The Free and Total *myo*-Inositol Contents of Early Lactation and Seasonal Bovine Milk

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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 hg^{-1} and 5.3–8.7 mg hg^{-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

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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.

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, Ellingson et al., 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 mL 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 x 250 mm analytical column preceded by a CarboPac MA1 4 x 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 x 0.45 mm PTFE syringe filters (Grace, Rowville, VIC, Australia).

Disposable, autoclavable 50 mL polycarbonate screw-capped centrifuge tubes were obtained from Thermo Fisher Scientific.

2.2. Statistical analysis

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 mg 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 mg 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 mL of filtrate was diluted with 980 mL 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 SRM NIST 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 at 120 °C for 6 h. The tubes were allowed to cool and the contents were adjusted to pH = 7.0 ± 0.2 with 50% (w/v) KOH. The neutralized samples were diluted to 50 mL with water and filtered through a glass fibre filter. If required, extracts could be stored at 4 °C for up to one week. An aliquot was filtered through a 0.45 µm PTFE syringe filter and 20 mL of filtrate was diluted with 980 mL of water (50-fold dilution) in an autosampler vial.

2.7. Chromatography

The chromatographic conditions were as reported previously (Ellingson et al., 2012, Ellingson et al., 2013) with minor modifications. The column was held at 30 °C and all injections were 25 mL. A constant isocratic flow rate of 0.4 mL min^{-1} was maintained across the gradient programme as described in Table 1. When not in use for lengthy periods, the CarboPac MA1 columns were stored in 600 mM NaOH. Pulsed integrated amperometric detection was employed using disposable gold working electrodes and a standard quad potential waveform, as described in Table 2. The *myo*-inositol concentration was interpolated from a six-level external standard least-squares linear regression calibration, and detector response stability confirmed with a single-level QC standard interspersed across each analytical run.

3. Results and discussion

3.1. Analytical method

Although gas-liquid chromatography and LC-mass spectrometry methods have proven reliability, derivatisation is required for the former, while the latter requires stable isotope-labelled internal standards and is a relatively demanding platform for routine deployment. In contrast, HPAEC-PAD is

now accepted as a robust analytical technique with inherent specificity for carbohydrates, does not require derivatisation, is well suited to routine application, and has been reported for the specific analysis of myo-inositol in human breast milk, animal feed and infant formulae (Cavalli et al., 2006; Dulinski et al., 2011; Ellingson et al., 2012; Schimpf et al., 2012).

The present study selected, with minor modifications, an HPAEC-PAD method recently described and comprehensively validated for bovine milk-based infant formulae, in view of its ability to quantify both free and total *myo*-inositol (Ellingson et al., 2012). Thus, the inherent free *myo*-inositol content was determined following dilute hydrochloric acid precipitation of proteins at the isoelectric point of casein, and the total *myo*-inositol content was estimated as free *myo*-inositol subsequent to an exhaustive high temperature autoclave extraction with concentrated hydrochloric acid, with both forms being quantified under identical chromatographic conditions, as illustrated in Figure 1.

The suitability of the chromatographic system was established by (i) ensuring baseline separation of glycerol from *myo*-inositol, (ii) an area precision of less than 5% relative standard deviation (RSD) for replicate injections (n = 5) of the top-level working standard and (iii) the absence of peak interferences in the reagent blank. Qualitatively, the principal chromatographic difference between determinations of free myo-inositol and total *myo*-inositol is the presence of the dominant late-eluting lactose peak when free *myo*-inositol is measured. Under the exhaustive acid digestion conditions required for the determination of total *myo*-inositol, endogenous lactose does not remain intact and is removed from subsequent analysis.

myo-Inositol exists in various bound forms within milk and their determination as a single entity, subsequent to the harsh extraction requirements for the analysis of total myo-inositol content, is of benefit for nutritional labeling declarations.

The structural stability of *myo*-inositol ensures that it remains intact under such extractive conditions, and its multiple phosphates and phosphatidylinositol are thereby quantitatively converted to free *myo*-inositol for subsequent determination (Ellingson et al., 2012; Woollard et al., 2014). Indeed, phytate-bound inositol has also been demonstrated to be quantitatively recovered by the autoclave procedure described, thereby facilitating analysis of soy-based infant formulae (Ellingson et al., 2012).

The reliability of the described analytical method was evaluated during the present study through estimation of the analytical precision for total *myo*-inositol determination with a QC infant formula sample and the NIST 1849a SRM infant formula. The within-run repeatability (RSD_r)was 0.80% for the QC sample (n = 7) and 0.60% for the NIST 1849a SRM (n = 7), and the between-run intermediate precisions (RSD_{IR}) were 4.01% (n = 14) and 5.51% (n = 10) for the same samples respectively. Analytical accuracy was evaluated by replicate analysis of the NIST 1849a SRM for both free and total *myo*-inositol content, which yielded values of 44.6 mg hg^{-1} (n = 6) and 55.7 mg hg^{-1} (n = 10)

respectively. The value obtained for free *myo*-inositol is comparable with the assigned reference value for free *myo*-inositol of 40.5 ± 0.8 mg hg^{-1} and, although there is no assigned value for total *myo*-inositol, the data are consistent with that reported (53.5 mg hg^{-1}) from an independent laboratory utilising the same methodology. These data confirm the reliability of the method used in the present study. Further, stable baseline resolution between the closely eluting glycerol and *myo*-inositol was achieved, and no peak interference was observed in the reagent blank.

3.2. Application to bovine milk

The described analytical methodology was applied to determining the variability and the distribution of myo-inositol between the free and bound forms in milk from extensively pasture-fed cows. The composition of ruminant milk is generally influenced by the physiological stage of lactation and, although colostrum is withheld from entering the commercial bovine milk supply, it was considered to be of value in the present study to investigate the distribution of myo-inositol during the transition from colostrum to mature milk. The described analytical method was therefore applied to quantify the free and total *myo*-inositol contents during this transition in a single pasture-fed lactating cow over 28 days subsequent to parturition (Table 3). There was a distinct, albeit minor, increase in free *myo*-inositol during the early colostral phase, followed by a relatively stable following the transition to mature milk, and the total *myo*-inositol content remained essentially constant across the entire 28 day post-partum period. The free to total *myo*-inositol ratio consequently increased to reach a constant value of approximately 0.4 in mature milk, a value that is consistent with an earlier study based on an HPLC determination of free *myo*-inositol and a microbiological assay of total *myo*-inositol (Indyk &Woollard, 1994). These data suggest that the expression of this component in bovine milk is influenced to a significant, albeit relatively minor, extent by the physiology of the early lactogenesis phase, a conclusion that is consistent with the few studies previously reported on the expression of *myo*-inositol in the colostrum and milk of the rat, human and cow (Byun & Jenness, 1982; Jóźwik et al., 2013; Ogasa et al., 1975; Pereira et al., 1990). Although incompletely understood, it has been unambiguously demonstrated that lacteal *myo*-inositol derives from both in vivo epithelial mammary gland biosynthesis and uptake via active transport, with the relative contribution from each difficult to define (Byun & Jenness, 1982; Jóźwik et al., 2013).

Dairy husbandry in New Zealand exploits extensive pasture grazing, which facilitates the investigation of natural seasonal changes in herd milk. The free and total *myo*-inositol contents in bulk raw bovine herd milk from the central North Island were quantified over an entire production season (Table 4). There was a significant, albeit minor, increasing seasonal trend in levels of both free *myo*-inositol and total *myo*-inositol and, consequently, a relatively constant free to total ratio of 0.49 (range: 0.41–0.57), which possibly suggests a greater influence from systemic active transport relative to mammary

biosynthesis across the season. This systematic trend may also be an outcome of the synchronised herd calving husbandry model that is intended to maximise herd lactation volume coincident with peak early summer grass growth in New Zealand. Two previous studies have reported trend data for total *myo*-inositol in New Zealand milk (Indyk, Lawrence, & Broda, 1993; Woollard et al., 2014). The mean levels of free and total *myo*-inositol in milk across the entire season were 3.4 mg hg^{-1} (range: 2.3–4.5) and 6.8 mg hg^{-1} (range: 5.3–8.7), respectively. The analytical method applied in the present study reports values for both free and total *myo*-inositol contents, whereas the limited number of previous studies have generally reported free *myo*-inositol only, with fewer determining the total *myo*-inositol content. Nonetheless, the data obtained for raw bovine milk by the described method are comparable with reported ranges of 2.1–5.4 mg hg^{-1} for free *myo*-inositol (Byun & Jenness, 1982; Indyk & Woollard, 1994; Recio et al., 1998; Ruas-Madiedo et al., 2000; Troyano et al., 1996) and 3.3–5.5 mg hg^{-1} for total *myo*-inositol (Clements & Darnell, 1980; Ogasa et al., 1975; Woollard et al., 2014).

Although human milk is correctly advocated by the World Health Organisation as the optimum food source for human infants, manufactured paediatric products formulated to achieve physiological benefits that are comparable with those obtained by breast-fed infants have a critical role where breast feeding is not implemented. The challenge for the global industry remains the on-going compositional adaptation of bovine milk to provide paediatric products that can progressively substitute for breast milk. In view of the evolutionarily adapted difference in the myo-inositol contents of human milk and bovine milk, any nutritionally credible formulation is dependent on available information regarding the variable concentration of this nutrient in cow's milk as a function of lactation and season. This study reveals new information regarding the temporal variability in endogenous myo-inositol content of milk derived from pasture grazed cows. The *myo*-inositol content of bovine milk contributes a significant proportion of that typically found in most paediatric products, and the presented seasonal data will therefore better facilitate formulation of nutritional products.

4. Conclusions

The analytical method for total *myo*-inositol determination utilised in the present study incorporates an exhaustive conversion of phosphatidylinositol and inositol phosphates to free inositol, thereby precluding speciation of bound forms. An advantage of the HPAEC-PAD technique described is its capability for determination of both free *myo*-inositol and total *myo*-inositol under identical chromatographic conditions, subsequent to two simple alternative sample preparation procedures. The method facilitated the determination of endogenous free and total *myo*-inositol in bovine milk and allowed, for the first time, an evaluation of the influence of lactation and season on the lacteal expression of this nutrient. Although the physiological significance of the distribution between free and bound forms in bovine milk both during early lactation and across a production season is

unknown, the data presented will augment understanding regarding the expression of this nutrient in bovine milk and further facilitate the enhanced control of infant formula manufacture with respect to the declared content of this conditionally essential nutrient.

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Table 1. Chromatographic conditions for quantification of *myo*-inositol: mobile phase gradient conditions

Time (min)	Flow rate (mL min^{-1})	Phase Composition	
		% A[a]	% B[b]
0	0.4	100	0
10.0	0.4	100	0
10.1	0.4	0	100
20.0	0.4	0	100
20.1	0.4	100	0
40.0	0.4	100	0

Table 2. Chromatographic conditions for quantification of *myo*-inositol: detector waveform conditions

Time (s)	Potential (V)	Integration
0	0.4	100
10.0	0.4	100
10.1	0.4	0
20.0	0.4	0
20.1	0.4	100
40.0	0.4	100

Table 3. Free and total *myo*-inositol contents in early lactational milk from a single cow[a]

Day post-partum	myo-Inositol contents		
	Free	Total	Free:Total
0	1.24 (0.08)	6.11 (0.23)	0.20
1	1.00 (0.04)	5.13 (0.18)	0.20
2	1.45 (0.03)	6.08 (0.17)	0.24
3	1.33 (0.07)	5.94 (0.07)	0.22
5	3.77 (0.11)	6.69 (0.45)	0.56
7	2.45 (0.02)	6.56 (0.19)	0.37
14	2.46 (0.10)	5.58 (0.05)	0.44
21	2.49 (0.02)	6.26 (0.14)	0.40
28	2.31 (0.08)	6.44 (0.08)	0.36

[a] Concentrations (mg hg^{-1}) are the mean of independent duplicate analyses with standard deviation in parentheses

Table 4. Free and total myo-inositol contents in early lactational milk from a single cow[a]

Month (2014–2015)	*myo*-Inositol contents		
	Free	Total	Free:Total
August	3.08 (0.01)	6.51 (0.05)	0.47
September	2.58 (0.07)	5.76 (0.44)	0.45
October	2.48 (0.02)	5.62 (0.49)	0.44
November	2.37 (0.09)	5.68 (0.57)	0.42
December	3.02 (0.15)	6.16 (0.78)	0.49
January	3.39 (0.06)	6.80 (0.85)	0.50
February	4.50 (0.07)	7.93 (0.30)	0.57
March	4.05 (0.13)	7.72 (0.76)	0.53
April	4.01 (0.19)	8.04 (0.94)	0.50
May	4.32 (0.04)	8.16 (0.67)	0.53

[a] Concentrations (mg hg^{-1}) are the mean of independent duplicate analyses with standard deviation in parentheses

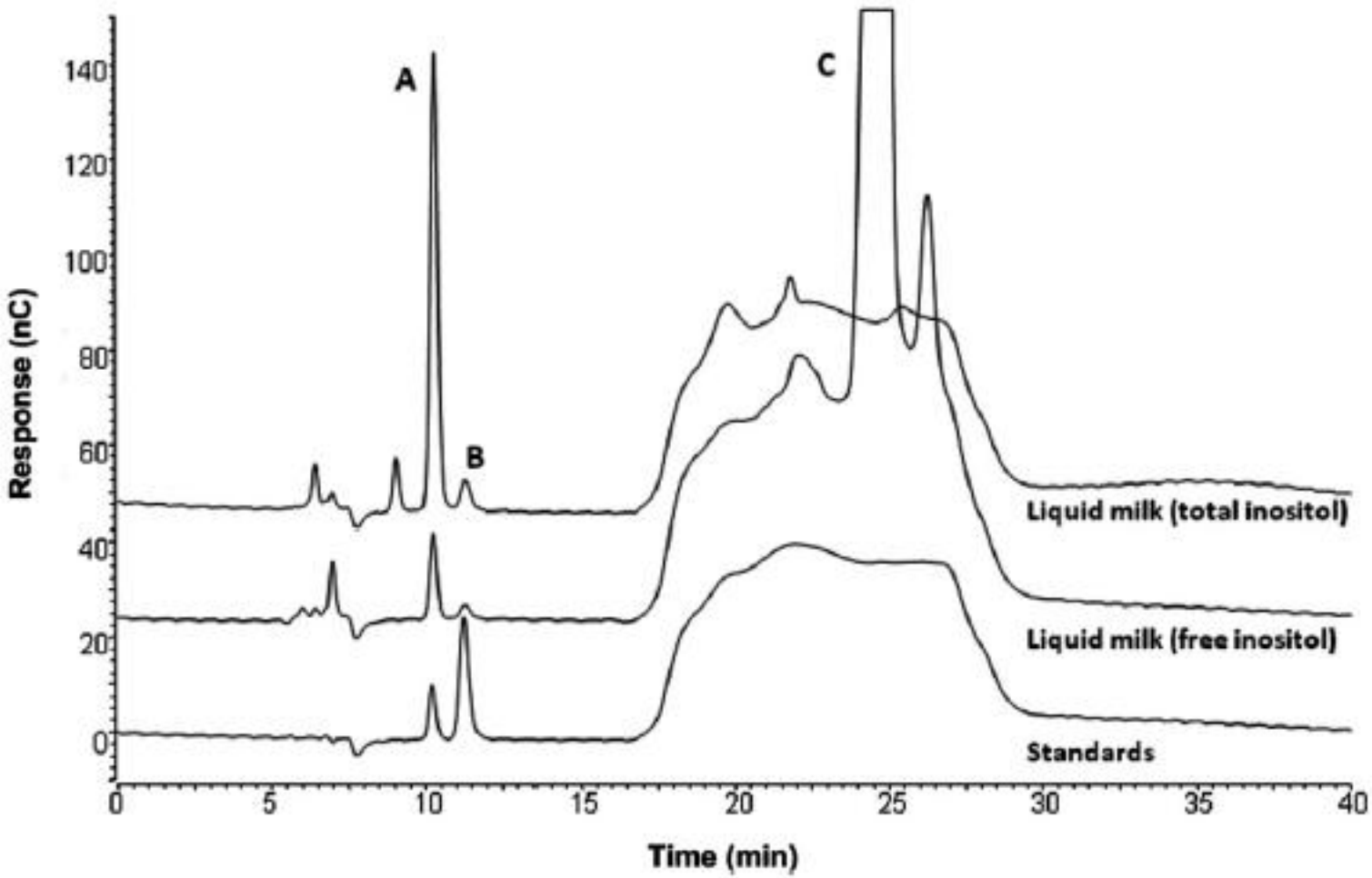

Figure 1. Chromatography of myo-inositol and glycerol authentic standards, bovine milk subsequent to preparation for free myo-inositol determination, and bovine milk subsequent to preparation for total myo-inositol determination: (A) glycerol; (B) *myo*-nositol; (C) lactose. HPAEC-PAD conditions as described in the text