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Analytical Methods

Fluorometric determination of free glucose and glucose 6-phosphate in cows' milk and other opaque matrices

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ABSTRACT

Analyses of free glucose and glucose 6-phosphate in milk have until now been dependent upon several time consuming and troublesome procedures. This has limited investigations in the area. The present article presents a new, reliable, analytical procedure, based on enzymatic degradation and fluorometric detection. Standards and control materials were based on milk that was stripped of intrinsic glucose and glucose 6-phosphate in order to obtain standards and samples based on the same matrix. The analysis works without pre-treatment of the samples, e.g. without centrifugation and precipitation of protein with acids.

1. Introduction

Carbohydrates are a natural constituent of milk along with proteins and fat. Together, the three components make up the vast majority of milk dry matter. Among the carbohydrates, the oligosaccharides dominate quantitatively and qualitatively and lactose is the principal component. Free monosaccharides in milk are dominated by glucose and galactose, although other monosaccharides are recognised. The concentration of free glucose in cows' milk is negligible compared to oligosaccharides and normally considered to be below 1 mmol/l (Davies, Holt, & Christie, 1983, chap. 3). Glucose 6-phosphate (Glu 6-P) is also a normal constituent of milk, although it is present at lower concentrations (e.g. 80–110 μmol/l, Hurtaud, Rulquin, & Verite, 1998; 30–60 μmol/l, Rigout, Hurtaud, Lemosquet, Bach, & Rulquin, 2003).

Several methods of glucose determination in milk are in use: Gas chromatography after derivatisation of glucose (e.g. Olano, Calvo, & Corzo, 1989; Troyano, Olano, Férnandez-Diaz, Sanz, & Martinez-Castro, 1991); electrochemical sensors, i.e. enzymatic conversion of glucose and subsequent amperometric determination (e.g. Pilloton & Mascini, 1990; Rajendran & Irudayaraj, 2002). The most often used method for glucose determination in milk is an enzymatic-colorimetric measurement after precipitation of milk protein and fat (the "hexokinase method", e.g. Boehringer Mannheim, 1995; Faulkner, Chaitabutr, Peaker, Carrick, & Kuhn, 1981).

The present article describes a new enzymatic-fluorometric method for determination of glucose and glucose 6-P. The basic principles are enzymatic oxidation of glucose and glucose 6-P with concomitant production of NAD(P)H_2 (the hexokinase method) and a subsequent reaction where enzymatic coupling of NAD(P)H_2 to a fluorophore develops a fluorescent product. The present method is useful in opaque matrices like milk and works without pre-treatment of the sample, e.g. high speed centrifugation and acid precipitation of proteins.

2. Materials and methods

2.1. Overall principle of the analyses

Glucose 6-phosphate was determined separately by enzymatic oxidation by glucose 6-P dehydrogenase using an $NADP^+$ dependent enzyme from *Saccharomyces* sp. (EC 1.1.1.49; Roche 10 127 655 001). The sum of free glucose and glucose 6-P (henceforth denoted total glucose) was determined by enzymatic oxidation by hexokinase (EC 2.7.1.1; Roche 11 426 362 001) and glucose 6-P dehydrogenase from *Leuconostoc* sp. (cofactor NAD^+ *and* $NADP^+$; EC 1.1.1.49; Roche 10 165 875 001). Free glucose was consequently estimated as the difference between the two results.

The enzymatic-fluorometric method of total glucose determination is a three-step enzymatic procedure to obtain the fluorescent product equivalent to the glucose content. The two first steps are identical to the widely used hexokinase and glucose-6-phosphatase mediated conversion of glucose to gluconate-6-phosphatase and NAD(P)H (Kunst, Draeger, & Ziegenhorn, 1988, chap. 2.4).

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The last step is an enzymatic coupling of the reducing equivalents from NAD(P)H to the non-fluorescent compound resazurin mediated by the enzyme diaphorase (EC 1.6.99. 1). Resazurin is reduced by NAD(P)H and the highly fluorescent substance resorufin is developed and measured fluorometrically (Larsen & Nielsen, 2005). Reducing equivalents (NADPH) from the separate glucose 6-P oxidation are in the same way coupled to resazurin in order to quantify glucose 6-P fluorometrically.

2.2. Reagents

2.2.1. Reagents, total glucose analysis

Reagent 1: Tris-buffer, 100 mmol/l, pH 7.6 with 10 mmol/l Mg^{++}, 3.6 mmol/l Na-oxamate (MW 111.03), 1.9 mmol/l ATP (Na_2-salt, MW 551.2) and 1.9 mmol/l NAD^+ (Na_2-salt, MW 717.5). Immediately before use the solution was supplied with hexokinase enzyme and glucose-6-phosphatase (Roche 10 737 275 001), 3.1 U and 1.6 U/ml, respectively.

Reagent 2: Tris-buffer, 60 mmol/l, pH 7.2, with 1.28 mmol/l resazurin (Sigma R-2127, MW 251.2), 0.01% Triton X-100, and 9.8 U/ml diaphorase (Toyobo Enzymes DAD-301). Ten drops of Tween 80 were added per 15 ml.

2.2.2. Reagents, glucose 6-P analyses

Reagent 1: Tris-buffer, 60 mmol/l, pH 7.2 with 3.6 mmol/l Na-oxamate and 1.9 mmol/l $NADP^+$ (di-Na-salt, MW 787.4). Immediately before use the solution was supplied with glucose 6-P dehydrogenase enzyme, 1.6 U/ml.

Reagent 2 was the same as reagent 2 used in the total glucose analysis (Section 2.2.1).

2.3. Procedure, both analyses

Dosage and dilution of sample and addition of reagents were performed in a robotic system (Biomek® 2000; Beckman Coulter). Milk samples (standards and control samples) and reagents were distributed in 96-well microplates and the plates transferred to the fluorometer (Fluostar, Galaxy, BMG Labtechnologies). The samples were excited with 544 nm monochromatic light and read at 590 nm light. Each plate contained $2 * 8$ standard solutions (glucose) and $2 * 4$ control solutions. The samples were read against a standard curve; control samples were used as internal control and day-to-day check. Standards and control samples were prepared (independently) from glucose monohydrate (MW 198.2) and glucose 6-P (MW 304.2), respectively, and milk free-from glucose and glucose 6-phosphate (basis). Standard concentrations used were 0; 0.24; 0.48; 0.72; 0.96; 1.20; 1.80; and 2.40 mmol/l (total glucose) and 0, 0.08, 0.16, 0.24, 0.32, 0.48, 0.64, and 0.80 mmol/l (glucose 6-P). Control samples were 0.5, 1.0, 1.5, and 2.0 mmol/l (total glucose) and 0.15, 0.30, 0.45, and 0.75 mmol/l (glucose 6-P), respectively. The sample (milk) made up 8.5% of the reaction medium.

2.4. Validation of the method

2.4.1. Standards and control material, detection limits

Measurements for standard curves and control samples from 30 microplates analysed on different days were collated. Intra and inter assay precision and accuracy (% bias) were calculated from the control samples; average slope and correlation coefficient were calculated from the standards. Results from eight replicate control samples, both 10 μmol/l and 5 μmol/l total glucose and glucose 6-P, respectively, were tested against eight replicates of zero samples in order to assess detection limits for the analyses.

2.4.2. Intra and inter assay precision, samples

Intra assay, total glucose and glucose 6-P: 56 milk samples were replicated three times within the same plate.

Inter assay, total glucose and glucose 6-P: 144 milk samples were analysed on separate plates on consecutive days.

2.4.3. Comparisons between spectrophotometric analyses and fluorometric analyses

2.4.3.1. Total glucose. Milk samples (50) plus milk-based standards and controls were analysed for total glucose using two different methods (1) and (2). Method 1 was an enzymatic spectrophotometric analysis performed in an autoanalyzer according to standard procedures (glucose hexokinase, Siemens Diagnostics® Clinical Methods for ADVIA 1650). This colorimetric method measures the sum of glucose 6-phosphate and glucose. Intra and inter assay precisions as well as accuracy for the spectrophotometric procedure were within 3 (CV)%. Milk samples, standards and controls were initially centrifuged to remove fat and precipitated with perchloric acid to remove protein, as suggested by Faulkner (1980). Method 2 was the present enzymatic-fluorometric method with no preceding precipitation of protein and fat.

2.4.3.2. Glucose 6-P. An equivalent comparison between spectrophotometric and fluorometric methods was established for glucose 6-P. A spectrophotometric endpoint analysis was established for glucose 6-P: Reagent 1 (R_1): Tris-buffer, 60 mmol/l, with 1.9 mmol/l $NADP^+$, pH 7.2; reagent 2 (R_2): Tris-buffer, 60 mmol/l, with 1.9 mmol/l $NADP^+$ and 6.7 U Glucose 6-P dehydrogenase per ml (Roche 10 127 655 001), pH 7.2. The absorbance was compared with a standard curve (0–0.8 mmol/l). These colorimetric analyses were performed using an autoanalyzer (ADVIA 1650, Siemens Diagnostics®). Intra assay precision and accuracy (bias%) were both within 2% for samples and control samples, respectively (0.15; 0.30; 0.45; and 0.75 mmol/l). Milk samples (50) plus standards and control materials for the milk analysis were treated according to Faulkner (1980, see above) and analysed by this spectrophotometric method and compared with the present fluorometric method.

2.4.3.3. Free glucose. Free glucose was also measured using the glucose oxidase method (glucose oxidase (Trinder), Siemens Diagnostics® Clinical Methods for ADVIA 1650). Fat- and protein-free milk samples (50) were analysed by this colorimetric method that measures glucose only (*not* glucose 6-P). Intra- and inter assay precisions as well as accuracy for the procedure were within 2.8% (CV).

2.4.4. Total glucose and glucose 6-P, the effect of addition of Na-oxamate

Milk samples (72) were analysed by the fluorometric method for both total glucose and glucose 6-P, with and without Na-oxamate in the reaction medium (1.9 mmol/l during 1. incubation and 1.0 mmol/l during 2. incubation, respectively).

2.4.5. Spiking of milk samples with milk samples

Milk samples (48) of low total glucose and glucose 6-P content (range 261–572 μmol/l and 8–58 μmol/l, respectively) were mixed 1:1 with 48 milk samples of relatively high total glucose and glucose 6-P content (range 488–914 μmol/l and 56–652 μmol/l) and analysed. The result was compared to the expected result (mean of low and high).

2.4.6. Spiking of milk samples with glucose or glucose 6-P

Milk samples (32) were analysed for total glucose and glucose 6-P according to the described procedure. The samples were furthermore spiked with 0.20, 0.40, or 0.60 mmol/l glucose or 0.064, 0.128, or 0.192 mmol/l glucose 6-P (in the dilution water). The