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RESISTANT OLIGOSACCHARIDES— ANALYTICAL METHODOLOGY

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A Sensitive and Reproducible Analytical Method to Measure Fructooligosaccharides in Food Products

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INTRODUCTION

Short-chain fructooligosaccharides (DP5) (Actilight®, Nutraflora®) are undigestible oligosaccharides industrially produced from sucrose. A recent survey (1) was conducted to get the views of professionals in the field concerning the definition of dietary fibers (DF). The majority felt that the definition of DF should

be revised to include oligosaccharides which are resistant to hydrolysis by the alimentary tract. The AOAC DF analytical method does not measure short-chain fructooligosaccharides (FOS) because of their ethanol/water mixture solubility.

In order to overcome this problem, we developed an analytical method to measure short-chain FOS in food products (small cakes, dairy products). This method is based on an extraction step, followed by anion exchange liquid chromatography (Dionex) associated with an hydrolysis step using invertase. This method has been applied to two types of small cake and three dairy products. In all cases 100% of added FOS has been recovered and measured. The results demonstrated the validity and reliability of the method for determining FOS in food products.

Fructooligosaccharides are naturally occurring molecules present in numerous edible plants. For example, they have been found in asparagus (2), banana (3) and onion (4). With a polymerization degree lower than 5, FOS are composed of one sucrose molecule to which is bound one to three fructose molecules. The resulting compounds are 1-kestose or GF2, nystose or GF3, and fructosylnystose or GF4. The production of these FOS from sucrose is protected by patents owned by the Japanese company Meiji Seika Kaisha. In Europe, these patents are exploited through a Béghin-Say-Meiji Seika Kaisha joint-venture : Béghin-Meiji-Industries.

The commercialization of such a new ingredient for human food involves the investigation of its nutritional properties. First of all, in vitro studies incubating FOS with human saliva, rat pancreas homogenates, rat and rabbit intestine mucosa (5), purified sucrase, α-amylase and isomaltase (3) have demonstrated that FOS are not hydrolyzed by digestive enzymes.

We did complementary in vitro and in vivo studies on humans (from stomach to stools). The first in vitro study showed the stability of FOS incubated with human gastric juice (personnal communication). The second in vitro study showed the stability of FOS incubated with small intestine mucosa (6). The third study, in vivo, concerning the adsorption of FOS along the small intestine, showed the non-adsorption in this part of the intestine and allowed us to determine the energy value of FOS : 2 Kcal/g.

In another we have demonstrated that FOS were fermented preferentially by bifidobacterium microflora and thus increased this population as well as the fructooligohydrolase activity of stools.

Therefore FOS, like dietary fibers, enter the large intestine without any change in their structure. At this stage, they are totally fermented by the resident microflora and not directly used as an energy source.

As a conclusion of all these studies, in 1994, the French ''Conseil Supérieur d'Hygiène Publique'' (Council for Public Health) admitted for the first time a functional claim for a food ingredient. Industrial users can now claim the stimu-

lating effect of FOS on the growth of bifidobacteria microflora in the human colon. It must be noted that the use of this claim is limited to G(F)n type molecules for which n is lower than 4.

Furthermore, a recent survey by Lee and Prosky (1) in 1995 conducted to obtain the view of professionnals concerning the definition of dietary fibers supports that dietary fibers should be re-defined to include short chain FOS resistant to hydrolysis by the alimentary tract.

The AOAC procedure for the determination of total dietary fiber involves three enzymatic digestion steps with (α-amylase, protease and amyloglucosidase.

These enzymatic treatments do not modify the FOS structure. The obtained solution then undergoes an ethanol precipitation. Only the resulting precipitate is used for the determination of total dietary fibers with the AOAC method. FOS are soluble in an ethanol/water mixture, and because of this, the AOAC dietary fiber analytical method does not measure short chain FOS.

This is why we needed to develop an analytical method to measure short chain FOS in food products. Our aim was to determine the validity and the repeatability of a method to quantify FOS in dairy products and cakes.

MATERIALS AND METHODS

Products

The concentrations of FOS which were determined in food products came from the Actilight* P powder which had a mass composition of quantifiable sugars as follows: glucose (G) 1.0%, fructose (F) 0.8%, sucrose (GF) 3.3%, GF2 38.8%, GF3 46.6% and GF4 9.5%.

Food Products

A Danone plain yogurt
Composition: milk of 13 g fat per liter, powder skimmed milk, yogurt starters. Mean nutritional value per 100 g: proteins 4.4 g, lipids 1.2 g, glucides 6.4 g, calcium 164 mg, sodium 57 mg, potassium 210 mg, phosphorus 114 mg.

A Danone Bio yogurt
''Plain fermented milk with active bifidus''. Composition: milk of 3.5% fat per liter, powder skimmed milk, Bifidobacterium (ACTIVE BIFIDUS). Mean nutritional value per 100 g: proteins 4.5 g, lipids 4.4 g, glucides 6.5 g, calcium 166 mg.

A Lactel fruit yogurt (strawberry)
Composition: full fat milk, sugar (11.5%), strawberry (8%), powder skimmed

milk, selected yogurt starters. Mean nutritional value per 100 g: proteins 3.4 g, lipids 2.6 g, glucides 15.4 g, calcium 129 mg.

A LU ''Petit Brun Extra'' biscuit
Composition: wheat flour, sugar, vegetable fat, invert sugar, powder skimmed milk, baking powder, salt, artificial aroma, antioxidant.

A J. PASQUIER Pâtissier ''Madeleine Superstar'' small cake
Composition: wheat flour, egg, sugar, butter, stabilizing agent E420, glucose, powder skimmed milk, baking powder, salt, artificial aroma.

Sample Preparation

The product was diluted 5 times (Md: final mass of dilute yogurt) with a solution of Actilight P (final concentration of approximately 1.5 g FOS per 100 g yogurt and 20 g FOS per 100 g bakery product). The suspension thus obtained was homogenized 5 min at 4°C with a high speed mixer (Ultraturax: Labortechnik, Saufer, Germany). Aliquots were taken (10 aliquots of a known mass Mk of approximately 10 g) and centrifuged 20 min, 2500 g at 4°C. A known volume, Vs, was filtered on a 0.45 μm porous filter, ultrafiltered by centrifuging 25 min at 2500 g on a 30 kD porous membrane (Microsep, Filtron, Northborough, MA), diluted, and then analyzed by anion exchange chromatography (Dionex). The pellet was weighed and dried in order to determine the volume (Vp) of liquid it contained.

Determination of Recovery of FOS Contained in Yogurt

The quantity of FOS determined is calculated as follows:

FOS quantity in g $= C(Vs + Vp)(Md/Mk)$

where

- C = FOS concentration in sample (g/L), determined by anion exchange chromatography
- Vs = volume of supernatant (L)
- Vp = volume of liquid in pellet (L)
- Md = mass of dilute yogurt (g)
- Mk = sample known mass (g)

The recovery corresponds to 100 times the ratio of the amount of FOS determined to the amount of FOS added to the yogurt.

Validity of the Analysis

To establish the sugar composition of products without FOS addition, they were diluted 5 times with ultra pure water. The suspension thus obtained was treated and analyzed as previously described.

To determine the nature of the oligosaccharides present in FOS containing samples, the supernatants obtained during sugar extraction were incubated 6 hours at 40°C in the presence of invertase (Max Invert, Gist Brocade, Delft, Netherlands, final activity: 1326 U/L reaction medium). One U of invertase activity was defined as the amount of enzyme which produced one mole of fructose per min at 60°C in a 0.5 mol/L sucrose solution in 0.1 mol/L acetate buffer at pH 4.5.

Invertase is an enzyme hydrolyzing the $\beta 1 \rightarrow 2$ linkage between glucose and fructose in sucrose, as well as the $\beta 1 \rightarrow 2$ linkages between fructoses in FOS. This step allows us to confirm the results of the direct analysis, by comparing them to the amount of glucose and fructose produced during hydrolysis. The solutions obtained before and after treatment by invertase were injected directly on the Dionex Ion Chromatograph.

Analysis of the Products by Dionex Ion Chromatograph

Principle of the Determination of Sugars by Dionex Ion Chromatograph

The determination of sugars was carried out on a Dionex (Sunnyvale, Ca, USA) chromatograph equipped with a gradient pump, a degas module and a pulsed amperometric detector (p.a.d.). The column used was a Dionex, Carbopac TM PA1 (4 × 250 mm) with a precolumn Carbopac PA (3 × 25 mm). The p.a.d. sensitivity was set at 3K. Results were recorded on a Hewlett-Packard H.P. 3365 integrator, series II. The volume of the injection loop was 25 μL. Detection was performed by a gold electrode under three alternating potentials. The values and duration of these potentials are: E1 = 0.05 V, t1 = 480 ms; E2 = 0.65 V, t2 = 120 ms; E3 = −0.65 V and t3 = 60 ms, respectively. The elution gradient was established by combining an eluent A (NaOH 150 mM) with an eluent B (NaOH 150 mM, NaOAc 600 mM). In the course of the analysis, sodium acetate concentration varied from 6 to 180 mM in 20 min with a constant flow rate of 1 mL/min. Eluents were prepared with ultra pure water which had been degassed by sparging with helium. Under these conditions, it was possible to separate all the products contained in Actilight*950P.

Calibration of the Pulsed Amperometric Response

FOS standard solutions were prepared from purified standards. Quantities of 300 μL were injected with a Spectra Physics SP8875 autosampler. A linear response

was obtained on the integrator for concentration values ranging from 0.15 to 70 mg/L. The lower detection limit was based on a peak height at least three times that of the background noise.

RESULTS AND DISCUSSION

Validity of the Method and Determination of the Recovery

In order to establish the sugar composition of the control dairy products, a sample of each yogurt without FOS addition was treated and analyzed as previously described.

For all yogurts, the determination showed only a simple sugar, galactose, and a disaccharide, lactose. The retention times of these sugars differed from those of FOS and therefore should not interfere with the determination of FOS in yogurt.

Inversely, the determination of sugars in the control cake with no FOS addition showed two simple sugars, glucose and fructose, one disaccharide, sucrose, and two starch residues, Rs1 and Rs2, the retention times of which correspond respectively to those of GF2 and GF3 peaks (Figure 1).

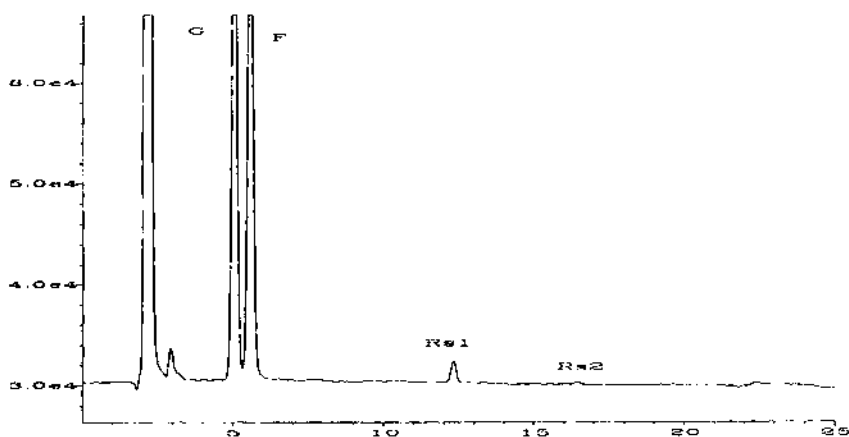As can be seen in Figure 2, the H.P.L.C. analysis of the supernatant of a

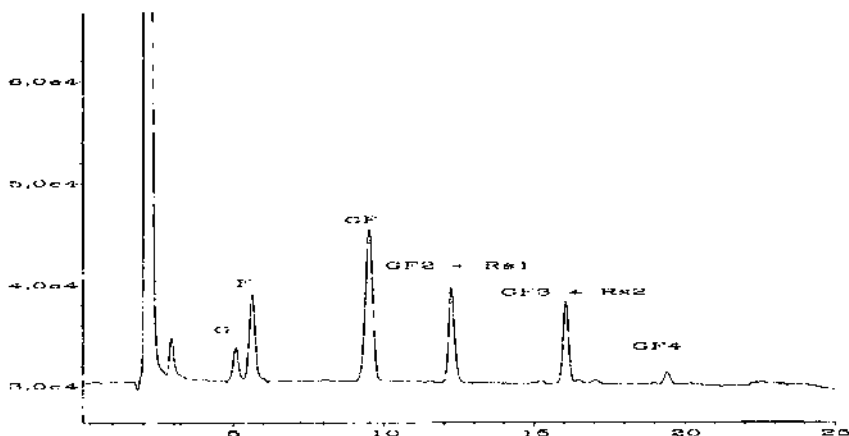**FIGURE 1** Determination of sugars in the control cake before FOS addition.

FIGURE 2 Determination of sugars in a small cake after FOS addition.

small cake (J. Pasquier pâtissier ''madeleine superstar'') after FOS addition showed glucose (G), fructose (F), sucrose (GF), GF2, GF3 and Rs1 and Rs2.

To investigate the nature of the oligosaccharides present in cakes containing FOS, the supernatants obtained during sugar extraction were incubated in the presence of invertase.

After the invertase action (Figure 3) the disappearance of oligosaccharides can be observed, correlated to an increase in glucose and fructose concentrations. On the other hand, Rs1 and Rs2 were not hydrolyzed, and appear on the chromatogram. If the specific areas of GF2 and GF3 were related to Rs1 and Rs2, respectively, the theoretical concentrations of these sugars in the supernatants analyzed could then be determined. The theoretical amounts (in g) of Rs1 and Rs2 in cakes can be calculated from the chromatogram corresponding to Figure 3. The recovery is then calculated as follows:

$$\frac{[(GF2 + RS1) - RS1] + [(GF3 + RS2) - RS2] + GF4}{(GF2i + GF3i + GF4i)} \times 100$$

where:

- GF2i = quantity (in g) of GF2 introduced in cake
- GF3i = quantity (in g) of GF3 introduced in cake
- GF4i = quantity (in g) of GF4 introduced in cake
- GF2 + RS1 = quantity (in g) of GF2 + RS1 determined in cake containing FOS
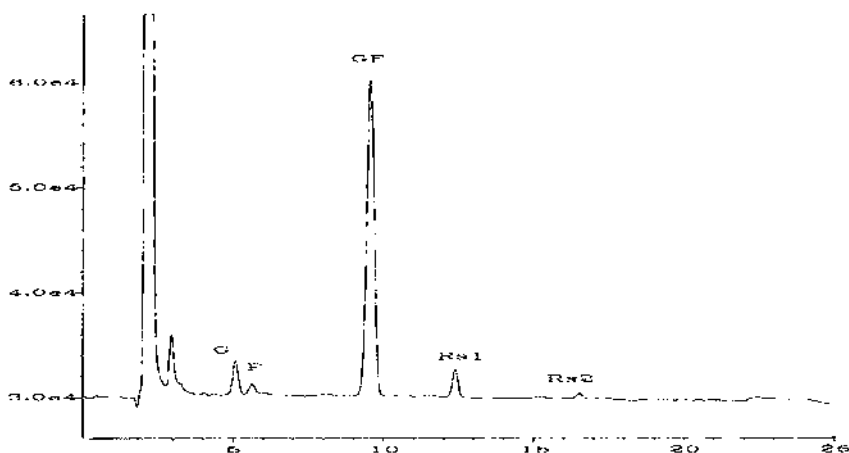
FIGURE 3 Determination of sugars in a supernatant of a small cake after invertase addition.

- GF3 + RS2 = quantity (in g) of GF3 + RS2 determined in cake containing FOS
- RS1 = quantity (in g) of RS1 determined in control cake without FOS
- RS2 = quantity (in g) of RS2 determined in control cake without FOS

With the invertase treatment, sucrose and FOS could be specifically hydrolyzed and the recovery of fructooligosaccharides calculated. The results obtained during direct analysis could also be verified by measurement of the glucose and fructose produced during hydrolysis (Table 1). Indeed, a quantitative study

TABLE 1 Sugar Concentrations in a Small Cake After FOS Addition

| | Calculated sugar concentration before inverse hydrolysis: | | | | | | | |
Sugars	G	F	GF	GF2 + RS1	GF3 + RS2	GF4	Total glucose	Total fructose
g/L	2.3	7.6	38	11	12.1	3.2		
mM	13.3	42.2	111	21.8	18.2	3.9	168.2	267.3

| Concentration of glucose and fructose produced during the hydrolysis step: | | |
Sugars	G	F
g/L	30.4	47.221
mM	168.7	261.9

demonstrated a direct correlation between the concentrations of FOS analyzed, and the quantities of glucose and fructose produced (Table 1).

The results demonstrated with certainty the validity of the method for determining FOS levels in cakes.

Recovery of FOS in Food Products

The final FOS concentration studied was 1.5 g fructooligosaccharide per 100 g of yogurt. The extraction rates of FOS are listed in the table. As can be seen, for each sample, the study of the repeatability of the extraction method was done by treating each sample ten times. For plain yogurt, the mean recovery is equal to 99.7% of initial FOS, with a standard deviation of 1.9%. The recovery of FOS from Bio yogurt is equal to 99.2% of initial FOS with a standard deviation of 2.4%. For fruit yogurt, the mean extraction rate is equal to 99.2% of initial FOS with a standard deviation of 1.3%. The results obtained for cakes can be observed in Table 3. Here, the final fructooligosaccharide concentration studied was 20 g FOS per 100 g of cake.

We studied the repeatability of the extraction method in the same way as for dairy products. For cookies ''Petit Brun'' LU, the mean recovery was 100.9% of the initial FOS with a standard deviation of 1.5%. For J. Pasquier pâtissier ''Madeleine Superstar'' cakes, the mean recovery was 99.7% of the initial fructooligosaccharide with a standard deviation of 1.1%.

The results obtained for yogurts can be observed in Table 2.

TABLE 2 Recovery of FOS Contained in Dairy Products

Extraction	Recovery of FOS in dairy products (%)		
	Plain yogurt	Bio yogurt	Fruit yogurt
1	97.4	100.4	101.1
2	99.1	102.6	99.9
3	97.6	97.8	100.4
4	98.0	97.5	99.7
5	100.3	102.1	100.0
6	103.9	98.1	97.7
7	100.9	99.8	99.0
8	99.8	94.4	100.0
9	98.9	98.4	98.3
10	101.2	101.4	96.4
Average	99.7	99.2	99.2
Standard deviation	1.9	2.4	1.3

TABLE 3 Recovery of FOS Contained in Bakery Products

| Extraction | Recovery of FOS in cookies and small cakes (%) | |
	Petit brun	Madeleine superstar
1	100.8	100.1
2	103.5	100.6
3	100.1	98.8
4	102.3	100.6
5	100.7	100.9
6	101.8	97.2
7	99.1	100.2
8	98.4	99.5
9	99.6	NA
10	102.5	NA
Average	100.9	99.7
SD	1.5	1.1

Sensitivity of the Method for Determining FOS in Cakes and Dairy Products

With the defined experimental protocol and with the sensitivity of the H.P.L.C. method used, the detection limit of quantifiable FOS is 0.04 g per 100 g of yogurt and 0.75 g per 100 g of cake. However, in these conditions, in cakes there is an excess of maltooligosaccharides in relation to FOS. In such a product, we introduced the extra step of hydrolysis of the maltooligosaccharides with α-glucosidase, followed by hydrolysis with invertase.

CONCLUSION

From the results of our work, we believe a valid, sensitive method, with good repeatability, has been established for the analysis of FOS in small cakes and dairy products. The methodology involves the analysis of the original product with the Dionex Ion Chromatograph, followed by analysis of the product after hydrolysis with invertase.

It should be a complementary method to the AOAC procedure for the determination of total dietary fibers, which does not take into account the water/ethanol soluble fibers such as short chain FOS.

REFERENCES

1. Lee S. S. & Prosky L. (1995) J. of AOAC, Vol. 78, 22–36.
2. Cairns A. J. (1992) New Phytol., 120, 463–473.
3. Fishbein L., Kaplan M. & Gough M. (1988) Vet. Mum Toxicol., 30(2), April, 105–107.
4. Shiomi N. (1978) Jour. Facul. Agri., Hokkaido Univ., Sapporo, 58, pt 4.
5. Oku T., Tokunaga T. & Hosoya N. (1984) J. Nut., 114, 1574–1581.