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The free and total *myo*-inositol contents of early lactation and seasonal bovine milk

Harvey E. Indyk [a, *], Sheila C. Saldo [a], Peter M. White [a], Mumtaz N. Dole [a], Brendon D. Gill [a], David C. Woollard [b]

[a] *Fonterra Co-operative Group Ltd, SH 26, Waitoa, New Zealand*
[b] *R J Hill Laboratories Ltd, 1 Clyde St, Hamilton, New Zealand*

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ABSTRACT

A high performance anion-exchange chromatographic method employing pulsed amperometric detection was applied to the determination of endogenous free and total *myo*-inositol in bovine milk, for which there is limited information. The contents and trend variability of *myo*-inositol in milk from extensively pasture-fed cows during early lactation and across a production season were therefore evaluated. Free and total *myo*-inositol in seasonal milk were within the ranges of 2.3–4.5 mg 100 g^{-1} and 5.3–8.7 mg 100 g^{-1}, respectively. This novel information will both improve understanding of the expression of innate *myo*-inositol in bovine milk, and provide manufacturers with information that can enhance formulation capability related to the production of cow's milk-based products

1. Introduction

Inositol is a cyclohexitol sugar alcohol that can exist in nine possible stereoisomeric forms, although only *myo*-inositol has been confirmed to have multiple cellular functions in mammalian cells. *myo*-Inositol forms the structural basis of biochemically significant compounds, including multiple inositol phosphates, phosphatidylinositol and phosphatidylinositol phosphates (Beemster, Groenen, & Steegers-Theunissen, 2002; Fisher, Novak, & Agranoff, 2002; Michell, 2008). Originally classified as a B-group vitamin in terms of human nutrition, it is now considered to be a conditionally essential nutrient, given that most mammals, including humans, are capable of its de novo synthesis from glucose-6-phosphate, although phosphatidylinositol is regarded as the principal metabolically active form (Michell, 2008).

Although it is normally present in food products as free *myo*-inositol, it also exists in multiple phosphorylated forms. Inositol hexaphosphate (phytate), which is present in many plant-based foods, is commonly considered to be nutritionally unavailable and is regarded as an anti-nutrient in humans, due to the absence of intestinal phytase activity and its binding of niacin and dietary multivalent transition metal cations. However, it has recently been reported that phytate is prevalent in mammalian neural tissue and that it may indeed contribute to human health, although its role within eukaryotic cell function remains poorly understood (Chen, 2004; Fisher et al., 2002). In contrast, several lower phosphates such as inositol 1,4,5-trisphosphate have confirmed physiological significance, as evidenced by their common occurrence in blood (Shears, 2004). Phospholipase activity within the human digestive tract facilitates the availability of *myo*-inositol bound within dietary phosphatidylinositol, and analytical methodology for food products should ideally include contributions from both phosphatidylinositol and the lower inositol phosphates, although there is currently a lack of consensus whether potential phytate content merits inclusion (Ellingson et al., 2012; Woollard, Macfadzean, Indyk, McMahon, & Christiansen, 2014).

Milk is a significant source of *myo*-inositol, both free and bound, as well as a galactopyranolsyl disaccharide form and, since human milk contains higher levels of *myo*-inositol than bovine milk, it is commonly added to infant formulae to ensure against potential early neonatal deficiency (Cavalli, Teng, Battaglia, & Bevilacqua, 2006; Indyk & Woollard, 1994). In view of the significant neonatal nutritional demand for this compound, there is an increasing requirement to declare levels of inositol in paediatric formulae. However, although the Codex Alimentarius Commission and other national regulatory bodies legislate a minimum level for infant formulae, they do not specify which of the multiple molecular species of inositol should be included for compliance.

* Corresponding author. Tel.: +64 78890899.
E-mail address: harvey.indyk@fonterra.com (H.E. Indyk).

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The analysis of free *myo*-inositol in foods, dairy products and biological tissues has generally been facilitated by simple protein precipitation with dilute acid, whereas methods targeting the sum of free and bound forms have followed an exhaustive, highly concentrated acidic or alkaline hydrolysis at high temperature. Analytical detection and quantitative techniques have included microbiological assays (Baker et al., 1990), enzymatic assays (Pereira, Baker, Egler, Corcoran, & Chiavacci, 1990), gas–liquid chromatography with prior derivatisation (Byun & Jenness, 1982; Clements & Darnell, 1980; de Koning, 1994; March, Forteza, & Grases, 1996; Ogasa, Kuboyama, Kiyosawa, Suzuki, & Itoh, 1975; Recio, Villamiel, Martínez-Castro, & Olano, 1998; Ruas-Madiedo, de los Reyes-Gavilán, Olano, & Villamiel, 2000; Sabater, Prodanov, Olano, Corzo, & Montilla, 2016; Troyano, Villamiel, Olano, Sanz, & Martínez-Castro, 1996; Woollard et al., 2014), high performance liquid chromatography (HPLC) employing UV and evaporative light scattering detection (Frieler, Mitteness, Golovko, Giener, & Rosenberger, 2009; Indyk & Woollard, 1994; Pazourek, 2014; Tagliaferri, Bonetti, & Blake, 2000; Wang, Safar, & Zopf, 1990; Yang & Ren, 2008) and HPLC or ultra-HPLC coupled with mass spectrometry (Flores, Moreno, Frenich, & Vidal, 2011; Kindt et al., 2004; Perelló, Isern, Costa-Bauzá, & Grases, 2004). In view of the electrochemical properties of *myo*-inositol, high performance anion-exchange chromatography coupled to pulsed amperometric detection (HPAEC–PAD) has become increasingly recognised as a highly sensitive and specific platform for its determination in food, infant formulae and human milk in free and/or bound forms (Cavalli et al., 2006; Dulinski, Starzyńska-Janiszewska, Stodolak, & Zyla, 2011; Ellingson et al., 2012, 2013; Jóźwik, Jóźwik, Teng, Jóźwik, & Battaglia, 2013; Schimpf, Thompson, & Baugh, 2012).

Currently, there is a paucity of data regarding the influence of lactation and season on the distribution in bovine milk of *myo*-inositol between its free and bound forms. Depending on the specific formulation of the paediatric product, the contribution of innate *myo*-inositol derived from milk is a significant proportion (>25%) of the total *myo*-inositol content of paediatric products that commonly include supplemental free *myo*-inositol and that derived from added whey protein and lecithin ingredients. The aim of the present study was therefore to provide knowledge of the temporal variation in endogenous *myo*-inositol content of bovine milk, which may be used to improve formulation of bovine milk-based paediatric products.

2. Materials and methods

2.1. Apparatus

A Dionex ICS 5000 high performance ion chromatographic system was used, which included an ICS 5000 + SP gradient pump, an AS-AP autosampler with a 25 μL injection loop, an ICS 5000 + DC column oven and detector compartment, and an ICS-5000 ED detector with Ag/AgCl reference cell and PTFE-backed Au disposable working electrode (Thermo Fisher Scientific NZ, Auckland, New Zealand). Instrument control and data analysis were performed with Chromeleon 7 software (version 7.2.0.4154).

Chromatographic analysis was performed using a CarboPac MA1 4 mm × 250 mm analytical column preceded by a CarboPac MA1 4 mm × 50 mm guard column (Thermo Fisher Scientific).

Sonication was accomplished with an Elmasonic S 60H (Elma, Singen, Germany) and the autoclave was an SX-300E (Tomy Seiko Co., Tokyo, Japan). Other apparatus included 90 mm grade A glass fibre filters (LabServ, Waltham, MA, USA) and 13 mm × 0.45 μm PTFE syringe filters (Grace, Rowville, VIC, Australia). Disposable, autoclavable 50 mL polycarbonate screw-capped centrifuge tubes were obtained from Thermo Fisher Scientific.

2.2. Reagents

myo-Inositol, ≥99% pure, was obtained from Sigma–Aldrich (St Louis, MO, USA). Potassium hydroxide pellets and 36% (v/v) hydrochloric acid were from Merck (Darmstadt, Germany) and 10 M carbonate-free sodium hydroxide ampoules were from Thermo Fisher Scientific NZ. Water of >18 MΩ resistivity was produced by a Barnstead E-Pure system (Dubuque, IA, USA).

2.3. Standards

A stock standard (1.0 mg mL^{-1}) was prepared by dissolving 0.10 g of accurately weighed *myo*-inositol in water and making to volume (100 mL), and was stable for 3 months at 4 °C. An intermediate standard (100 μg mL^{-1}) was prepared by diluting the stock standard 10-fold with water and was stable for 1 month at 4 °C. Six calibration standards (0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 μg mL^{-1}) were prepared daily by serial dilution with water.

2.4. Mobile phase

Eluents were prepared with helium-degassed high purity water and were stored in polypropylene containers under low pressure nitrogen while in use. Mobile phase B (1.0 M NaOH) was prepared by diluting the contents (100 mL) of a 10 M ampoule to 1.0 L with water. Mobile phase A (40 mM NaOH) was prepared by diluting 40 mL of mobile phase B to 1.0 L with water. The eluents were used for up to 1 month when stored under nitrogen.

2.5. Sample collection

Early lactation samples included raw colostrum, transitional milk and mature milk (mid-flow, same quarter) acquired at ten intervals from a single Jersey cow (4th calving) over the first 30 days post-partum. Ten raw milk samples were acquired from a bulk composite herd milk processing silo across the 2014–2015 season at a frequency of one per month.

At each time interval, duplicate 10.0 g samples were accurately weighed into 50 mL disposable centrifuge tubes assigned for the analysis of either free or total *myo*-inositol and were stored at −80 °C until all samples had been collected.

2.6. Sample preparation

Samples stored at −80 °C were thawed overnight at 4 °C, vortexed, and subjected to analysis in independent runs on two different days.

2.6.1. Free myo-inositol

Each liquid milk sample was diluted to approximately 25 mL with water. An in-house quality control (QC) infant formula powder (1.0 g), NIST 1849a SRM (National Institute of Standards and Technology, Gaithersburg, MD, USA) and a reagent blank were included in each analytical run. Tubes were vortex mixed and then sonicated for 30 min. Each sample was pH adjusted to 4.5 ± 0.2 (with 0.1 M HCl), further diluted to 50 mL with water and then filtered through a glass fibre filter. An aliquot was filtered through a 0.45 μm PTFE syringe filter and 20 μL of filtrate was diluted with 980 μL of water (50-fold dilution) in an autosampler vial.

2.6.2. Total myo-inositol

Each 10 mL liquid milk sample, 1 g powdered reference samples (in-house QC and NIST SRM 1849a dissolved in 10 mL water) and reagent blank were vortex mixed and sonicated for 30 min. To each sample, 10 mL of 36% HCl was added and the tubes were autoclaved