Short communication: Determination of inulin in milk using high-performance liquid chromatography with evaporative light scattering detection

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ABSTRACT

The objective of this study was to develop an HPLC method for the determination of inulin in a dairy matrix. Inulin is often added to dairy products to act as a source of dietary fiber as well as to provide technological functionality (e.g., water-holding or fat-replacing functions). The method includes hydrolysis of inulin with inulinase enzyme and determination of released fructose and glucose by HPLC coupled with evaporative light scattering detection, using water as the mobile phase. The effect of the milk background was investigated, preparing standards in milk, whey, or permeate and subjecting them to inulinase treatment. The developed method showed satisfactory linearity ($R^2 = 0.98$ for glucose and 0.99 for fructose), good repeatability (relative standard deviation, RSD, ranging from 2.2 to 7.3% for glucose and from 0.6 to 2.3% for fructose), satisfactory reproducibility (RSD from 8.1 to 12.9% for glucose and from 3.1 to 4.9% for fructose), and good recovery (98.4 to 103.6%).

Key words: inulin, evaporative light scattering detection, milk

Short Communication

Inulin is defined as a polydisperse carbohydrate, mostly composed of β (2 $\rightarrow$ 1) fructosyl-fructose links and one α (1 $\rightarrow$ 2) glucose moiety linked at the end of each fructose chain. The degree of polymerization of inulin ranges from 2 to 60, depending on the source and the processing history (Niness, 1999). Detailed information about chemistry, functionality, and health benefits of inulin can be found elsewhere (see, for example, Flamm et al., 2001; Kalyani Nair et al., 2010).

The incorporation of inulin in dairy products has shown an increase in the past few years, mostly because of the data related to the health benefits associated with its consumption. Inulin is water soluble, has texture-enhancing properties, and is often used to

fortify dairy products with fiber as well as to replace fat and provide creamy mouthfeel in low-fat dairy products such as milk drinks, yogurts, dips, cream cheese, and process cheese (Franck, 2002). The determination of inulin in these products remains a challenge.

Few methods are available in the literature on quantification of inulin-type fructans. These methods are based on the quantification of fructose and glucose released after the enzymatic hydrolysis of inulin. High-performance anion exchange chromatography with pulsed amperometric or refractive index detection (RID; Quemener et al., 1994; Hoebregs, 1997; Vendrell-Pascuas et al., 2000) and spectrophotometry (McCleary et al., 2000) are usually used.

The objective of this work was to develop an alternative method of inulin quantification in a dairy matrix. Compared with the HPLC methods currently available (see for example, La Pera et al., 2007), this method does not require any organic solvents because HPLC-grade water is used as the mobile phase and detection is achieved using evaporative light scattering detector (ELSD). Evaporative light scattering detection has been used recently in quantification of low molecular weight carbohydrates in various matrices such as wine (La Pera et al., 2007), beer (Nogueira et al., 2005), and other beverages (Wei and Ding, 2000). It has shown higher sensitivity compared with RID, the most commonly used detector in quantification of sugars with HPLC (Megoulas and Koupparis, 2005).

Reagents (fructose, glucose, acetic acid, sodium acetate, TCA, and HPLC-grade water) were purchased from Fisher Scientific (Mississauga, ON, Canada). Inulin samples used were Frutafit CLR (Sensus America LLC, Monmouth Junction, NJ) with a degree of polymerization (DP) of 7 to 8; Oligo-Fiber XL with a DP of about 20 (Lomas Limited, Brampton, ON, Canada), and Beneo HPX with DP ≥ 23 (Orafti SA, Tienen, Belgium).

Aliquots of inulin were dissolved in raw skim milk (Gay Lea Foods Co-op Ltd., Guelph, ON, Canada) containing sodium azide (0.02% wt/vol) as a bacteriostatic. The milk mixtures were homogenized by using a tissue homogenizer (PowerGen 125, Fisher Scientific, Pittsburgh, PA) at 30,000 rpm for 5 min. Milk coagu-

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lation was induced using chymosin (Chy-Max Ultra, Chr. Hansen Inc., Milwaukee, WI) at a concentration of 0.017 international milk clotting units (IMCU)/mL, and the samples were incubated at 30°C for 3 h. The milk coagulum was then cut, the content was transferred into a mesh, and the whey was collected for 3 h. The inulin solubilized in the whey was quantified.

Aliquots (2 mL) of whey solutions were mixed with 5 mL of acetate buffer (0.1 M, pH 4.5). Then, 100 μ L of inulinase from *Aspergillus niger* (EC 3.2.1.7) with enzymatic activity of 1,740 inulinase units (INU)/g (Sigma-Aldrich Inc., St. Louis, MO) was added into the mixture and incubated at 60°C overnight. The mixture was transferred quantitatively to a 10-mL volumetric flask, 1.5 mL of 20% TCA was added, and the volume was made up with HPLC water. After mixing well, a small amount of the treated whey was centrifuged at $13,224 \times g$ for 8 min and the supernatant was filtered through 0.22- μ m syringe filter (Millex-GV, Millipore, Billerica, MA) before injection into the column.

The HPLC system consisted of Waters 600E pump system, Waters 700 Satellite WISP autosampler (Waters, Milford, MA) equipped with a 20- μ L loop and a SEDEX75 ELSD (Sedere, Alfortville, France). Separation was achieved with ion exclusion Rezex RCM-Monosaccharide column (300 $\times$ 7.8 mm, 8- μ m particle size) and a SecurityGuard Carbo-Ca²⁺ guard column (4 $\times$ 30 mm) from Phenomenex (Torrance, CA), both maintained at 80°C during runs. The mobile phase was HPLC-grade water at 0.5 mL/min. The pressure of the carrier gas (compressed nitrogen, Linde Canada Ltd., Guelph, ON, Canada) in ELSD was 200 kPa, drift tube temperature was 40°C, and detector gain was 8. Calibration was performed using external standards. A mixture of glucose and fructose was prepared at 5 concentrations ranging from 0.2 to 2 mg/mL for glucose and from 0.5 to 5 mg/mL for fructose by dissolving the sugars in different matrices; namely, water, milk whey, and milk permeate. Milk whey was prepared by centrifugation of skim milk at $25,000 \times g$ for 1 h at 20°C in a Beckman Coulter Optima LE-80 K ultracentrifuge with rotor type 45 Ti (Beckman Coulter Canada Inc., Mississauga, ON, Canada), and permeate was collected from ultrafiltration of pasteurized skim milk using a pilot-plant UF unit equipped with a 30-kDa membrane (Smart Flow Technologies Inc., Apex, NC). Inulin content was obtained by

$$I = k(G + F), \text{ with } k = [180 + 162(n - 1)]/180 n,$$

where n = average DP, and I , G , and F are inulin, glucose, and fructose contents, respectively.

The whey released from rennet curd was used as a model for the quantification of inulin released from cheese-type matrices. It is assumed that during the making of a rennet-type cheese, part of the inulin added to the original milk is solubilized in the whey. The amount of inulin lost in the whey was reported as percentage of the amount of inulin that was added in milk. The extent of the inulin loss in the whey may depend on the DP of the fructans. Figure 1 shows representative chromatograms of a standard mixture that contained 0.5 and 1.2 mg/mL glucose and fructose, respectively. The chromatograms of the standard mixture prepared in water and either not submitted to treatment with inulinase (untreated) or treated with inulinase (treated) are shown in Figure 1a. Glucose eluted at about 14.6 min and fructose at about 18 min. Subjecting standards to the same treatment as the samples accounts for all changes that may happen to sugars due to treatment. Indeed, treatment with inulinase may introduce variations in the chromatograms, as shown in the large peak (eluting between 9 and 12 min), which may be attributed to the presence of the other compounds used in the sample preparation; namely, the inulinase enzyme, sodium acetate salts, or TCA. This can be clearly seen when comparing the untreated and treated standards prepared in water (Figure 1a). Furthermore, components present in the whey fraction of milk can also be affected with the addition of inulinase. Figure 1b shows the chromatogram of the same standard mixture (glucose and fructose) solubilized in whey. The large peak eluting at about 13 min, observed in solutions both untreated and treated with inulinase, was attributed to lactose and confirmed by running an aqueous lactose solution standard. When the standard mixture in whey was subjected to the same treatment steps as inulin samples (i.e., the addition of acetate buffer, inulinase treatment, and TCA precipitation), the lactose eluted in a narrower peak, and the glucose peak was larger than that of the untreated standard mixture. In addition, galactose eluted at about 16 min, as confirmed with standard galactose solutions. These changes indicated a partial hydrolysis of lactose into glucose and galactose during inulinase treatment, which resulted in a higher than expected glucose peak. This implies that the amount of glucose will be overestimated in samples of inulin in milk whey if the lactose breakdown with inulinase is not taken into account. A similar effect of lactose from the matrix was observed in treated and untreated standards prepared in permeate or milk (data not shown).

To correct for the amount of glucose released from lactose hydrolysis in dairy matrices, the calibration

Figure 1. Chromatograms of standard mixtures of glucose and fructose prepared in a) water and b) milk whey and either subjected to inulinase treatment (treated) or not (untreated).

Figure 2. Chromatograms of standards with various levels of sugars (S1 to S5) prepared in milk whey and subjected to inulinase treatment. The amount of sugars per injection in the standards S1, S2, S3, S4, and S5 was 4, 12, 20, 32, and 40 μg of glucose and 10, 30, 50, 80, and 100 μg of fructose, respectively.

curves were based on standard mixtures prepared in whey and treated with inulinase in parallel with the samples. Figure 2 shows representative chromatograms of standard mixtures containing glucose from 0.2 to 2 mg/mL and fructose from 0.5 to 5 mg/mL. The standard mixtures were dissolved in whey and treated with inulinase. Both glucose and fructose peak areas increased proportionally to the amount of sugar added, whereas the peak area of galactose was the same in all cases, confirming a similar extent of hydrolysis for the

lactose contained in the whey. The calibration curves were obtained by plotting the peak areas as a function of amount (μg) of sugar injected. The calibration curves were linear in the range from 4 to 40 and from 10 to 100 μg of sugar injected for glucose and fructose, respectively. Satisfactory coefficients of determination of 0.98 for glucose and 0.99 for fructose in all cases (standards prepared in water, whey, or permeate and submitted or not to inulinase treatment), and for the concentration ranges studied, were obtained. Further-

Table 1. Recovery and repeatability of the method

Sample	Recovery ¹ (%)	Repeatability ² RSD (%)		Reproducibility ³ RSD (%)	
		Glucose	Fructose	Glucose	Fructose
Water	98.4	2.2	2.3	8.1	4.9
Whey	98.4	3.3	0.6	12.9	3.1
Permeate	103.6	7.3	1.7	ND ⁴	ND

¹Calculated as (amount found/amount added) $\times$ 100; Frutafit CLR inulin 3% (wt/vol; Sensus America LLC, Monmouth Junction, NJ) solution in water, milk whey, or permeate was used as model solution.

²Repeatability is expressed as relative standard deviation (RSD) of the amount of each sugar obtained from 4 runs of Frutafit CLR inulin 3% (wt/vol) solution in water, milk whey, or permeate.

³Reproducibility is expressed as RSD of the amount of each sugar obtained from 8 runs of Frutafit CLR inulin 1% (wt/vol) solution in water or milk whey over 2 wk.

⁴Not determined.

Figure 3. Chromatograms of whey released from renneted milk curd containing inulin of 3 different degrees of polymerization (DP): CLR (Frutafit CLR, Sensus America LLC, Monmouth Junction, NJ) with DP 7 to 8, XL (Oliggo-Fiber XL, Lomas Limited, Brampton, ON, Canada) with DP ≥20, and HPX (Beneo HPX, Orafit SA, Tienen, Belgium) with DP ≥23. The concentration of inulin in the original milk mixture was 3% (wt/wt).

more, the detection limits (**LOD**) and quantification limits (**LOQ**) of glucose and fructose were estimated using the root mean square error method as described by Corley (2003). Limit of detection values of 0.34 and 0.09 µg (corresponding to 17 and 4.6 mg/L) and LOQ of 0.31 and 1.13 µg (corresponding to 56.5 and 15.4 mg/L) were obtained for glucose and fructose, respectively. The LOD values of fructose and glucose were within the range of values published by others (Wei and Ding, 2000; Nogueira et al., 2005; La Pera et al., 2007) who used a similar method of detection (ELSD). Such low LOD and LOQ values, particularly for fructose, indicate a high sensitivity of this method that allows for low concentrations of both sugars to be measured.

To determine the recovery, repeatability, and reproducibility of the method, model solutions of inulin (Frutafit CLR) at 3% (wt/vol) concentration were prepared in water, milk whey (centrifugal supernatant), or permeate (separated from milk using ultrafiltration). The accuracy of the method was expressed as recovery of inulin added (i.e., the ratio of inulin determined experimentally to inulin added). The method showed good recovery (98.4 to 103%) independently of the matrix used to dissolve the inulin samples (Table 1). Repeatability, expressed as relative standard deviation of 4 replicate determinations of the same sample of Frutafit CLR 3% (wt/vol), ranged from 2.2 to 7.3% for glucose and from 0.6 to 2.3% for fructose depending on

Table 2. Concentration of inulin recovered in the whey of rennet milk

Sample ¹	Inulin concentration in whey (mg/mL)	Inulin recovered in whey (%)
Frutafit CLR	29.9 ± 1.0	69.3 ± 3.1
Oliggo-Fiber XL	13.9 ± 1.2	25.2 ± 3.6
Beneo HPX	18.7 ± 1.0	32.2 ± 4.1

¹Frutafit CLR from Sensus America LLC (Monmouth Junction, NJ); Oliggo-Fiber XL from Lomas Limited (Brampton, ON, Canada); Beneo HPX from Orafit SA (Tienen, Belgium).

the solvent used. Reproducibility of the method during extended storage time was determined in a model solution of Frutafit CLR 1% (wt/vol) as relative standard deviation of the amount of each sugar obtained from 8 runs over 2 wk. It was satisfactory for fructose (3.1 and 4.9% for solutions prepared in whey and water, respectively) and higher for glucose (12.9 and 8.1% in whey and water, respectively), but in both cases was within the range reported for food ingredients (Huber, 2007). These results indicated that the method was appropriate for measuring inulin in dairy matrices.

To test further the applicability of this method in determining the amount of inulin recovered in a cheese curd, the amount of inulin released in the whey drained from rennet curd was measured in samples prepared by rennet coagulation of a model system containing milk fortified with inulin. The chromatograms of 3 whey samples containing inulin with 3 different DP are shown in Figure 3. Smaller amounts of Oligo-Fiber XL (DP 20) and Beneo HPX (DP ≥ 23) were found in the whey compared with Frutafit CLR (DP 7–8), a difference that can be explained with the fact that Oligo-Fiber XL and Beneo HPX have greater DP, which results in a greater amount trapped in the curd and less inulin released in the whey. Table 2 summarizes the amount of inulin recovered in the whey separated from the milk curd after renneting.

In conclusion, inulin present in model dairy beverages and whey was successfully quantified by hydrolysis with inulinase followed by ion exclusion column chromatography and ELSD. The method showed good linearity, high sensitivity, and satisfactory precision. Consideration of background interferences such as lactose in whey was important for accurate quantification of inulin. Therefore, future applications of the method in similar matrices require careful considerations of such interferences. This method may help food product

developers in the evaluation of inulin recovery in dairy matrices.

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