Quantification of prebiotics in commercial infant formulas

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ABSTRACT

Since breastfeeding is not always possible, infant formulas (IF) are supplemented with prebiotic oligosaccharides, such as galactooligosaccharides (GOS) and/or fructooligosaccharides (FOS) to exert similar effects to those of the breast milk. Nowadays, a great number of infant formulas enriched with prebiotics are disposal in the market, however there are scarce data about their composition. In this study, the combined use of two chromatographic methods (GC-FID and HPLC-RID) has been used for the quantification of carbohydrates present in commercial infant formulas. According to the results obtained by GC-FID for products containing prebiotics, the content of FOS, GOS and GOS/FOS was in the ranges of 1.6-5.0, 2.8-3.8 and 4.8-5.3 g/100 g of product, respectively. HPLC-RID analysis allowed quantification of maltodextrins with DP up to 19. The methodology proposed here may be used for routine quality control of infant formula and other food ingredients containing prebiotics.

Keywords: infant formula, prebiotics, GOS, FOS, maltodextrins

1. Introduction

Although human milk is considered the best food to satisfy the nutritional newborn infant's needs, there are some situations where breastfeeding must be interrupted and infants are fed with infant formula (IF) or cases where mothers cannot produce enough milk to supply all of their baby's nutritional needs and they combine breast and formula feeding. The knowledge gained on chemical composition and biological properties of human milk allows adapting the composition of IFs to meet the nutritional needs of the newborns.

Human milk contains 8-13 g/L of a complex mixture of oligosaccharides which is about 20-fold higher than those of bovine milk (Urashima, Taufik, Fukuda, & Asakuma, 2013). This is one of the factors that explain, at least in part, the higher level of intestinal bifidobacteria and lactobacilli, as well as, the lower incidence of bacterial infections found in breast-fed infants (Barile & Rastall, 2013).

Since Gibson and Roberfroid (1995) introduced the concept of prebiotics as "non-digestible oligosaccharides that reach the colon without being hydrolysed and are selectively metabolized by health-positive bacteria such as bifidobacteria and lactobacilli thereby exerting a beneficial effect on the host health", extensive research has been carried out to identify prebiotic components. A growing number of *in vitro* and *in vivo* studies also show that prebiotics could induce beneficial physiological effects in the colon and also in extra-intestinal compartments or contribute towards the reduction of the risk of dysbiosis and associated intestinal and systemic pathologies (Roberfroid et al., 2010). Several other studies strongly suggest that human milk oligosaccharides (HMOs) act as prebiotics (Bode, 2009; Engfer, Stahl, Finke, Sawatzki, & Daniel, 2000) and have a wide range of biological activities. However, as HMOs are very complex glycans, their production at industrial scale is very difficult (Bode, 2009).

Only the enzymatic synthesis of some prebiotic such as galactooligosaccharides (GOS) and fructooligosaccharides (FOS) is a feasible alternative to produce them in the food industry. Particularly, it has been shown that the addition of different amounts of GOS, FOS or GOS/FOS mixtures to IF stimulates the growth of bifidobacteria and lactobacilli (Ben et al., 2008; Boehm et al., 2002; Moro et al., 2002), produces changes in the short chain fatty acids making the profile of these acids closer to that observed in breast-fed infants (Knol et al., 2005), improves the stool characteristics (frequency, pH and softening) (Ben et al., 2008; Fanaro et al., 2005; Moro et al., 2002) and reduces the incidence of allergic manifestations and infections during the first two years of life (Arslanoglu et al., 2008).

IFs are composed basically by carbohydrates (54 – 61 g/100 g of product) and proteins (11 - 15 g/100 g of product). Depending on the type of IF, sugars such as lactose, corn syrup, sucrose or starches have been successfully used as a source of carbohydrates (Morales, Olano, & Corzo, 2004). Besides, in the last years, prebiotic oligosaccharides have been added to IFs to mimic the benefits attributed to HMOs (Barile & Rastall, 2013; Braegger et al., 2011; Cilla, Lacomba, Garcia-Llatas, & Alegria, 2012). Despite the increasing use of prebiotics in IF production, there are scarce data about their prebiotic composition. In this work, a study on the carbohydrate composition of 24 commercial IFs, selected as representative of the Spanish market, has been carried out. Special attention has been paid to the determination of prebiotic carbohydrates.

2. Materials and methods

2.1. Reference substances and samples

Analytical reference substances such as fructose, galactose, glucose, *myo*-inositol, lactose, maltulose, maltose, kestose, nystose and maltodextrins with a degree of polymerization (DP) from 2 to 5 were purchased from Sigma (St. Louis, MO, USA). Raftilose® and Wako® FOS were from Orafti (ORAFTI, Barcelona, Spain) and Wako (Chemical Industries, Osaka, Japan), respectively. Vivinal® GOS were from Domo (Friesland Campina Domo, Amersfoort, Netherlands).

Twenty four IFs were purchased from several Spanish chemist's, corresponding to starting and follow-up formulas without prebiotics (n=8), 4 with lactose (C) and 4 lactose free (LF), and prebiotic-enriched IF (n=16), 3 with FOS (PFOS), 7 with GOS (PGOS), and 6 with mixtures of GOS/FOS (PGOS/FOS). Table 1 shows their carbohydrate composition as indicated on the product labels. All samples were analysed before their expiry date and all determinations were done in duplicate.

2.2. Determination of carbohydrates

Before chromatographic analysis, fat and protein interfering materials were removed by precipitation, using Carrez reagents (Moreno, Olano, Santa-Maria, & Corzo, 1999).

Carbohydrate (monosaccharides, disaccharides, GOS and FOS) quantification was carried out by GC-FID, following the method of Montilla, van de Lagemaat, Olano and del Castillo (2006). Analysis of maltodextrins were performed by HPLC-RID according to Corzo-Martínez, Copoví, Olano, Moreno and Montilla (2013).

To quantify GOS in the IFs containing maltodextrins and to avoid interference in the GC analysis, samples were incubated at 37°C for 24 h with α-amyloglucosidase (*Megazyme*©3300, Bray Co. Wicklow, Ireland) (330 U/mL IF reconstituted at 100 mg/mL) in order to remove them.

3. Results and discussion

The GC method of Montilla et al. (2006) has been used in this study to determine carbohydrate composition of commercial IFs. The repeatability was calculated using an IF containing prebiotics. Four replicates of this formula were analysed daily during the following 4 days (Table 2). As it can be observed, the repeatability was acceptable for all carbohydrates measured with relative standard deviations below 10%, with the exception for hexa and heptasaccharides (12,3%), similar results were obtained by Montilla et al. (2006). GC profiles of the carbohydrates found in the starting and follow–up IFs with prebiotics are depicted in Figure 1. The used GC method allowed the quantification of the monosaccharides fructose, galactose and glucose, *myo*-inositol, the disaccharides sucrose and lactose, and the prebiotic oligosaccharides GOS and FOS with a degree of polymerization (DP) of up to 7. Figure 1 also shows the carbohydrate profiles of commercial Vivinal®GOS (Figure 1A) and Raftilose®FOS (Figure 1B), commonly added to IF products. It can be observed that the chromatographic profiles of commercial GOS and FOS were similar to those found in IFs, containing these carbohydrates. The chromatographic profiles of IFs containing GOS and maltodextrins after hydrolysis using α-amyloglucosidase are also shown in Figure 2. In this case, the previous hydrolysis of maltodextrins allowed the quantification of GOS.

Table 3 shows the carbohydrate composition of the analysed commercial IFs. In general, fructose, glucose and galactose, were detected in the majority of the studied IFs. Their concentrations were quite similar to those previously found in other IFs (Morales et al., 2004). Among them, glucose was the major monosaccharide with highly variable contents. Regarding to fructose, only three samples showed contents higher than 0.1 g/100 g, probably due to the presence of free fructose in the products with added FOS. The galactose content was higher than those found by Morales et al. (2004) and similar to those obtained by Troyano, Villamiel, Olano, Sanz and Martinez-Castro (1996) in commercial sterilized milks. *Myo*-inositol, a polyalcohol that may play a significant role in the prevention of bronchopulmonary dysplasia and retinopathy in premature infants (Hallman, Saugstad, Porreco, Epstein, & Gluck, 1985), was present in all IFs at concentrations significantly higher than those reported for Spanish commercial milks (Troyano et al., 1996), but similar to the found by Woollard, Macfadzean, Indyk, McMahon and Christiansen (2014). As expected, with the exception of the lactose-free IFs, lactose was the main carbohydrate in all studied products, with amounts varying between 19.2 and 70.1 g/100 g. Maltose was found in most of the analysed IFs in a range from 0.2 to 7 g/100 g. This compound is usually not added as ingredient in IFs and its presence may be related to the partial hydrolysis of the added maltodextrins during processing. Some low amounts of sucrose were found in the formulas enriched with FOS and in those enriched with GOS/FOS (0.13 – 0.19 and 0.01 – 0.05 g/100 g, respectively). Maltulose was also detected in five IFs in a range from 0.05 to 0.23 g/100 g (data not shown). This isomer of maltose was detected for the first time in IF by Morales et al. (2004), who found a wide variability from 0.13 to 0.8 g/100 g product, attributing this to differences in processing conditions.

The analysis of maltodextrins by HPLC-RID allowed the quantitative determination of oligosaccharides with DP of up to 19. As it can be seen from the Table 3, maltodextrins were the second major component (after lactose) of most of the studied products with contents varying from 0.84 to 45.7 g/100 g of product. Among them, the most abundant compounds were those with DP between 3 and 6 (data not shown).

In the IF containing prebiotic oligosaccharides the content of FOS and GOS were in the ranges of 1.6-5.0 and 1.7-3.2 g/100 g, respectively. In the products enriched with a mixture of FOS and GOS the content was in the range 0.08–0.25 and 2.3-3.8 g/100 g, respectively. Some of these values and especially those referred to the products enriched with FOS were below the officially declared values on the labels. These differences are probably due to the difficulties of quantification of oligosaccharides with a high DP (inulins), since it was specified on the label that these formula contained these polysaccharides.

The analysed IFs presented prebiotic oligosaccharide contents in the range of 2.0 to 6.4 g/L (considering IF reconstitution of 12.5% w/v). In Europe, IFs are supplemented with prebiotic oligosaccharides according to Directive 2006/141/CE, which allows addition of these carbohydrates in amounts of up to 8 g/L. Different studies have shown that this concentration limit is strictly respected and that the most commonly assayed prebiotics used in IFs are FOS, GOS and GOS/FOS mixtures (in a 9:1 ratio) at concentrations ranging between 1.5 - 8 g/L (Braegger et al., 2011). Several experimental studies have revealed that at these concentrations, prebiotics had a significant bifidogenic effect (Ben et al., 2008; Euler, Mitchell, Kline, & Pickering, 2005), modulates the intestinal flora and the immune system as human milk does (Fanaro et al., 2005) and provides beneficial effects for formula-fed infants (Boehm et al., 2005). Also, the amounts of prebiotics added to IF were generally well tolerated and they did not produce adverse side effects (crying, regurgitation or vomiting) (Boehm et al., 2002; Closa-Monasterolo et al., 2013).

4. Conclusion

This study shows that the analysed IFs present carbohydrate contents within the range indicated on the package labels. The combined utilization of GC and HPLC techniques allows an overall quantification of the carbohydrate fraction, including prebiotic oligosaccharides. GC-FID and HPLC-RID are rapid, simple, cheap, and powerful analytical techniques commonly found in academic and industrial laboratories. Beside, GC presents high resolving power, sensitivity and selectivity which enables the determination of higher oligosaccharides in foods that are often present at low concentrations.

The results have demonstrated the usefulness of GC as a powerful tool for the analysis of mono-, and disaccharides, as well as GOS, FOS and their mixtures in IFs. Furthermore, the methodology proposed here may be used for routine quality control of IF and other food ingredients containing prebiotics.

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Figure legends:

Figure 1. GC-FID profiles of trimethylsilyl oximes (TMSO) of the carbohydrates present in (A): Commercial Vivinal®GOS (red) and infant formula with GOS (blue). Peaks: 1: Galactose, 2: Glucose, 3: *Myo*-inositol, I.S.: Internal standard (phenyl-β-D-glucoside), 4: Lactose, 5: 6-Galactobiose, 6: 4'Galactosyl-lactose, 7: 6'Galactosyl-lactose, 8: 4'Digalactosyl-lactose; 9: 4'Trigalactosyl-lactose; and (B) Commercial Raftilose®FOS (red) and infant formula with FOS (blue). Peaks: 1: Fructose, 2: Galactose, 3: Glucose, 4: *Myo*-inositol, 5: Sucrose, 6: Lactose, 7: Maltose, 8: Kestose, 9: Trifructosaccharides, 10: Maltotriose, 11: Nystose, 12: Tetrafructosaccharides, 13: Maltotetraose, 14: Fructosyl-nystose, 15: Pentafructosaccharides, 16: Maltopentaose, 17: Difructosyl-nystose, 18: Hexafructosaccharides, 19: Trifructosyl-nystose, 20: Heptafructosaccharides. DP: Degree of polymerisation.

Figure 2. GC-FID profile of TMSO derivatives of carbohydrates present in a commercial infant formulas with maltodextrins and GOS before (blue) and after (red) of α-amyloglucosidase treatment. (1) GOS; (2) Maltodextrins. DP: Degree of polymerisation.

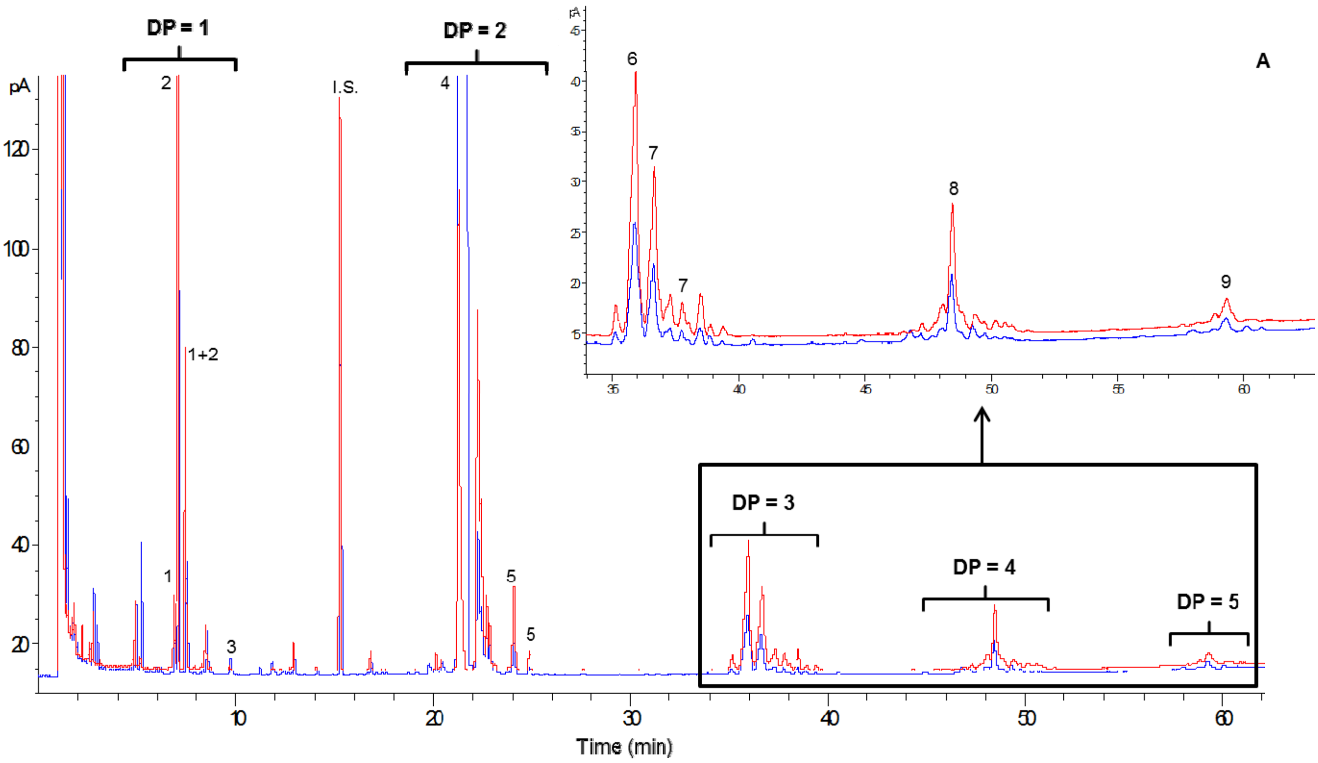


Figure 1A.

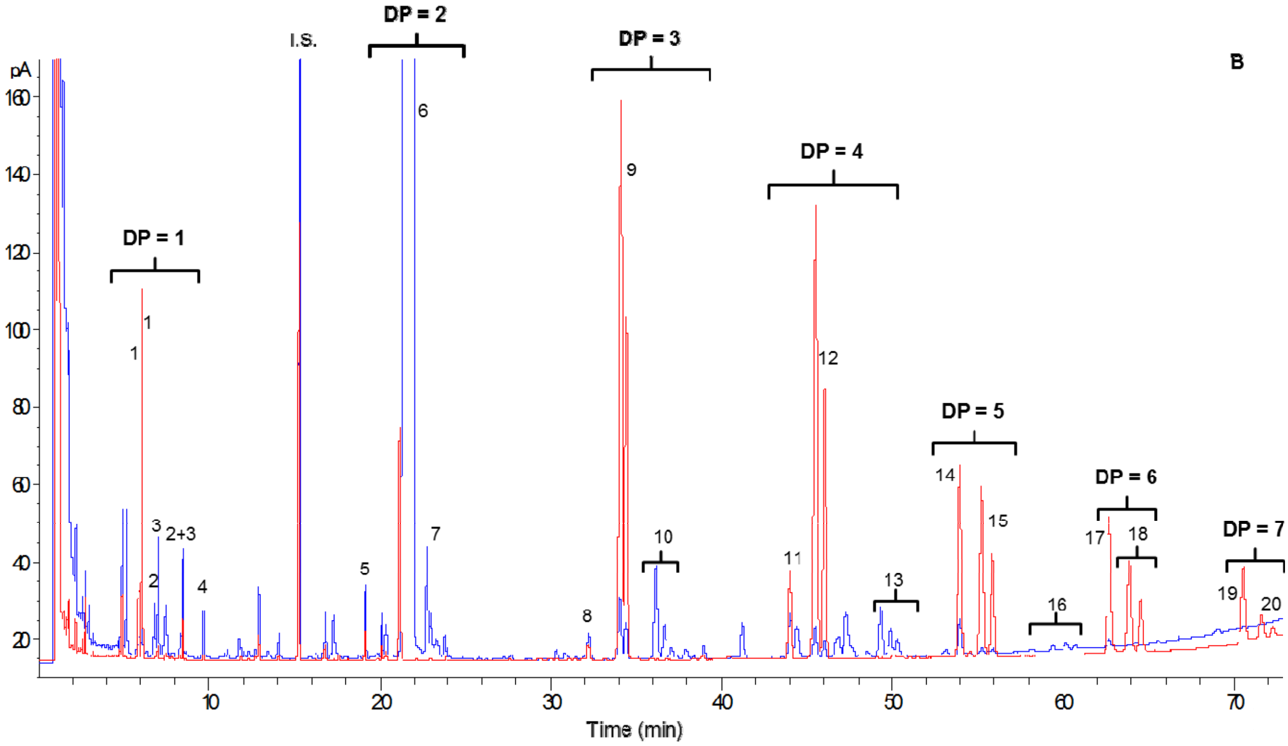
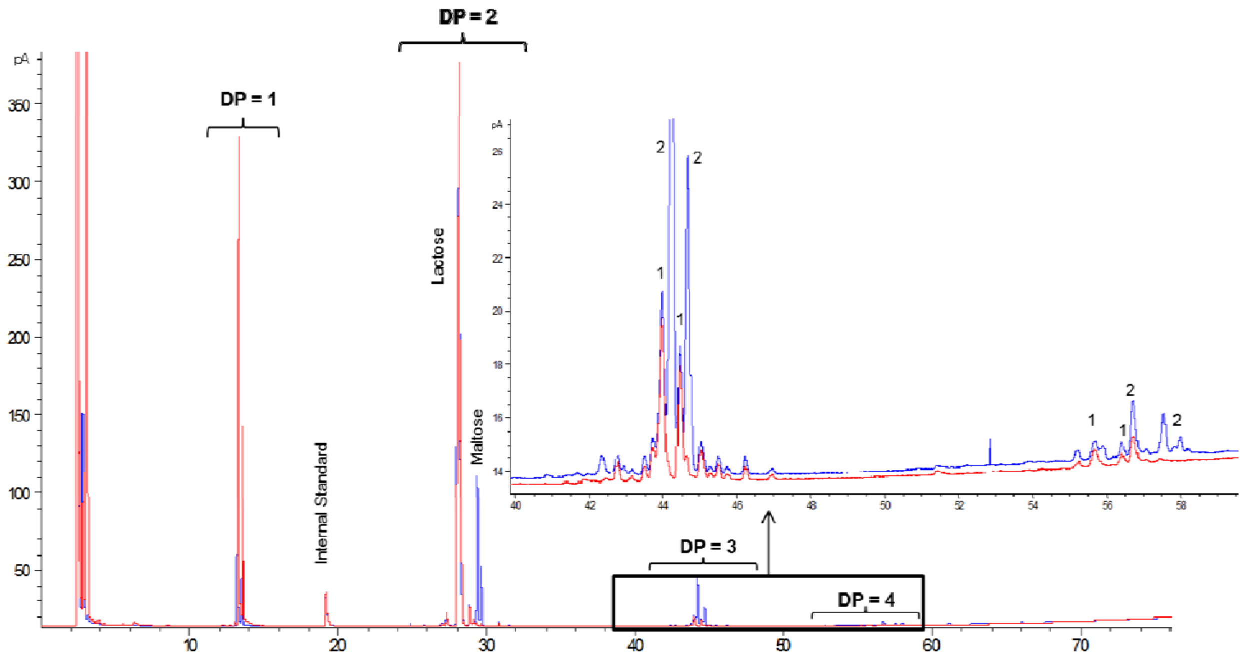


Figure 1B



DP = 2
pA
360
300
250
200
150
100
50
DP = 1
Internal Standard
Lactose
Maltose
DP = 3
DP = 4
10
20
30
40
50
60
70
2
2
1
1
1
1
2
2

Figure 2

Table 1. Carbohydrate composition of infant formulas available at the Spanish market, according to their package labels.

Product code	Total carbohydrates	Lactose	Prebiotics	Maltodextrins	Inositol	Observations
		(g/100 g product)			(mg/100 g)	
Infant formula without prebiotics						
Conventional ***(C)***						
C1	57.8	57.8			80.0	
C2	61.7	-[1]		-	-	mdx
C3	60.0	-		12.3	-	starch
C4	55.6	-		-	9.9	mdx
Lactose free ***(LF)***						
LF1	58.6			-	36.0	mdx
LF2	60.8			60.8	9.9	mdx
LF3	57.6			36.1	47.0	mdx
LF4	55.0			-	30.0	mdx
Infant formula with prebiotics						
With fructooligosaccharides (PFOS)						
PFOS1	53.3	-	5.7	-	45.0	FOS/inuline
PFOS2	56.0	-	3.0	-	45.0	FOS/inuline
PFOS3	50.8		5.7	50.8	25.0	without lactose
With galactooligosaccharides (PGOS)						
PGOS1	50.1	46.0	3.5	2.5[2]	32.0	GOS
PGOS2	55.0	-	1.5	1.5	51.0	GOS.mdx[3,4]
PGOS3	62.9	-	2.2	-	25.0	GOS.mdx
PGOS4	56.4	-	1.9	-	30.0	GOS.mdx
PGOS5	61.7	-	2.6	-	26.0	GOS.mdx
PGOS6	55.6	35.6	2.5	19.4	24.4	GOS.mdx
PGOS7	50.9	45.3	2.8	5.0	27.2	GOS.mdx
With galactooligosaccharides and fructooligosaccharides (PGOS/FOS)						
PGOS/FOS1	59.0	56.5	3.9		25.0	GOS/FOS
PGOS/FOS2	59.3	41.9	3.8	-	24.7	GOS/FOS.mdx[4]
PGOS/FOS3	59.3	41.9	3.8	-	24.7	GOS/FOS.mdx
PGOS/FOS4	59.3	41.9	3.8	-	24.7	GOS/FOS.mdx
PGOS/FOS5	55.7	19.7	3.8	-	23.0	GOS/FOS.mdx
PGOS/FOS6	53.6	-	2.9	-	-	GOS/FOS.mdx

[1] The data did not appear on the label; [2] High molecular mass dextrin; [3] mdx: maltodextrins;
[4] Infant formula containing GOS.mdx and GOS/FOS.mdx were treated with α-amyloglucosidase.

Table 2. Repeatability of the used GC method for determination of carbohydrates in a commercial infant formula containing prebiotics, prepared four times and analysed on the same day and on different days (four days).

Carbohydrate	Concentration (mg/100 g of product)			
	Same day (**four** replicates)		Daily (during 4 days) (one replicate)	
	Average value	RSD* (%)	Average value	RSD (%)
Fructose	0.33	4.4	0.33	4.6
Galactose	0.02	8.3	0.02	8.8
Glucose	0.18	4.7	0.19	5.3
Myo-inositol	0.06	4.3	0.05	4.5
Sucrose	0.18	5.6	0.17	4.4
Lactose	41.61	3.9	41.7	3.6
Maltose	0.30	7.3	0.32	6.8
Trisaccharides	0.60	6.3	0.59	7.3
Tetrasaccharides	0.59	7.3	0.56	7.6
Pentasaccharides	0.25	6.9	0.27	7.4
Hexa and heptasaccharides	0.26	9.9	0.23	12.3

*RSD: relative standard deviation

Table 3. Carbohydrate content encountered (g/100 g) in the commercial infant formula under study (data shown as average value ± SD).

Product code	Fructose	Galactose	Glucose	*Myo*-inositol	Sucrose	Lactose	Maltose	GOS	FOS	Maltodextrins (DP 3-19)[1]	TCH[2]
C1		0.21 ± 0.00	0.27 ± 0.01	0.07 ± 0.00		70.1 ± 1.2					70.77
C2	0.02 ± 0.00	0.25 ± 0.01	0.39 ± 0.02	0.03 ± 0.00		30.7 ± 1.7	1.43 ± 0.06			11.7 ± 2.7	44.62
C3	0.01 ± 0.00	0.26 ± 0.00	0.15 ± 0.00	0.05 ± 0.00.		45.5 ± 0.3	0.20 ± 0.00			0.8 ± 0.2	46.96
C4	0.05 ± 0.00	0.05 ± 0.00	1.59 ± 0.04	0.08 ± 0.01		22.2 ± 0.6	4.98 ± 0.11			22.5 ± 0.2	51.53
LF1	0.05 ± 0.00		1.27 ± 0.04	0.01 ± 0.00			5.73 ± 0.20			41.0 ± 0.6	48.14
LF2	0.04 ± 0.00	0.07 ± 0.00	0.99 ± 0.00	0.01 ± 0.00			3.01 ± 0.01			27.8 ± 5.7	31.88
LF3	0.03 ± 0.00		3.96 ± 0.04	0.03 ± 0.00			7.02 ± 0.05			45.7 ± 2.1	56.84
LF4	0.03 ± 0.00		1.53 ± 0.01	0.03 ± 0.00			5.11 ± 0.01			30.1 ± 1.6	36.85
PFOS1	0.34 ± 0.17	0.02 ± 0.00	0.19 ± 0.10	0.05 ± 0.00	0.17 ± 0.06	41.6 ± 1.7	0.30 ± 0.03		1.75 ± 0.07	4.3 ± 0.5	48.74
PFOS2	0.13 ± 0.01	0.08 ± 0.00	0.17 ± 0.01	0.06 ± 0.00	0.13 ± 0.01	47.5 ± 2.7	0.37 ± 0.00		1.57 ± 0.06	5.3 ± 0.7	55.27
PFOS3	0.05 ± 0.00	0.13 ± 0.01	0.69 ± 0.03	0.03 ± 0.00	0.19 ± 0.01		2.87 ± 0.13		5.00 ± 0.25	35.9 ± 0.2	44.90
PGOS1	0.03 ± 0.01	0.16 ± 0.01	1.29 ± 0.04	0.05 ± 0.00		44.5 ± 1.0	0.39 ± 0.01	3.16 ± 0.14			49.58
PGOS2	0.03 ± 0.00	0.23 ± 0.01	0.92 ± 0.02	0.06 ± 0.00		45.2 ± 0.9	0.32 ± 0.01	1.70 ± 0.04		1.7 ± 0.3	50.18
PGOS3	0.04 ± 0.00	0.13 ± 0.00	1.16 ± 0.00	0.04 ± 0.00		38.5 ± 0.0	1.57 ± 0.02	2.24 ± 0.05		11.3 ± 0.7	55.05
PGOS4	0.03 ± 0.00	0.12 ± 0.02	0.81 ± 0.09	0.04 ± 0.00		49.1 ± 0.0	0.54 ± 0.07	1.86 ± 0.14		2.3 ± 0.1	54.80
PGOS5	0.03 ± 0.00	0.15 ± 0.01	1.28 ± 0.13	0.04 ± 0.00		36.4 ± 3.5	1.57 ± 0.17	2.35 ± 0.05		11.1 ± 0.3	52.9
PGOS6	0.05 ± 0.00	0.13 ± 0.01	1.09 ± 0.09	0.02 ± 0.00		35.0 ± 3.1	0.82 ± 0.09	2.39 ± 0.07		14.5 ± 1.9	53.99
PGOS7	0.05 ± 0.00	0.16 ± 0.00	1.34 ± 0.02	0.05 ± 0.00		46.3 ± 3.2	0.72 ± 0.02	2.68 ± 0.14		4.2 ± 0.7	55.49
PGOS/FOS1	0.03 ± 0.00	0.12 ± 0.01	1.59 ± 0.14	0.03 ± 0.01	0.01 ± 0,00	51.1 ± 4.0		3.67 ± 0.11	0.13 ± 0.02		56.70
PGOS/FOS2	0.04 ± 0.00	0.20 ± 0.04	1.83 ± 0.16	0.04 ± 0.00	0.01 ± 0.00	39.9 ± 3.8	1.28 ± 0.24	3.73 ± 0.16	0.16 ± 0.00	26.2 ± 2.3	73.39
PGOS/FOS3	0.16 ± 0.21	0.17 ± 0.01	0.64 ± 0.14	0.03 ± 0.00	0.05 ± 0.05	38.5 ± 2.0	1.14 ± 0.12	3.64 ± 0.18	0.13 ± 0.01	15.0 ± 1.8	60.09
PGOS/FOS4	0.03 ± 0.00	0.12 ± 0.01	1.60 ± 0.16	0.03 ± 0.00	0.01 ± 0.00	33.9 ± 3.1	1.22 ± 0.09	3.79 ± 0.15	0.09 ± 0.00	11.1 ± 0.3	52.40
PGOS/FOS5	0.07 ± 0.01	0.18 ± 0.01	2.06 ± 0.10	0.03 ± 0.00	0.01 ± 0.00	19.2 ± 0.9	2.15 ± 0.11	3.70 ± 0.03	0.08 ± 0.01	19.4 ± 2.3	46.81
PGOS/FOS6	0.04 ± 0.00	0.11 ± 0.00	1.22 ± 0.02	0.07 ± 0.00	0.04 ± 0.00	29.5 ± 0.6	1.69 ± 0.03	2.28 ± 0.20	0.25 ± 0.05	21.5 ± 1.7	56.74

[1]Values obtained by HPLC analysis.

[2]TCH: Total carbohydrates. Each value is the sum of fructose, galactose, glucose, *myo*-inositol, sucrose, lactose, maltose, GOS, FOS and maltodextrins (DP 3-19).

Product code: like in table 1.