%)
Albumin	64.59
Globulin	92.24
Prolamin	49.94
β-Glutelin (supernatant)	56.11
α-Glutelin (precipitate)	99.47
Residue (supernatant)	23.77
Residue (precipitate)	33.30

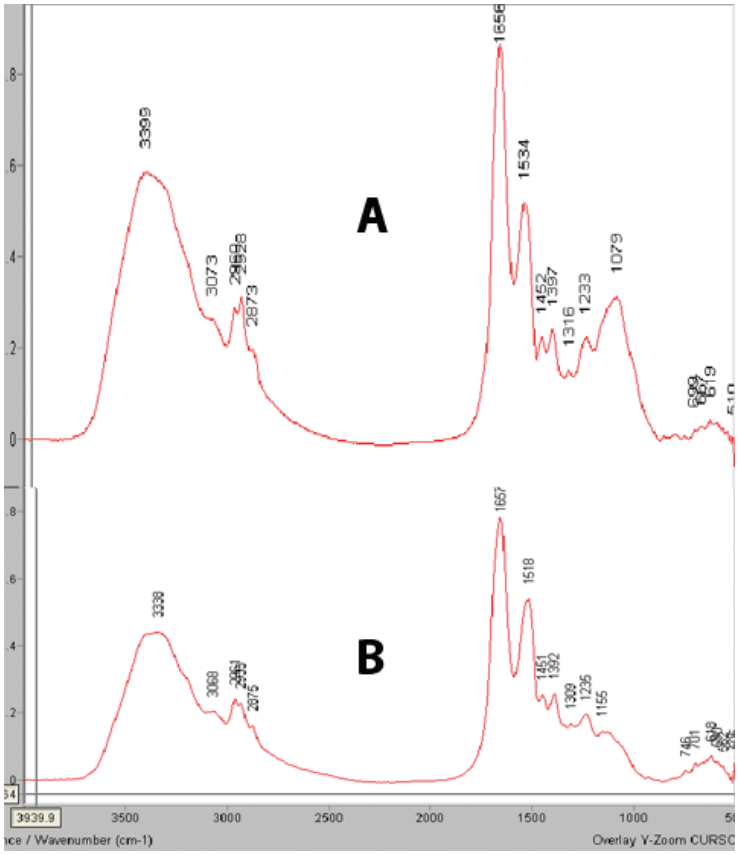

Fig. 2. FT-IR spectra of (A) sicklepod albumin fraction; (B) globulins fraction.

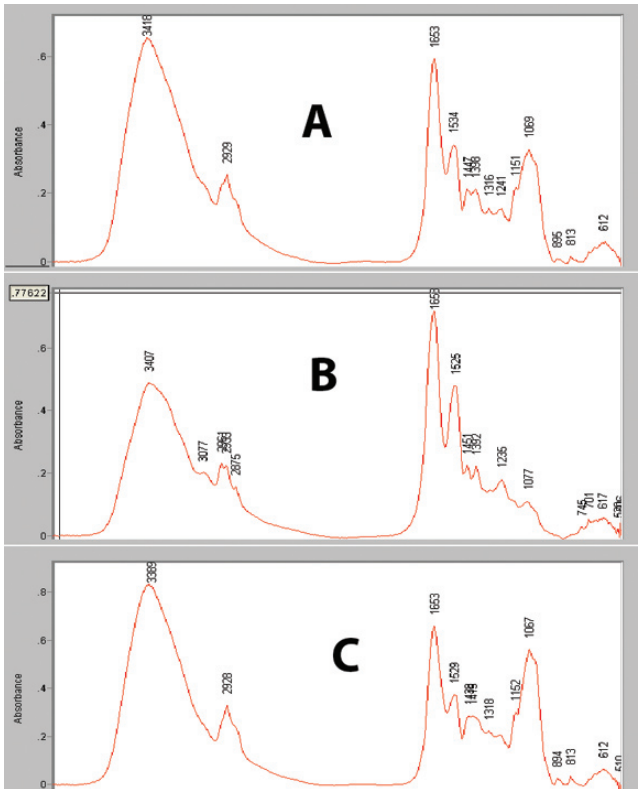

Fig. 3. FT-IR spectrum of (A) sicklepod prolamine fraction; (B) sicklepod glutelins fraction; (C) sicklepod glycoprotein.

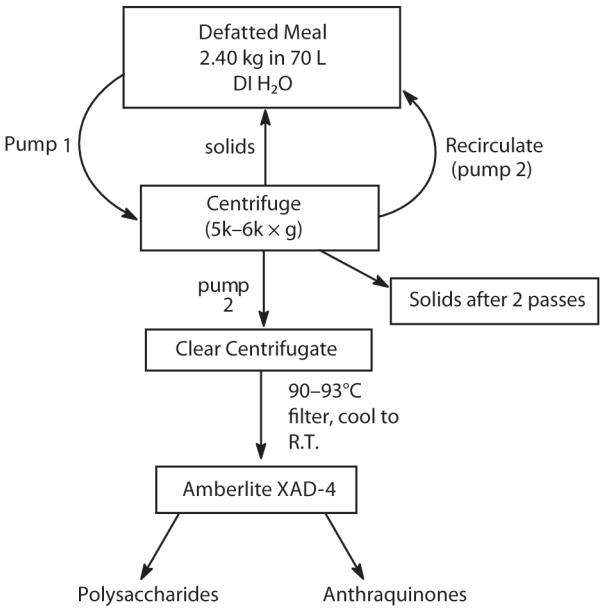

Fig. 4. Flow chart of scale-up of sicklepod aqueous processing.

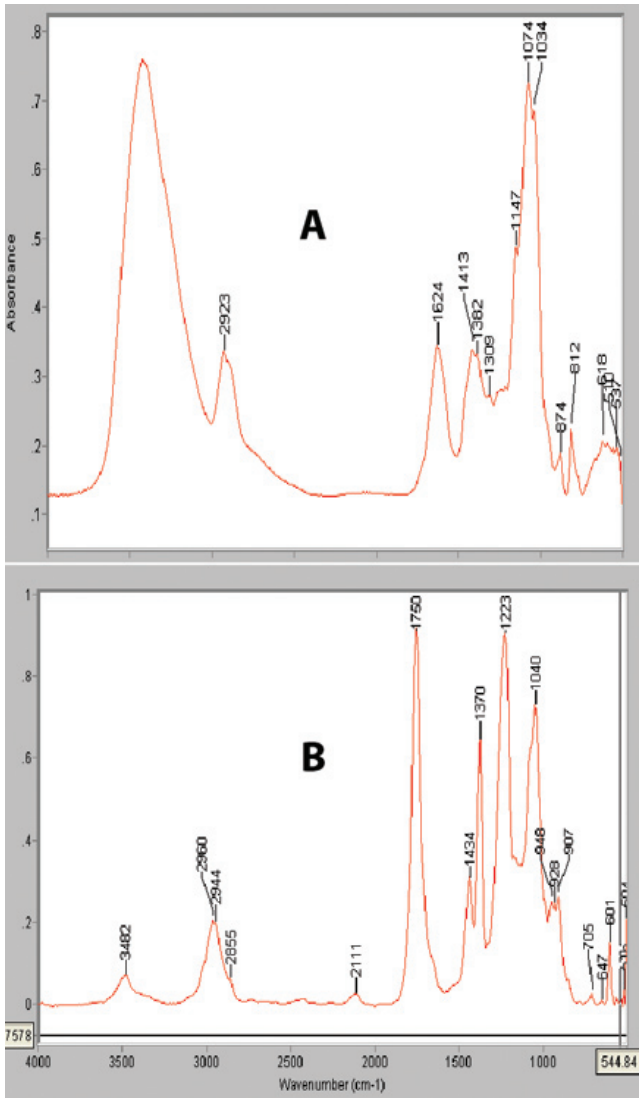

Fig. 5. FT-IR Spectra of: (A) extracted sicklepod polysaccharide; (B) its acetylated derivative.

tion is summarized in the flow chart (Fig. 4). Stirring of the dry defatted meal into water allowed solubilization of the polysaccharides and water-soluble proteins and anthraquinones. The process allowed monitoring of the centrifugate for particulates so the eluate could be recycled until free of solids. The particulate-free centrifugate was saved and the pellet in the centrifuge bowl was fed back into the stirring holding tank with a fresh volume of water for a second pass through the centrifuge. Portions of the extract were heated as indicated to coagulate soluble proteins for removal from the polysaccharide by filtration. The cooled, filtrate was then passed through (amberlite XAD-4) a resin bed to separate the polysaccharides from the anthraquinones. The freeze dried polysaccharide gave an IR spectrum typical of carbohydrates (Fig. 5A). As observed, a very strong OH stretch broadened due to hydrogen bonding centered at 3405 cm^{-1} was observed; while a much weaker alkyl band (CH_2 stretch) occurs at 2923 cm^{-1}; a broad, medium intensity band is seen at 1624 cm^{-1} overlapping the region where the OH bending mode of water usually occurs; a weak, broad band at 1409 cm^{-1} is assignable to the CH_2 deformation, whereas a very strong CC-O stretching modes were observed at 1074 and 1034 cm^{-1}. Acetylation of this solid gave an IR spectrum (Fig. 5B) with new features: a disappeared OH band as expected and the appearance of a very strong 1750 cm^{-1} acyl C=O stretching mode for the ester; a strong 1370 cm^{-1} band corresponding to the $-CH_3$ deformation mode of the acetyl ester relative to the much weaker CH_2 bending mode at 1434 cm^{-1}. A very strong 1223 cm^{-1} stretching absorption band is observed for the C-C-O mode of the ester and a strong 1040 cm^{-1} $C-CH_2-O$ band are evident. These spectral features are characteristics expected in an acetylated carbohydrate. Additionally, the ^{13}C NMR spectrum was also consistent with what would be expected of an acetylated polysaccharide. The spectrum contained resonance lines for the ester carbonyls in the range of 168.5–170.5 ppm; the anomeric carbons of the polysaccharide are represented by resonances between 100.2–88.72 ppm; whereas the C-4, C-2, C-3, C-5 resonances are seen between 70.07–67.64 ppm; the C-6 resonances occurring between 63.38–59.93 ppm, and the $-CH_3$ of the acyl groups are observed between 19.99–20.54 ppm. Further analysis of the freeze dried polysaccharide by HPLC-GPC technique using a Synchropak GPC 100 column eluted with water at 0.5 mL/ minute with refractive index detection indicated a molecular mass range of 25–27 kD, using dextrans as standards.

REFERENCES

Abbott, T.P., S.F. Vaughn, P.F. Dowd, H. Mojtahedi, and R.F. Wilson. 1998. Potential uses of sicklepod (*Cassia obtusifolia*). Indust. Crops Prod. 8:77–82.

Bayerlein, F., N. Keramaris, N. Kottmair, M. Khun, and M.M. Maton. 1989. Process for producing colorless, odorless and tasteless cassia endosperm flour. U.S. Patent 4,840,811.

Crawford, L., G.M. McDonald, and M. Friedman. 1990. Composition of sicklepod (*Cassia obtusifolia*) toxic weed seed. J. Agr. Food Chem. 38:2169–2175.

Csonka, F.A. and D.B. Jones. 1927. Studies on glutelins. I. The α- and β-glutelins of wheat (*Triticum vulgare*). J. Biol. Chem. 73:321–329.

Csonka, F.A. and D.B. Jones. 1929. Studies on glutelins. V. The glutelins of rye (*Secale cereale*), and of barley (*Hordeum vulgare*). J. Biol. Chem. 82:17–21.

Harry-O'kuru, R.E. Y.V. Wu, R. Evangelista, S.F. Vaughn, W. Rayford, and R.F. Wilson. 2005. Sicklepod (*Senna obtusifolia*) seed processing and potential utilization. J. Agr. Food Chem. 53:4784–4787.

Khanna, S.N. and P.C. Gupta. 1967. The structure of a galactomannan from the seeds of *Ipomoea muricata*. Phytochemistry 6:605–609.

Osborne, T.B. 1895. The proteids of the rye kernel. J. Am. Chem. Soc. 17:429–448.

Varshney, S.C., S.A.I. Rizvi, and P.C. Gupta. 1973. Structure of a polysaccharide from *Cassia tora* seeds. 1. J. Agr. Food Chem. 21:222–226.