Vol. 7, No. 1, 2016
ISSN 2233-4203/ e-ISSN 2093-8950
LETTER www.msletters.org | Mass Spectrometry Letters

Survey of Inositol in Infant Formula

A. Patel, M. Ditiatkovski, L. Kennedy, A. Oglobline, N. Choi, G. Richardson*

Dairy Technical Services Ltd, 3/63-71 Boundary Road, North Melbourne, 3051, Victoria, Australia

Received November 12, 2015; Revised December 14, 2015; Accepted December 21, 2015
First published on the web March 31, 2016; DOI: 10.5478/MSL.2016.7.1.12

Abstract : Results of free and bound *myo*-inositol in infant formula (IF) are presented. Inositol was analyzed by HILIC ultra-performance liquid chromatography coupled with mass spectrometer. The levels of free *myo*-inositol in 27 Australian and 4 EU originated IF samples were 300-600 mg/kg of powder or 1.6-3.1 mg/100 kJ. The amount of bound inositol in lipid fraction of IF was, on average, 10% of free *myo*-inositol.

Keywords : infant formula, *myo*-inositol, survey, UPLC-MS/MS.

Introduction

Inositol is an important nutrient present naturally in human milk and cow's milk but in a lesser quantity. It exists predominantly in the free *myo*-inositol form. *myo*-Inositol is found in large amounts in plant materials such as soy beans but is in the bound form, predominantly as polyphosphate and in a lesser extent as phosphatidylinositol.

The levels of inositol in infant formula are regulated. CODEX[1] defines lower and upper levels of inositol at 1 and 9.5 mg/100 kJ. In the USA there is no prescribed limits[2] for inositol in dairy-based IF. The minimum amount of inositol in non-dairy based IF according to the Code of Federal Regulations (CFR) is 4 mg/100 kcal (~1 mg/100 kJ) and the upper limit is not regulated. The levels of inositol in IF in Australia and New Zealand are regulated by the FSANZ 2.9.1 and in China by the GB 10765-2010 Food Standards, Table 1.

There are nine isomers of inositol $C_6H_{12}O_6$ with the *myo*-inositol form being the most abundant and physiologically relevant. The structures of the inositol isomers are presented in the Fig. 1. There is limited data on inositol levels in dairy products. The most substantial survey was done in Japan[3]. The content of total *myo*-inositol in human milk was reported as 32.7±15.2 in colostrum, 17.8±1.9 in transitional milk, and 14.9±3.1 mg/100 mL in mature milk. In cow's milk, it was 10.6±1.0, 7.0±1.1, and 4.1±1.0 mg/100 mL, respectively. Levels of lipid bound *myo*-inositol in human and cow's milk were 0.22±0.09 and 0.36±0.10 mg/100 mL, respectively. A small amount of *scyllo*-inositol was

Table 1. Chemical identity and regulatory limits of inositol

IUPAC name	Common name	CAS	Regulation[a] mg/100 kJ	
			min	max
cis-1,2,3,5-*trans*-4,6-Cyclohexane-hexol	*myo*-Inositol	87-89-8	1	9.5

[a] Identical in Australia, China and New Zealand

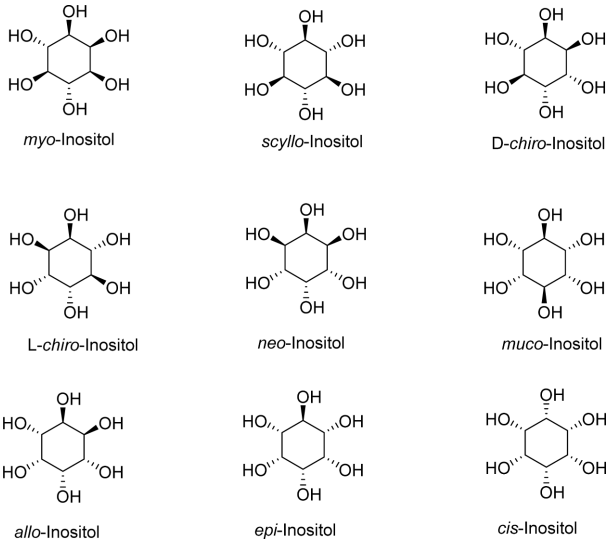

Figure 1. Nine isomeric forms of inositol.

*Reprint requests to G. Richardson
E-mail: grichardson@dtsfoodlabs.com.au

Open Access

All MS Letters content is Open Access, meaning it is accessible online to everyone, without fee and authors' permission. All MS Letters content is published and distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org /licenses/by/3.0/). Under this license, authors reserve the copyright for their content; however, they permit anyone to unrestrictedly use, distribute, and reproduce the content in any medium as far as the original authors and source are cited. For any reuse, redistribution, or reproduction of a work, users must clarify the license terms under which the work was produced.

found in both human and cow's milk, while *D-chiro*-inositol was not found in either. The percent of lipid bound to total *myo*-inositol in cow's milk reported was 6.3±1.3%.

There are several testing methods for inositol - GC/FID[4,5] LC[6,7,8,9] anion exchange chromatography[10] and microbiological.[11,12] The performance requirements set by the Association of Analytical Communities (AOAC International) with respect to inositol methods describe the determination of the free *myo*-inositol and phosphatidylinositol, but exclude methyl ethers, glycosides, phosphorylated forms and phytate in all forms of infant, adult, and/or pediatric formula (powders, ready-to-feed liquids, and liquid concentrates). In this report we present results of a LCMSMS method for the determination of free and bound *myo*-inositol in Australia- and EU-originated commercial IF samples.

Experimental

Instruments and Reagents

All reagents were of analytical grade unless otherwise specified. Solvents and reagents for UPLC mobile phases were of *LCMS* grade. Water for UPLC mobile phases was with TOC <2 ppb and resistivity > 16MΩ·cm, double purified on *Integra*, Siemens of Barsbüttel, Germany from institute water produced by Millipore, Billerica, MA, USA. Acetonitrile and methanol were from Fisher Scientific and the hydrochloric acid was from Merck KGaA of Darmstadt, Germany. The reference material of *myo*-inositol cat. No. I5125-50G was from Sigma-Aldrich of St. Louis, MO, USA. *myo*-Inositol-D_6 was from TRC Toronto, Canada.

The *Acquity H*-class UPLC instrument coupled with *Xevo TQ MS* mass spectrometer were from Waters of Milford, MA, USA. The chromatographic column used was *Acquity* UPLC BEH Amide, 2.1 mm × 100 mm × 1.7 μm also from Waters.

Analytical procedure

Dairy-based IF dry powder products were purchased from different retail outlets of the metropolitan Melbourne during June-August 2014. Before analysis samples were stored according to the label requirements. Free and bound inositols were extracted according to AOAC official methods 2012.12[13] and bound inositols were hydrolyzed to the free form according to 2011.18.[14] Both AOAC protocols were followed precisely in order to have consistency with data of other laboratories. Extracts were analyzed by UPLC-MS/MS. *myo*-inositol-D_6 (100 μL, 5.0 μg/mL) was added to 200 μL of the filtered sample which was further diluted with 1.7 mL of acetonitrile to facilitate HILIC separation. The injection volume was 10 μL. The analytical system was calibrated from 0.25 to 5 ppm using six standards in solvent. Table 2 shows the UPLC gradient used.

Quality assurance and quality control was performed by analysis of reagent blanks, duplicate samples and SRM 1849a, NIST USA.

Results and discussion

The extraction of free and bound inositol was based on official AOAC procedures, and the LCMS determination was developed and validated in-house based on publications of J.-H. Ahn[15] *et al*. and K.-Y. Leung[16] *et al*. The run time of the chromatographic separation is 8 min, Fig. 2.

Table 2. UPLC Gradient

Time (min)	MP A (Water)	MP B (Acetonitrile)
0.0	5	95
0.5	5	95
1.0	15	85
3.0	25	75
4.0	25	75
4.1	5	95
8.0	5	95

Table 3. Results of *myo*-inositol in SRM1849a analyzed on 7 separate occasions

Days	Found, mg/kg	Difference to reference value
1	397.2	-2%
2	425.9	5%
3	409.7	1%
4	419.2	3%
5	417.1	3%
6	426.8	5%
7	427.2	5%

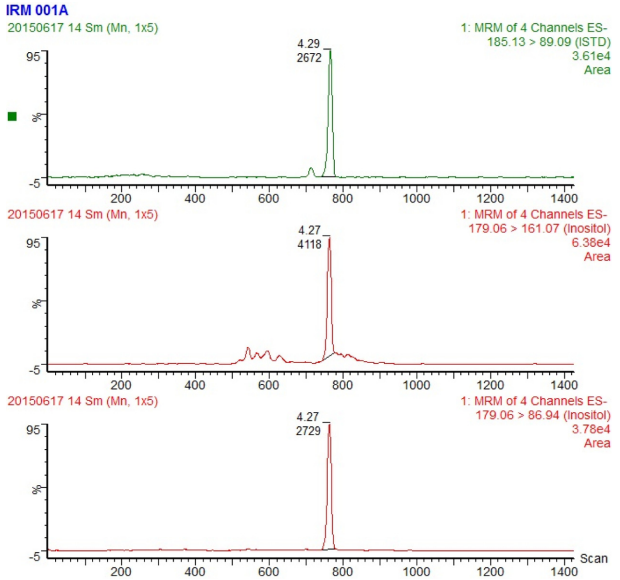

Figure 2. Chromatograms of the dairy-based IF extract, t_R of inositol is 4.29 min. Top spectrum is quantitative peak, middle is qualitative peak and bottom is internal standard reference.

Table 4. Content of inositol in IF samples, SD is indicated for n=2.

Sample	Country of origin	Free *myo*-inositol mg/100g ± SD	*myo*-Inositol in lipid fraction
IF1	AU	41.1	-
IF2	AU	40.6	-
IF3	AU	41.2	-
IF4	AU	44.0	-
IF5	AU	41.4	-
IF6	AU	31.6	-
IF7	AU	40.3	-
IF8	AU	42.0	-
IF9	AU	43.2	-
IF10	AU	41.4	-
IF11	AU	42.9±0.9	-
IF12	AU	44.2	-
IF13	AU	44.1	-
IF14	AU	43.5	-
IF15	AU	50.2	-
IF16	AU	50.4	3.0±0.1
IF17	AU	43.2	6.7±1.4
IF18	AU	42.4	2.4±0.1
IF19	AU	44.2	-
IF20	AU	45.2±0.4	-
IF21	AU	75.2	-
IF22	AU	45.5	-
IF23	AU	44.5	-
IF24	AU	45.4	-
IF25	AU	44.3	-
IF26	AU	45.4	-
IF27	AU	48.7	-
IF28	FR	30.7±1.6	4.7±0.1
IF29	NL	62.4±5.5	2.9±0.2
IF30	DE	34.8±2.4	0.8±0.1
IF31	IE	44.3±2.1	3.0±0.5
SRM1849a	US	41.8±1.5*	-
Skim milk	AU	3.42	-
UHT milk**	AU	3.00	-

* - SD calculated for n=7; ** - full cream UHT milk.

Reference material, SRM1849a with stated *myo*-inositol level of 405.2±7.6 mg/kg was repetitively analyzed to determine accuracy and precision of the assay, the results are listed in Table 3.

Table 4 shows the result of the survey. The results showed a mean value of 43.5 mg/100 g for Australian IF, excluding one outlier (IF21). Thermo[17] recently published brief results and method. The microbiological and liquid chromatography methods of total inositol determination have been compared during analysis of dietetic milk powders. Several authors reported systematically higher concentrations of total inositol determined by the LC/PAD technique (420 to 1340 mg/kg) than by the microbiological procedure (270 to 1120 mg/kg). The difference between values given by two methods is significant considering mild extraction conditions applied for free inositol determination of both methods. To the contrary, Indyk and Woollard reported[18] similar amounts of free inositol in skimmed milk powders, whole-milk powders and milk infant formulas determined by HPLC and microbiological procedures. Soy beans are known to contain up to 0.9 % of pinitol, a methylated form of *D-chiro*-inositol that may be separated[19,20] from *D-chiro*- and *myo*-inositol by gas chromatography. The high fraction of free to total inositol in samples of soya bean meal determined by the chromatographic procedure was not confirmed in the microbiological assay. These findings suggest a stereo selectivity of the latter. Pinitol and *D-chiro*-inositol, the predominant inositols of soya bean husk, most probably co-eluted from the analytical column with *myo*-inositol while microbiological assay determined only the *myo*-inositol. The use of the better chromatographic resolution of UPLC and selectivity of mass spectrometry is critical to reducing these errors.

Following the protocols of NATA's Technical Note 33,[21] the MU for *myo*-inositol results was determined to be 25 mg/kg (7.7%) at the levels present in infant formula. The inositol of lipid fraction was found to be about 10% of the total *myo*-inositol content.

Conclusions

A UPLC-MS/MS method was developed for the analysis of free *myo*-inositol which was single-laboratory validated and used for the survey of dairy-based IF samples originated in Australia and EU. The mean±SD of free *myo*-inositol of 27 IF samples originated in Australia was 43.5±3.6 mg/100 g (excluding one outlier) in dry powders.

Acknowledgment

Authors would like to thank Dr. Simone Rochfort and her group from the DEPI, Vic. for the valuable discussions during the project.

Abbreviations

CFR - Code of Federal Regulations
FSANZ - Food Standards Australian and New Zealand
GB - Guobiao, Chinese National Standard
NATA - National Association of Testing Authorities
PAD - Pulse Amperometric Detector
SRM - Standard Reference Material
UHT - Ultra Heat Treated
EU - European Union

References

1. Standard For Infant Formula And Formulas For Special Medical Purposes Intended For Infants. CODEX STEN 72-1981, rev 2011
2. 21CFR107.100. Chapter I. Food and Drug Administration, Department Of Health and Human Services, USA 2000.
3. Ogasa, K.; Kuboyama, M.; Kiyosawa, I.; Suzuki, T.; Itoh, M. *J. Nutr. Sci. Vitaminol*. **1975**, 21, 129.
4. Koning, A. J. *Analyst* **1994**, 119, 1319.
5. March, J. G.; Forteza, R.; Grases, F. *Chromatographia* **1996**, 42, 329.
6. Lauro, P. N.; Craven, P. A.; DeRubertis, F. R. *Anal. Biochem*. **1989**, 178, 331.
7. Katayama, M.; Matsuda, Y.; Kobayashi, K.; Kaneko, S.; Ishikawa, H. *Biomed. Chromatogr*. **2006**, 20, 440.
8. Perell, J.; Isern, B.; Costa-Bauz, A.; Grases, F. *J. Chromatogr. B* **2004**, 802, 367.
9. Flores, M. I. A.; Moreno, J. L. F.; Frenich, A. G.; Vidal, J. L. M. *Food Chem*. **2011**, 129, 1281.
10. Tagliaferri, E. G.; Bonetti, G.; Blake, C. J. *J. Chromatogr. A* **2000**, 879, 129.
11. Kouzuma, T.; Takahashi, M.; Endoh, T.; Kaneko, R.; Ura, N.; Shimamoto, K.; Watanabe, N. *Clin. Chim. Acta* **2001**, 312, 143.
12. Ashizawa, N.; Yoshida, M.; Aotsuka, T. *J. Biochem. Biophys. Methods* **2000**, 44, 89.
13. Free and Total Myo-Inositol in Infant Formula and Adult/ Pediatric Nutritional Formula. AOAC Official Method 2012.12.
14. Myo-Inositol (Free and Bound as Phosphatidylinositol) in Infant Formula and Adult Nutritionals. AOAC Official Method 2011.18.
15. Ahn, J. H.; Lee, M. S.; Kwak, B. M.; Park, J. S.; Park, J. M.; Kim, J. M. *2013 China-Japan-Korea Symposium on Analytical Chemistry*, Fukuoka and Nagasaki (Japan), 22-25 August 2013, p PL25.
16. Leung, K. Y.; Mills, K.; Burren, K. A.; Andrew, J. *J. Chromatogr. B* **2011**, 879, 2759.
17. Chen, L.; Borba, B.; Rohrer, J. Determination of Myo-Inositol (Free and Bound as Phosphatidylinositol) in Infant Formula and Adult Nutritionals. Application note 1083, *Thermo Fisher Scientific* 2014.
18. Indyk, H. E.; Woollard, D. C. *Analyst* **1994**,119, 397.
19. Lee, Y. K.; Park, P. B.; Park, Y. I.; Chung, N. *Agr. Chem. Biotechnol*. **2003**, 46, 148.
20. Gomes, C. I.; Obendorf, R. L.; Horbowicz, M. *Crop Sci*. **2005**, 45,1312.
21. Guidelines for estimating and reporting measurement uncertainty of chemical test results. Technical Note 33, NATA, Australia 2013.