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# Quantification of Xylitol in Foods by an Indirect Competitive Immunoassay

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Sugar alcohols are widely used as food additives and drug excipients. D-Xylitol (INS 967), an important five-carbon sugar alcohol, is a natural constituent of many fruits and vegetables. The critical reagent for an immunoassay of haptens is the requirement of hapten-specific antibodies. Here, affinity-purified xylitol-specific antibodies generated earlier [Sreenath, K.; Venkatesh, Y. P. Reductively aminated D-xylose-albumin conjugate as the immunogen for generation of IgG and IgE antibodies specific to D-xylitol, a haptenic allergen. *Bioconjugate Chem.* 2007, *18*, 1995−2003] have been utilized for developing an indirect competitive ELISA for xylitol. With xylitol−BSA conjugate as the coating antigen, a working range of 5−400 ng of xylitol could be determined in the immunoassay; the limit of detection was 1 ng of xylitol. Onion (*Allium cepa*) and strawberry (*Fragaria nilgerrensis*) were selected as the food sources containing D-xylitol. The amount of D-xylitol was found to be 12.6 and 44 mg/100 g fresh weight of onion and strawberry, respectively, and the results are in good agreement with the reported values by HPLC and GC. The recovery analyses showed that added amounts of D-xylitol were recovered fairly accurately with recoveries in the range of 89.2 to 94.9% in the case of onion, and 88.4 to 95.9% in the case of strawberry. The indirect competitive ELISA for xylitol quantification is a simple method using a 3 kDa ultrafiltrate of whole food extract, and does not require extensive sample preparation and derivatization as in the case of GC and HPLC analyses. This is the first immunoassay developed for the sugar alcohol, xylitol.

**KEYWORDS: Food additive; immunoassay; indirect competitive ELISA; INS 967; onion; strawberry; sugar alcohol; xylitol**

## INTRODUCTION

Xylitol is a five-carbon polyol or pentitol that has been used as a food additive and sweetening agent since 1960s. It is a natural constituent of many fruits and vegetables (*1*, *2*). Although xylitol levels are usually less than 1%, it has been a natural component of the modern human's diet (*3*). The human body also produces 5−15 g of xylitol/day during normal carbohydrate metabolism in the liver. The biochemistry, biosynthesis, biotechnological production and applications of xylitol have been reviewed by Granström et al. (*4*, *5*). There have been many studies conducted on the beneficial effects of usage of xylitol in prevention of caries and acute otitis media (*6*−*10*). Xylitol (INS 967) and polyols in general are increasingly utilized as replacements for fermentable carbohydrates in a wide range of foods, where their inherent noncariogenic natures combined with a low glycemic index are beneficial in many situations (*11*). The US Food and Drug Administration (FDA) has approved the use of a "does not promote tooth decay" health claim in labeling for sugar-free foods that contain xylitol or other polyols.

Xylitol is not reactive, and hence not metabolized to reactive metabolites (*2*, *4*, *5*). Therefore, it is not known to undergo any covalent binding to proteins. However, we used an indirect approach for the preparation of monospecific polyclonal antibodies to xylitol in laboratory animals (rabbits) by utilizing the reductive amination product of D-xylose and bovine serum albumin (BSA) as the immunogen (*12*). The affinity-purified xylitol-specific antibodies have been characterized in terms of specificity and cross-reactivity (*12*).

Generally, HPLC and GC methods are available for the quantification of xylitol in natural and processed foods (*1*−*3*). Recently, a one-step chiral HPLC technique has been developed for the separation of anomers and enantiomers of some monosaccharides (*13*); such separations have not been developed for sugar alcohols. An enantioselective immunoassay using antibodies as chiral molecules which can bind the enantiomers of a chiral analyte has been developed as an emerging technique for the enantioselective analysis of analytes or drugs in foods and biological fluids (*14*). An immunoassay for erythritol in foods was recently described by the authors using erythritol-specific antibodies (*15*), and this represented the first immunoassay for

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any sugar alcohol. However, an immunoassay for xylitol has not been developed so far. In view of the generation of xylitol-specific antibodies earlier (*12*), it appeared interesting to develop an immunoassay for xylitol utilizing rabbit anti-xylitol IgG antibodies. This paper describes the development of an indirect competitive ELISA (IC-ELISA) to detect and quantitate D-xylitol in foods by making use of affinity-purified hapten-specific, anti-xylitol antibodies. Onion (*Allium cepa*) and strawberry (*Fragaria nilgerrensis*) are chosen as foods for analysis since they contain moderate (12.91 mg/100 g fresh weight of onion) to high (44.16 mg/100 g fresh weight of strawberry) amounts of xylitol (*1*, *2*).

## MATERIALS AND METHODS

**Materials.** D-Xylose, Sepharose CL-6B (6% beaded agarose, wet bead diameter: 40−165 $\mu$m), Dowex-50 W (200−400 dry mesh, 8% cross-linked), borane−pyridine complex solution ($C_5H_5N \cdot BH_3$; $BH_3$ concentration ~8 M; excess pyridine, ~20%), anthrone, acetic anhydride and pyridine were obtained from Sigma−Aldrich Chemical Co., St. Louis, MO. Maxisorp ELISA microtiter plates (flat-bottom) were a product of Nunc A/S, Roskilde, Denmark. Chromotropic acid was a product of Riedel-de Haën, Seelze, Germany. Goat anti-rabbit IgG−alkaline phosphatase conjugate was a product of Bangalore Genei, Bangalore, India. DIAFLO YM3 disk membrane was a product of Millipore Corp., Bedford, MA. Onions, strawberries, and apple juice (without preservatives; Tetrapak) were purchased from the local market. All other chemicals were of analytical grade.

**Preparation of Samples.** Onions and strawberries (edible portion) were crushed (without the addition of any buffer) separately to make the respective extracts. Apple juice was used as such. These two extracts as well as apple juice were separately subjected to ultrafiltration in an Amicon-stirred cell using DIAFLO YM3 disk membrane having a molecular weight cutoff (MWCO) of 3,000 to obtain the corresponding 3K-ultrafiltrates. The extracts and their 3K-ultrafiltrates were stored at 4 °C until analysis. Alternatively, a miniconcentrator like Centricon-3 (molecular weight cutoff = 3 kDa) having a sample capacity of 2 mL should suffice for sample preparation. Apple juice was chosen as a negative control as it is known not to contain xylitol; however, it contains high amounts of sorbitol (203 mg/100 mL) (*16*).

**Methods**

**Indirect Competitive Enzyme-Linked Immunosorbant Assay (IC-ELISA) for Detection and Quantification of D-Xylitol.** IC-ELISA (*17*, *18*) was employed to detect and quantitate D-xylitol from onion and strawberry (**Figure 1**). Flat bottom polystyrene microtiter wells were coated with high hapten density, xylitol−BSA conjugate (32 haptens/mol) at 100 ng per well in 0.1 M carbonate−bicarbonate buffer, pH 9.6 by incubating at 4 °C overnight. The wells were washed thrice between steps using PBS containing 0.05% Tween-20 (PBS-T). Blocking was done using 0.5% gelatin in PBS-T at 37 °C for 30 min. Affinity-purified anti-xylitol antibodies (4 ng in 50 $\mu$L), and different dilutions of 3K-ultrafiltrates or whole food extracts (onion or strawberry), or apple juice, (1:0 to 1:100; 50 $\mu$L) were added to the wells, and the plates were incubated at 37 °C for 1 h. Indirect competition was created between the xylitol-specific antibodies (which are supposed to bind to the xylitoyl epitopes of xylitol−BSA conjugate coated on the wells of microtiter plate) and free xylitol present in the food extracts or their 3K-ultrafiltrates. Goat anti-rabbit IgG−alkaline phosphatase (ALP) secondary antibody conjugate (1:5000 dilution in blocking buffer; 100 $\mu$L/well) was added and incubated at 37 °C for 1 h. Color development was done using *p*-nitrophenyl phosphate (1 mg/mL; 100 $\mu$L/well) in 1% diethanolamine buffer, pH 9.8, at 37 °C for 30 min. The reaction was stopped by adding 3 M NaOH (40 $\mu$L/well), and the absorbance was read at 405 nm in an ELISA microplate reader. Absorbance values obtained were compared with the calibration curve prepared for D-xylitol, and the concentration of D-xylitol in onion and strawberry were calculated. Apple juice and its 3K-ultrafiltrate were used as negative controls.

All analyses were performed in triplicate. Results of three independent experiments were analyzed by statistical analyses (mean ± SEM) using SigmaStat-3.5 software (Systat Software Inc., Chicago, IL).

A calibration curve was prepared using different known amounts of standard D-xylitol in the competition. D-Xylitol (0.1−100,000 ng) was added to microtiter plate wells along with affinity-purified anti-xylitol antibodies to follow the dose-dependent inhibition of D-xylitol in preventing xylitol-specific antibodies to bind to the xylitoyl epitopes in the microtiter plate that were coated with high hapten density xylitol−BSA conjugate (32 haptens/mol). All other steps were performed as described above under IC-ELISA.

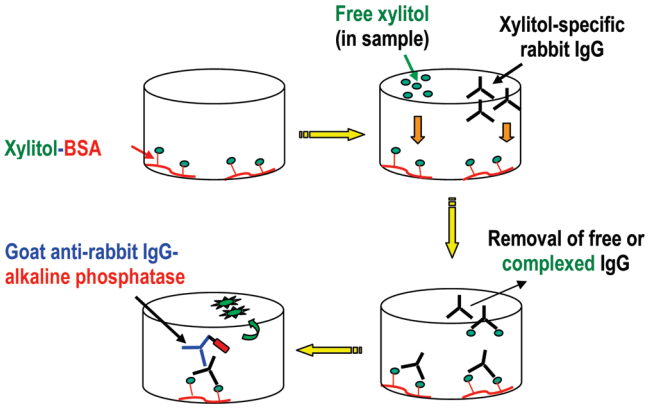


**Figure 1.** Indirect competitive ELISA for quantification of xylitol. Coating antigen: xylitol−BSA conjugate prepared by reductive amination of D-xylose with BSA. Xylitol groups on BSA compete with free xylitol in food sample for binding to xylitol-specific rabbit IgG (affinity purified) or rabbit polyclonal antiserum to xylitol−BSA. Following removal of free or complexed IgG, the bound rabbit anti-xylitol IgGs are detected using goat anti-rabbit IgG−alkaline phosphate conjugate. The magnitude of absorbance is inversely proportional to the amount of free xylitol present in food sample for a given set of coating antigen amount and xylitol-specific antibody.

From the optimized ELISA conditions, IC-ELISA in serial concentrations of xylitol standard was repeated four times at different times in order to work out the linear detection range, and evaluate the precision (intraassay variability and interassay variability) and sensitivity.

**Recovery Analysis.** Flat bottom polystyrene microtiter wells were coated with high hapten density, xylitol−BSA conjugate (32 haptens/mol) at 100 ng per well in 0.1 M carbonate−bicarbonate buffer, pH 9.6 by incubating at 4 °C overnight. The wells were washed thrice between steps using PBS containing 0.05% Tween-20 (PBS-T). Blocking was done using 0.5% gelatin in PBS-T at 37 °C for 30 min. Affinity-purified anti-xylitol antibodies (4 ng in 40 $\mu$L), onion 3K-ultrafiltrate (1:100 dilution; 50 $\mu$L, containing ~108 ng xylitol) or strawberry 3K-ultrafiltrate (1:1000; 50 $\mu$L, containing ~31 ng of xylitol) and different known amounts of standard D-xylitol (10−290 ng) were added and the plates were incubated at 37 °C for 1 h. Goat anti-rabbit IgG−ALP secondary antibody conjugate (1:5000 dilution in blocking buffer; 100 $\mu$L/well) was added and incubated at 37 °C for 1 h. Color development was done using *p*-nitrophenyl phosphate (1 mg/mL; 100 $\mu$L/well) in 1% diethanolamine buffer, pH 9.8, at 37 °C for 30 min. The reaction was stopped by adding 3 M NaOH (40 $\mu$L/well) and the absorbance was read at 405 nm in a microplate reader.

**Quantification of D-Xylitol from Onion by Immunoaffinity Chromatography and GC.** (*a*) *Preparation of Rabbit Anti-xylitol IgG−Sepharose CL-6B Matrix*. Immunization of rabbits with xylitol−BSA conjugate (32 haptens/mol) and purification of xylitol-specific antibodies from immune serum by hapten affinity chromatography were performed as described earlier (*12*). The affinity-purified xylitol-specific antibodies were used within 10 days of purification. The antibodies were immobilized onto Sepharose CL-6B as described by Stults et al., (*19*). Briefly, Sepharose CL-6B was activated with 25 mM sodium periodate ($NaIO_4$) at 25 °C for 30 min; unreacted $NaIO_4$ was inactivated by the addition of an equimolar amount of ethylene glycol. Next, coupling of activated gel beads (2 g of Sepharose CL-6B at a concentration of 0.2 g/moist gel) with affinity-purified rabbit anti-xylitol antibodies (~1.5 mg) was carried out by the direct addition of borane−pyridine complex (25 mM final concentration) essentially as described for the preparation of D-xylitol−keyhole limpet hemocyanin (D-xylitol−KLH)−Sepharose CL-6B affinity matrix (*12*).

(*b*) *Immunoaffinity Purification of D-Xylitol from Onion*. This was carried out at 4 °C. Five milliliters of 1:10 diluted onion 3K-ultrafiltrate

were passed through the anti-xylitol IgG−Sepharose CL-6B column (0.8 cm i.d. × 4 cm; 2 mL bed volume), pre-equilibrated with PBS. The flow-through was recycled thrice through the column. After washing the column with 50 mL of PBS, the bound component was eluted in 1 mL fractions using 0.1 M glycine-HCl buffer, pH 2.9. The eluate was neutralized immediately using 0.5 M Tris base.

The reconstituted, lyophilized eluate (pooled fractions 1−5) from the immunoaffinity column and standard D-xylitol (10 mg) were subjected to gas−liquid chromatography (*20*). They were taken separately in 0.5 mL of deionized water for acetylation. Dry and distilled acetic anhydride and pyridine (0.5 mL each) were added and kept in a boiling water bath for 2 h after tightly stoppering the tubes. Excess reagents were removed by codistilling with water thrice (1 mL) and toluene thrice (1 mL). After thorough drying, contents were dissolved in chloroform, filtered through glass wool, and dried by passing a stream of nitrogen gas over them. The residues were dissolved in chloroform for analysis.

**Detection of Sugar Alcohol by Polyol Assay.** The fractions from the anti-xylitol IgG−Sepharose CL-6B column (immunoaffinity column) were analyzed by polyol assay (*21*), which involves periodate oxidation followed by estimation of the formaldehyde formed using chromotropic acid. Sugar alcohols produce a characteristic pink color. Other than sugar alcohols, fructose is the only sugar that is somewhat reactive in this assay (*21*). Each fraction (50 μL) was diluted to 1 mL with water, and to these were added 0.5 mL of 10 mM sodium metaperiodate in 1 N sulfuric acid. After mixing, the solutions were allowed to stand for at least 10 min at 25 °C. Next, 0.2 mL of 10% (w/v) aqueous chromotropic acid solution was added. Finally, 3 mL of concentrated sulfuric acid was added from a buret. The solutions were mixed well by vortexing, and the tubes were placed in a boiling water bath for 30 min. The tubes were cooled, and the absorbance was read at 570 nm.

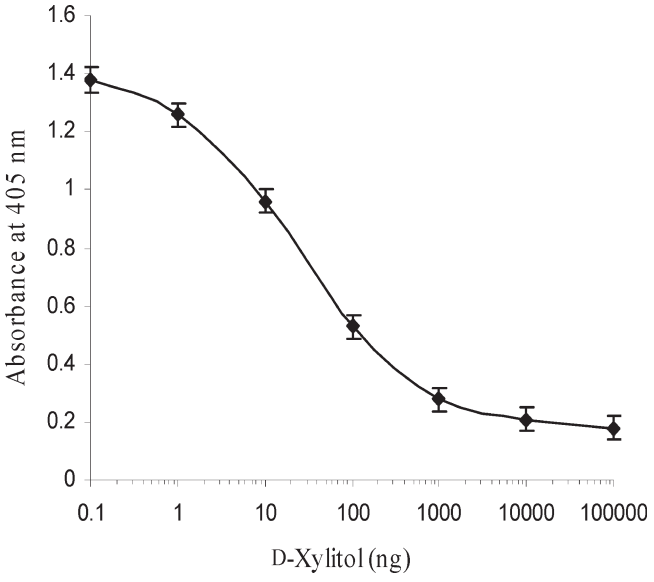


**Figure 2.** Coating antigen: xylitol−BSA conjugate (32 haptens/mol, 100 ng). Primary antibody: affinity-purified anti-xylitol IgG (4 ng). Competitor: standard D-xylitol at different concentrations (0.1−100,000 ng). Secondary antibody: goat anti-rabbit IgG−ALP conjugate (1:4000 dilution).

**Quantification of Xylitol in Onion by Ion-Moderated Cation-Exchange Chromatography.** This was carried out as described for determination of polyols in chewing gums and confections (*22*), using a cation exchange resin ($H^+$ form) after converting it to $Ca^{2+}$ form as outlined earlier in the analysis of sugars and sugar alcohols in mushroom (*23*). Onion 3K-ultrafiltrate (200 μL), reconstituted/lyophilized eluate (pooled fractions 1−5) from the immunoaffinity column (300 μL), and standard D-xylitol (4 mg/mL, 250 μL) were individually subjected to ion-moderated cation-exchange chromatography on a calcium form ($Ca^{2+}$) of Dowex-50 W column (0.5 cm i.d. × 46 cm; 9 mL bed volume) pre-equilibrated with water, and run at 25 °C at a flow rate of 7.2 mL/h. Fractions were subjected to polyol assay as described above.

**Fructose Assay.** Fructose was detected in the onion 3K-ultrafiltrate using cold anthrone reagent (*24*). This reagent was prepared fresh by dissolving 150 mg of anthrone in 100 mL of 71.7% sulfuric acid. Each fraction (50 μL) from the Dowex-50 W column (sample: onion 3K-ultrafiltrate) was mixed with 1.5 mL of cold anthrone reagent, and incubated at 25 °C for 1 to 1.5 h. The absorbance was measured at 620 nm.

## RESULTS

**Indirect Competitive Enzyme-Linked Immunosorbant Assay (IC-ELISA) for Quantification of D-Xylitol.** Standard D-xylitol (A. R. grade) was used as a competitor to the xylitol epitopes of xylitol−BSA conjugate that was coated on microtiter plate wells. These two compete for the antigen-binding sites of anti-xylitol antibodies. The calibration curve for D-xylitol is shown in **Figure 2**.

IC-ELISA was employed to detect and quantitate D-xylitol in two foods, viz., onion and strawberry. The absorbance values obtained using onion and strawberry extracts, and their 3K-ultrafiltrates as the source of D-xylitol in the competition assay were compared with the calibration curve for D-xylitol to obtain xylitol amount. The concentrations of D-xylitol in onion and strawberry were calculated, and the results are presented in **Tables 1** and **2**, respectively.

**Sensitivity and Precision.** Under the optimized conditions, the IC-ELISA procedure was conducted in quadruplicate with a set of standard concentration of xylitol at different times (within 3 days). In this optimized IC-ELISA, the linear range for xylitol quantitation was found to be 5−400 ng, and the limit of detection was 1 ng (**Figure 2**). The variability of intra-assay and interassay of the ELISA curve for xylitol was used to show the precision of this protocol. The intra-assay variability was given by the average of 4 replicated wells in one microplate. The interassay variability was given by the average of 4 replicated microplates at different times. The intra-assay average variation coefficient was 3.2%, and the interassay average variation coefficient was 6.1%, which showed that it was feasible to determine xylitol using the IC-ELISA.

**Recovery Analysis.** Recovery analysis was performed with known amounts of added D-xylitol (92, 192, or 292 ng to a sample of onion 3K-ultrafiltrate and 10, 20, or 30 ng to a sample of strawberry 3K-ultrafiltrate), and the recoveries were calculated. The results are presented in **Tables 3** and **4**. It is observed from the results that the recovery ranged from 89.2 to 94.9% in the case of

**Table 1.** Concentrations of D-Xylitol in Onion, Calculated from the Calibration Curve for D-Xylitol by Indirect Immunoassay[a]

| onion 3K-ultrafiltrate (50 μL) | A405 (nm) | amt of xylitol (ng ± SEM) | concn of xylitol in onion 3K-ultrafiltrate (μg/mL ± SEM) |
|---|---|---|---|
| 1:0 | 0.220 | 9012.8 ± 17.50 | 180.24[b] |
| 1:25 | 0.346 | 551.8 ± 3.28 | 275.90 ± 1.62 |
| 1:50 | 0.422 | 260.4 ± 1.98 | 260.40 ± 1.97 |
| 1:100 | 0.480 | 109.2 ± 2.96 | 218.40 ± 5.91 |
| | | mean: | 251.56 ± 3.18 |

[a] Amount of anti-xylitol Ab used in the immunoassay = 4 ng. [b] Not included for calculation of the mean as the amount of xylitol in column 3 falls outside the linear range.

**Table 2.** Concentrations of D-Xylitol in Strawberry, Calculated from the Calibration Curve for D-Xylitol in Indirect Competitive Immunoassay[a]

| strawberry 3K-ultrafiltrate (50 $\mu$L) | A405 nm | amt of xylitol (ng ± SEM) | concn of xylitol in strawberry 3K-ultrafiltrate ($\mu$g/mL ± SEM) |
|---|---|---|---|
| 1:250 | 0.46 | 113.9 ± 1.94 | 569.5 ± 09.7 |
| 1:500 | 0.65 | 61.8 ± 1.62 | 618.0 ± 16.2 |
| 1:750 | 0.76 | 41.5 ± 0.98 | 622.5 ± 14.7 |
| 1:1000 | 0.84 | 31.7 ± 1.44 | 634.0 ± 28.8 |
| | | mean: | 611.0 ± 17.4 |

[a] Amount of anti-xylitol Ab used in the immunoassay = 4 ng.

**Table 3.** Recovery Analysis for Quantitation of D-Xylitol in Onion 3K-Ultrafiltrate by Indirect Competitive ELISA

| onion 3K-ultrafiltrate (1:100; 50 $\mu$L) (ng) | D-xylitol added (ng) | expected value (ng) | obtained value (ng ± SEM) | % recovery (± SEM) |
|---|---|---|---|---|
| 108 | 92 | 200 | 178.4 ± 1.15 | 89.20 ± 0.57 |
| 108 | 192 | 300 | 280.4 ± 3.31 | 93.46 ± 1.10 |
| 108 | 292 | 400 | 379.4 ± 2.51 | 94.85 ± 0.62 |
| | | | mean: | 92.50 ± 0.76 |

**Table 4.** Recovery Analysis for Quantitation of D-Xylitol in Strawberry 3K-Ultrafiltrate by Indirect Competitive ELISA

| strawberry 3K-ultrafiltrate (1:1000; 50 $\mu$L) (ng) | D-xylitol added (ng) | expected value (ng) | obtained value (ng ± SEM) | % recovery (± SEM) |
|---|---|---|---|---|
| 31 | 10 | 41 | 37.1 ± 2.04 | 90.48 ± 4.97 |
| 31 | 20 | 51 | 48.9 ± 1.77 | 95.88 ± 3.47 |
| 31 | 30 | 61 | 53.9 ± 1.58 | 88.36 ± 2.59 |
| | | | mean: | 91.57 ± 3.67 |

onion 3K-ultrafiltrate, and 88.4 to 95.9% in the case of strawberry 3K-ultrafiltrate. The mean recoveries were found to be 92.5% in the case of onion 3K-ultrafiltrate and 91.6% in the case of strawberry 3K-ultrafiltrate.

**Purification of D-Xylitol from Onion 3K-Ultrafiltrate by Immunoaffinity Chromatography.** The immunoaffinity column was utilized to purify, specifically, D-xylitol from onion 3K-ultrafiltrate. The eluate obtained from the immunoaffinity column was subjected to chromotropic acid assay for the presence of polyol. It is seen that only the first 5 fractions showed a positive response in the polyol assay (**Figure 3**). Since the bed volume of the column is only 2 mL, the first fraction having a volume of 1 mL is expected to contain the eluted hapten (xylitol); in fact, this was what was observed.

**Ion-Moderated Cation-Exchange Chromatography.** The polyol assay-positive eluate (fractions 1−5) from the immunoaffinity chromatography was pooled, lyophilized and subjected to $Ca^{2+}$-moderated cation exchange chromatography on Dowex-50 W column. The Dowex-50 W elution profile as analyzed by polyol assay appears as a small peak, and is shown in **Figure 4** (panel **C**). Standard D-xylitol and onion-3K ultrafiltrate were also independently subjected to cation exchange chromatography under identical conditions. The results obtained as followed by the polyol assay are shown in **Figure 4** [panels **A** and **B** (□, A570 nm)]. It is seen from the chromatographic profiles that the lyophilized eluate from the immunoaffinity column showed a peak in the polyol assay (**Figure 4**, panel **C**) at exactly the same position as that of standard D-xylitol run separately under identical conditions.

However, in the case of onion 3K-ultrafiltrate, an identical peak (though larger) appears [**Figure 4**, panel **B** (□, A570 nm)]. This may contain other sugars/polyols besides xylitol. Fructose somewhat interferes in the polyol assay; hence fructose assay has been performed to check for the presence of fructose in the Dowex-50 W fractions. The result of fructose assay is shown in **Figure 4** (panel **B**, ◆, A620 nm), which shows an overlapping peak around the elution position of D-xylitol. The fructose present in

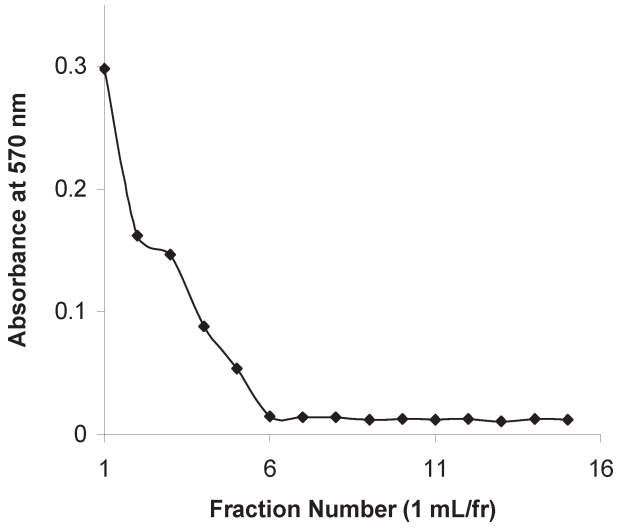


**Figure 3.** Column: anti-xylitol IgG−Sepharose CL-6B immunoaffinity (0.8 × 4 cm; bed volume 2 mL). Sample: 5 mL of onion 3K-ultrafiltrate (1:10 dilution). Eluant: 0.1 M glycine-HCl buffer, pH 2.9. Absorbance at 570 nm indicates the presence of polyol in the eluate by chromotropic acid assay.

onion might have contributed to the larger peak in the polyol assay for xylitol.

The lyophilized eluate (initial 5 fractions from immunoaffinity column) was analyzed by GC to confirm the specificity of the immunoaffinity column to bind, specifically, to D-xylitol. The results of GC analysis are shown in **Figure 5**. GC analysis clearly shows a single peak in the eluate from immunoaffinity column with retention time of 17.68 min (**Figure 5**, panel **B**) as compared to that of standard D-xylitol (retention time = 17.70 min) (**Figure 5**, panel **A**).

**Comparison of the Results Obtained for Quantification of D-Xylitol in Onion.** The theoretical amount of D-xylitol that can bind

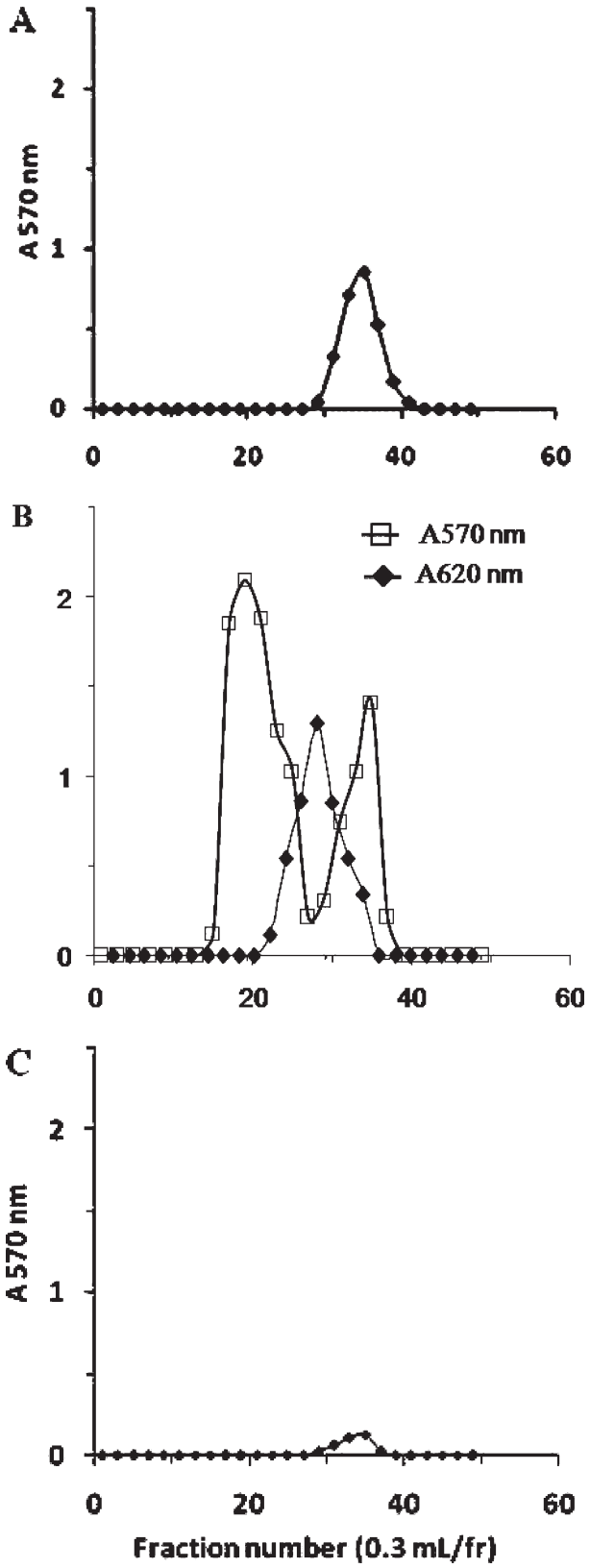


**Figure 4.** Ion-moderated Dowex-50 W (0.5 × 46 cm; 9 mL bed volume) cation-exchange chromatography of (**A**) standard D-xylitol [sample load: 250 $\mu$L of 4 mg/mL xylitol (1 mg)]; (**B**) onion 3K-ultrafiltrate [sample load: 200 $\mu$L; polyol detection (□, A570 nm); fructose detection by cold anthrone assay (◆, A620 nm)] and (**C**) the eluate (fractions 1−5 pooled and lyophilized) obtained from anti-xylitol IgG−Sepharose CL-6B immunoaffinity chromatography shown in **Figure 3** [sample load: 300 $\mu$L]. In panels **A** and **C**, A570 nm represents polyol detection by chromotropic acid assay. Eluant: distilled water.

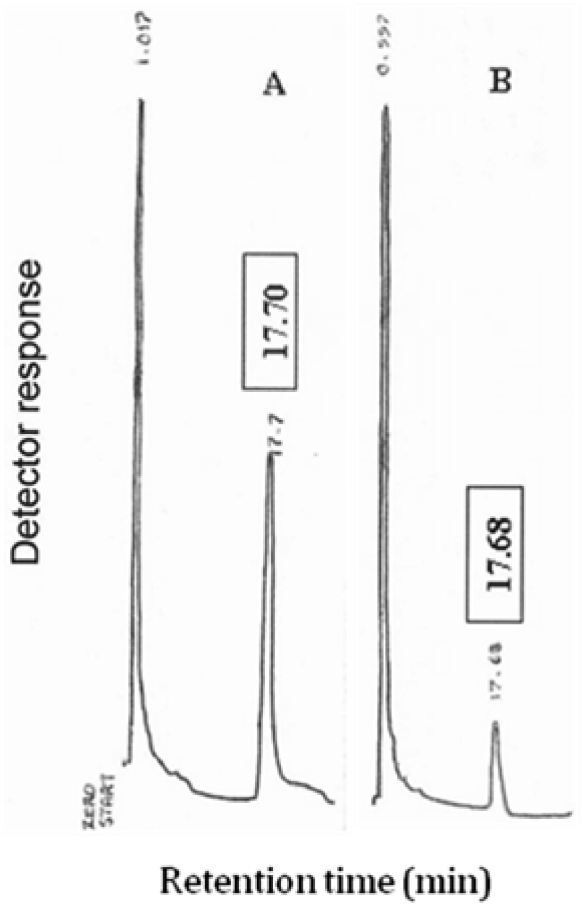


**Figure 5.** Gas−liquid chromatography (GC) analysis of (**A**) standard D-xylitol, 10 mg/mL; 1500 dilution; 1 $\mu$L sample volume (6.7 ng); (**B**) lyophilized eluate (1: 2600 dilution) from the immunoaffinity chromatography (anti-xylitol IgG−Sepharose CL-6B) of onion 3K-ultrafiltrate; 1 $\mu$L sample volume. Retention times (min) are shown in boxes. GC conditions: Shimadzu GC-15A. Column: OV 225 (1/8 in. × 8 ft; 50% cyanopropylphenyl methylpolysiloxane). Mesh range: 80/100. Injector block temp: 200 °C. Column temperature: 180 °C. Detector temperature: 200 °C. Detector: flame ionization detector (FID). Carrier gas: nitrogen (40 mL/min).

to the immunoaffinity column has been calculated based on the xylitol content in onion as 89 mg/100 g dry weight (*1*) and moisture content of onion as 85% (*25*). This translates to a xylitol content of 12.91 mg/100 g fresh weight onion. In our experiments described here, 100 g of raw onion yields 50 mL of onion extract (contains 12.91 mg of D-xylitol); therefore, 1 mL of onion extract (~equivalent to 2 g of onion) contains 258 $\mu$g of D-xylitol. Anti-xylitol antibodies (1.5 mg, 9.375 nmol) can bind 18.75 nmol of D-xylitol assuming that both the antigen-binding sites bind to D-xylitol which indicates that 1.5 mg of immobilized antibodies can theoretically bind to 2.85 $\mu$g of D-xylitol.

(*a*) *Immunoaffinity Chromatography Method in Conjunction with GC*. Results from the immunoaffinity column indicate that only 1.74 $\mu$g of D-xylitol was eluted from the immunoaffinity column and its capacity was found to be only 68% of the calculated capacity. The observed capacity may be due to some antibody molecules being covalently linked to Sepharose CL-6B, at the antigen-binding sites and hence nonavailability of antigen-binding sites for binding free xylitol. One milliliter of onion extract contains 258 $\mu$g of xylitol; 5 mL of this 1:10 diluted onion extract, therefore, contains 128 $\mu$g of xylitol.

GC analysis of the eluate from the immunoaffinity column showed a single peak at 17.68 min, which is in close agreement

with the retention time of standard D-xylitol (17.70 min) analyzed under identical conditions. Based on GC quantitation, it was found that ~1.74 μg of D-xylitol was bound to the immunoaffinity column.

(*b*) *Indirect Competitive Immunoassay*. The amount of D-xylitol calculated from indirect competitive immunoassay was 12.6 mg/100 g fresh weight onion, which appears to be close to the expected value (12.91 mg/100 g fresh weight onion) as reported by other investigators (*1*, *2*) by analytical techniques like HPLC and GC.

## DISCUSSION

The present article describes the development of an indirect competitive ELISA for the quantification of xylitol in natural foods using xylitol-specific antibodies. Calibration curve obtained for the indirect competitive immunoassay showed a linear range between 5 and 400 ng of standard D-xylitol, which is used as a source of competing analyte. The results of indirect competitive immunoassay showed xylitol concentrations of ~251.6 μg/mL for onion (**Table 1**), and 611 μg/mL for strawberry (**Table 2**). The amount of D-xylitol was calculated and found to be 12.6 mg/100 g fresh weight of onion and 44 mg/100 g fresh weight of strawberry by taking into account the volume of the extracts obtained from 100 g fresh weight of the foods (50 mL/100 g onion and 72 mL/100 g strawberry fresh weights, edible portions). The results obtained from the immunoassay are in close agreement with the values reported previously using HPLC: 12.91 mg/100 g fresh weight of onion and 44.16 mg/100 g fresh weight of strawberry (*1*, *2*). The advantage of the IC-ELISA described here is that the sugar alcohols need not be derivatized for detection compared to GC, GC−MS (*20*, *26*) or HPLC methods (benzoylation/nitrobenzoylation) described previously (*27*−*29*).

However, the absorbance values in the immunoassay did not show any change from the reagent blank values when different dilutions of the whole extracts of onion and strawberry were used. This certainly appears to be due to the contribution of matrix effect of the whole extracts since they contain many other biomolecules/components such as proteins of varying molecular weights, carbohydrate polymers, fatty acids and their esters, and nucleic acids, etc. Apple juice or its 3K-ultrafiltrate [known to contain only sorbitol at a concentration of 203 mg/100 mL (*16*)] used in the immunoassay as a competing analyte did not show any change in absorbance in the IC-ELISA compared to buffer control indicating that apple does not contain D-xylitol.

The recovery analysis was performed to check the accuracy of the indirect competitive immunoassay for D-xylitol. The results of the recovery analysis showed that added amounts of D-xylitol to 3K-ultrafiltrate of onion or strawberry were recovered fairly accurately with recoveries in the range of 89.2 to 94.9% in the case of onion, and 88.4 to 95.9% in the case of strawberry. Although spiking experiments using whole extracts have not been carried out, we are of the view that such experiments would also have given good recovery since the initial ultrafiltration step for sample preparation provides 3K-ultrafiltrates not containing biomolecules above 3 kDa.

Purification of D-xylitol from onion 3K-ultrafiltrate by anti-xylitol IgG−Sepharose CL-6B (immunoaffinity) chromatography showed that only the first 5 fractions contained polyol as determined by chromotropic acid assay. Since the bed volume of the column was only 2 mL, the bound material eluted in the first 4−5 fractions. However, at this point it was not clear whether the eluted component from the immunoaffinity column was only D-xylitol or D-xylitol along with other sugars or sugar alcohols. The lyophilized polyol assay-positive fractions of the eluate from the immunoaffinity column were subjected to $Ca^{2+}$ ion-moderated cation exchange chromatography on Dowex-50 W column and eluted with distilled water. Onion 3K-ultrafiltrate and standard D-xylitol were subjected to Dowex-50 W chromatography in separate runs under identical conditions. Polyol assay of the Dowex-50 W fractions (from immunoaffinity column eluate of onion 3K-ultrafiltrate) showed a peak at exactly the same elution position as that of standard D-xylitol, indicating that the bound component in the immunoaffinity column represents D-xylitol. A similar peak was also seen in the Dowex-50 W chromatography of onion 3K-ultrafiltrate. The characteristic peak obtained for D-xylitol in all three runs on Dowex column (**Figure 4**) indicates that the purified component from the immunoaffinity column is D-xylitol. However, the fructose analysis (by cold anthrone assay) of the Dowex-50 W fractions revealed the presence of fructose in onion 3K-ultrafiltrate which contributes significantly to the increased absorbance in the polyol assay. Onion also contains small amounts of mannitol, and moderate amounts of sucrose and other sugars [glucose, 1.97 g; fructose, 1.29 g; and sucrose, 0.99 g (all expressed per 100 g fresh weight of edible portion (*30*))]; fructose is known to contribute to the absorbance in the polyol assay (*21*). In this study, a significant peak (prior to the xylitol peak) is observed in the Dowex-50 W elution profile of onion 3K-ultrafiltrate (**Figure 4**, panel **B**), strongly indicating the presence of fructose. The moderate resolution of various sugars and sugar alcohols on Dowex-50 W thus appears to be not suitable for accurate determination.

The presence of D-xylitol in the eluate of the immunoaffinity column was further confirmed and quantitated by an analytical method, viz., GC. The result of the GC analysis showed a peak at exactly the same retention time (17.68 min) as that of standard D-xylitol (17.70 min) run under identical conditions, indicating that the immunoaffinity column specifically binds only D-xylitol. This is in agreement with our earlier observations that the anti-xylitol antibodies have low cross-reactivity with other structural analogues (D-sorbitol, 3.7%; *meso*-erythritol and L-arabinitol, 3.8%; D-xylose, 3.9%; D-mannitol, 4.4%) as determined by inhibition ELISA (*12*). Similar magnitudes of low cross-reactivity toward other sugar alcohols are also true of other antibodies specific to some sugar alcohols generated earlier, viz., *meso*-erythritol and D-mannitol (*31*, *32*). It may be recalled here that D-mannitol-specific antibodies were found to exhibit $<1\%$ cross-reactivity toward L-mannitol (*32*), indicating the potential utility of hapten-specific antibodies in enantioselective immunoassays.

Quantitation of D-xylitol from the immunoaffinity column using GC peak area showed that only 1.74 μg of xylitol binds to the immunoaffinity column when compared to the theoretically calculated maximum column capacity of 2.85 μg for xylitol. This may be due to the random coupling of affinity-purified anti-xylitol antibodies to the activated Sepharose CL-6B in which a proportion of the antibody molecules are linked by the ε-amino groups of lysine present in the antigen-binding sites, and hence unavailable for hapten binding. It appears that immunoaffinity chromatography, though operating well as per the principles of affinity chromatography, is practically not feasible for routine analysis of food samples due to its limited capacity and labor-intensive approach.

In conclusion, IC-ELISA developed by making use of specific antibodies to xylitol should serve as a sensitive analytical tool to detect and quantitate nanogram amounts of D-xylitol in various biological samples, and natural/processed foods. Since the linear range of the IC-ELISA for xylitol quantitation is 5−400 ng, this method is also useful for xylitol analysis in low-abundance foods. Here, we have essentially shown that the quantification of

xylitol in foods by the IC-ELISA method works well for quantifying xylitol in comparison to the reported results in the literature, and the results are in very good agreement. It is our view that if the IC-ELISA for xylitol in foods were to be developed as an official method, then this method certainly has to be validated by two other methods so that the IC-ELISA for xylitol may be an alternative technique to HPLC or GC or enzymatic methods for routine use in an analytical or food laboratory. The immunoassay may also find utility in the detection of xylitoyl moieties in various modified proteins in health and disease as well as in processed foods or during their preparation. The IC-ELISA developed here for xylitol quantitation may be suitably adapted for routine food analysis using polyclonal antiserum *in lieu* of affinity-purified xylitol-specific antibodies. To our knowledge, this is the first report of an immunoassay for the sugar alcohol, xylitol.

## ABBREVIATIONS USED

ALP, alkaline phosphatase; BSA, bovine serum albumin; IC-ELISA, indirect competitive enzyme-linked immunosorbent assay; INS, international numbering system (food additives); MWCO, molecular weight cutoff; PBS, phosphate-buffered saline; 3K, 3 kDa molecular weight cutoff.

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