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Determination of inulin in meat products by high-performance liquid chromatography with refractive index detection

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Abstract

Inulin is a naturally occurring carbohydrate with beneficial nutritional and technological properties. A high-performance liquid chromatographic (HPLC) method was developed for the quantitative determination of these β-fructans in meat products, containing this type of additive. The method includes extraction of inulin with hot water, followed by hydrolysis with inulinase enzyme, and determination of the released fructose by HPLC with refractive index detection. An internal standard of rhamnose was used to quantify fructose. The method incorporates a sample blank (without inulinase hydrolysis) for each specimen to subtract contributions of free fructose and fructose from sucrose. The results showed good precision with average RSDs of 2.4% for repeatability and 5.2% for reproducibility. Analytical recovery ranged from 102 to 106%. Satisfactory linearity ($r=0.999$) was obtained.

Keywords: Meat; Food analysis; Inulin; Carbohydrates; Fructans; Fructose

1. Introduction

The term dietary fibre covers those polysaccharides plus lignin that are not hydrolysed by the endogenous secretion of the human digestive tract [1]. Fibre addition in meat products is on the increase nowadays, due to its technological use [2–4] and its benefits to human health [2–7].

Inulin has specific technological assets, being soluble upon heating and bland in taste. It can be blended into a large number of different food products, which retain their intrinsic flavour without alteration of texture or of appearance. Furthermore, inulin helps to provide body, good mouthfeel and appearance, thus it can be used as a fat replacer for the emerging sector of lower energy food products. Thanks to its water-binding capacity, it displays gelling and thickening properties [2].

Inulin is a fructose polysaccharide that is not hydrolysed by the enzymes of the human small intestine [2,6,7]. In quantity it reaches the large bowel, where it is fermented into volatile fatty acids and lactic acid [2,6,7], so it is often considered a dietary fibre [2,6,8]. The fermentation of inulin selectively promotes the growth of the bifidobacteria populations [2,4,5,7,9], making inulin-type fructans the prototype prebiotic [7,10,11]. Furthermore, its caloric value is significantly lower (about 1 kcal/g) than that of digestible carbohydrates (4 kcal/g) [2,4–6,8].

The inulin molecule is a polydisperse polymer that contains an important fraction of oligosaccharides generally referred to as oligofructose, with a degree

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of polymerisation ranging from 3 to more than 60 [6,8,12,13]. It belongs to the fructan group, consisting of (2-1)-linked β-D-fructofuranosyl units terminating in glucose [2,6–8,12,14–17]. An important physico-chemical property of pure inulin is that it is almost insoluble in cold water, but easily soluble in hot water [2]. As its molecular mass is lower than that of current dietary fibre [2,15], it is not completely precipitated by 95% ethanol, so it cannot be assessed by the standard AOAC method used for dietary fibre analysis [2].

It is generally accepted that the most quantitative methods for measurement of inulin-type fructans, involve enzymatic hydrolysis of all fructan materials to fructose and glucose, followed by measurement of these sugars by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) [7,12], high-performance liquid chromatography with refractometer detection (HPLC–RI) [7] and colorimetric method [18]. Inulin determination has also been integrated into the AOAC method for the measurement of the soluble dietary fibre fraction [16] by inclusion of an enzymatic treatment to remove the residual β-fructans from the soluble fibre fraction [16].

The aim of the present investigation is to develop a reliable method to be used in routine quantitative and qualitative determination of inulin, and β-fructans in general, in meat products. This method consists of aqueous extraction of inulin followed by enzymatic hydrolysis with inulinase and the final quantification of fructose obtained, by high-performance liquid chromatography with refractive index detection, using rhamnose as internal standard.

The method was used to quantify the amount of inulin in meat products.

The validation parameters were established.

2. Experimental

2.1. Reagents and standards

All chemicals used for sample preparation were of analytical reagent grade: HPLC-grade acetonitrile (SDS, Peypin, France), HPLC-grade methanol (SDS), deionised water, water purified by passage through a Milli-Q apparatus (Millipore, Bedford, MA, USA). Acetic acid 96% and trihydrate sodium acetate (both analytical-reagent grade) were from Probus (Badalona, Spain).

Carrez I reagent comprised 24 g zinc acetate dihydrate (analytical-reagent grade; Sigma, St. Louis, MO, USA) and 3 g glacial acetic acid (analytical-reagent grade; Panreac, Barcelona, Spain) to 100 ml with water; Carrez II reagent comprised 10.6 g potassium hexacyaneferrate II trihydrate (analytical-reagent grade; Probus, Badalona, Spain) to 100 ml with water. The acetate buffer (0.1 *M*, pH 4.5) comprised 280 ml 0.1 *M* acetic acid and 220 ml 0.1 *M* sodium acetate, made up to 1000 ml with water.

D(−)Fructose and sucrose were used as standard sugars and L-rhamnose was used as internal standard, all 99% pure (Sigma).

Commercial inulin (Raftiline GR) from chicory root was a product of Orafti (Belgium).

The inulinase used (Fructozyme, which is a mixture of exo-inulinase EC 3.2.1.80 and endo-inulinase EC 3.2.1.7) was from Novo Nordisk (Denmark). Fructozyme is a liquid with a density of approximately 1.2 g/ml and an activity of 2000 INU/g. One Novo Nordisk inulinase unit (INU) is the amount of enzyme which forms 1 μmol of reducing sugar (calculated as glucose) per minute under the conditions of 40°C and pH 4.7.

The samples consisted of different commercial meat products (ham, mortadelle, chopped and chub-packed breast turkey) from Campofrío (Burgos, Spain), to which inulin was added as a food additive. These foods are heated meat products which have been prepared in two different ways: heated as a whole piece (ham and turkey breast) or heated as a mixture of different ingredients, emulsified products (mortadelle and chopped turkey). Each sample was carefully homogenised prior to analysis.

A C_{18} Sep-Pak Plus Cartridge (Waters, Milford, MA, USA) was used to purify the analyte before analysis.

2.2. Instrumentation

The chromatographic equipment consisted of a Hewlett-Packard (Waldbronn, Germany) Model 1050 pump system, a Rheodyne (Cotati, CA, USA) Model 7125 injector with a 20-μl sample loop, a refractive index detector (Model LC-25, Perkin-Elmer, Nor-

walk, CT, USA), and an HP 3365 series II Chemstation which acquired data from the refractive index detector.

The analytical column used was a Kromasil 100 NH_2 (250×4.6 mm I.D., 5 μm particle size) from Tracer (Teknokroma, Barcelona, Spain). A precolumn NH_2(13×3 mm I.D.) from Tracer was also used.

2.3. Chromatographic conditions

Chromatographic separation was achieved with a mobile phase of acetonitrile–water (80:20, v/v). The flow-rate of the eluent was 1.8 ml/min. The volume of the sample injected was 20 μl (filling the loop completely); 15 min were needed to complete the analysis. All measurements were done at room temperature.

Peak areas were used for quantitative analysis. Rhamnose was used for internal standardisation. Calibration curves were prepared between six levels from 0.14 to 3.01 mg/ml of fructose in water, six levels from 0.14 to 3.07 mg/ml of sucrose in water, and five levels from 0.41 to 3.25 mg/ml of inulin in water. All the standards contained the same known amount of rhamnose (3.25 mg/ml).

2.4. Sample preparation

2.4.1. Sample extraction

As the samples are solid, we needed to mill about 300 g of the food product in a hammer mill and mix well in order to homogenise correctly. Then the sample was accurately weighed into a dry Pyrex beaker (400 ml capacity).

Then, 130 ml of 0.1 *M* acetate buffer solution, pH 4.5, and 5 ml of aqueous rhamnose solution (120 mg/ml) were added. The beaker and contents were stirred on a plate magnetic stirrer at a room temperature for approximately 15 min (until sample was completely dispersed). After homogenisation, the beaker was covered with aluminium foil and was placed in a shaking water-bath at 85°C for 20 min to extract the inulin.

2.4.2. Enzymatic hydrolysis

After the extraction, the beaker was removed from the bath and was cooled to 60°C. At this temperature, 100 μl (41.67 INU) of Fructozyme enzyme were added to the beaker and incubated in the shaking water-bath at 60°C for 30 min for total digestion of inulin.

The beaker was cooled to room temperature and was transferred quantitatively to a 200-ml volumetric flask. Three ml of Carrez I reagent and 3 ml of Carrez II reagent were added to precipitate the protein, and the mixture was made up to 200 ml with water. After homogenisation, the flask was left for 1 or 2 h for a complete precipitation of proteins.

The extract was filtered through filter paper and passed through a C_{18} Sep-Pak Plus cartridge, previously conditioned with 10 ml of methanol (HPLC-grade) and 10 ml of Milli-Q water. The filtered extract was transferred into a 1.5-ml Eppendorf until HPLC analysis was performed. Samples were stored in darkness at −20°C. All the samples were filtered through a 0.45-μm nylon filter from Tracer (Teknokroma, Barcelona, Spain) before injection into the HPLC system.

The quantity of fructose obtained by HPLC (F_1) represents the total amount of fructose in the sample.

At the same time, a blank analysis, proceeding exactly the same as before, but without addition of Fructozyme enzyme, was done. The quantity of fructose obtained (F_2) in this case represents the amount of free fructose in the sample.

For samples containing sucrose, the blank analysis also enabled fructose to be determined from sucrose (F_3).

All calibration standards and experimental samples were prepared in duplicate and analysed in triplicate.

2.5. Calculation and expression of results

The amount of inulin dietary fibre (IN) is given by:

$$\%\text{IN} = [A \times (F_1 - F_2)/P] \times 100$$

for a sample containing no sucrose, and

$$\%\text{IN} = [A \times (F_1 - F_2 - F_3]/P) \times 100$$

for a sample containing sucrose, with $F_3 = S/B$ where: F_1 is concentration of the total fructose (g/l); F_2 is concentration of the free fructose (g/l); F_3 is concentration of fructose from sucrose (g/l); S is

concentration of sucrose (g/l); P is mean mass (g/l) of the test samples; $A=1.03$ (empirical conversion factor for fructose to inulin, obtained from different dilutions of inulin hydrolysed with Fructozyme enzyme and quantified using rhamnose as internal standard); $B=2.13$ (empirical conversion factor for fructose to sucrose, obtained from different dilutions of sucrose hydrolysed with Fructozyme enzyme and quantified using rhamnose as internal standard).

3. Results and discussion

It must be emphasised that inulin or oligofructose cannot be quantitatively measured in the soluble fibre fraction of foods obtained by the standard AOAC dietary fibre method [6,12]. Therefore, β-fructans have to be directly determined in products [6].

High-performance liquid chromatography with a refractive index detection was the method we chose. This method has been widely used for the determination of sugars and small oligosaccharides.

The amount of sample needed for the analysis is very high due to the low quantity of inulin added (1–2.5 g/100 g food) to this kind of product, unlike other foods such as yoghurts, mousses, puddings, ice creams, etc.

Many standards were tested for their ability to be detected and to quantify fructose correctly. Rhamnose was selected as internal standard because its retention time is very close to that of fructose without interfering with it, and because it has good resolution.

After testing different reagents to precipitate protein in sample, the combination of Carrez I reagent and Carrez II reagent showed the best precipitation for this kind of sample.

The hydrolysis conditions were 60°C, 30 min and pH 4.5, as described [2,6]. It is supposed that under these conditions there is total hydrolysis of inulin.

We did not need to preheat the Fructozyme enzyme to avoid its pectolytic activity because our samples did not contain pectins. If there were pectins in samples, the enzyme would have to be heated at 60°C for 2 h just before use to remove pectolytic activity without affecting inulinase activity [16].

Fig. 1 shows a typical chromatogram for a meat product (mortadelle). The resolution was satisfactory.

3.1. Validation of proposed method

3.1.1. Linearity

A plot of the peak area ratios of fructose to rhamnose versus amount of fructose gave a linear response in the range 2.8–60.0 μg of fructose ($r=0.9989$, $a=-0.019$, $b=0.489$). Thus, the mean relative response factor of fructose between the six levels was calculated ($f_x=0.670$).

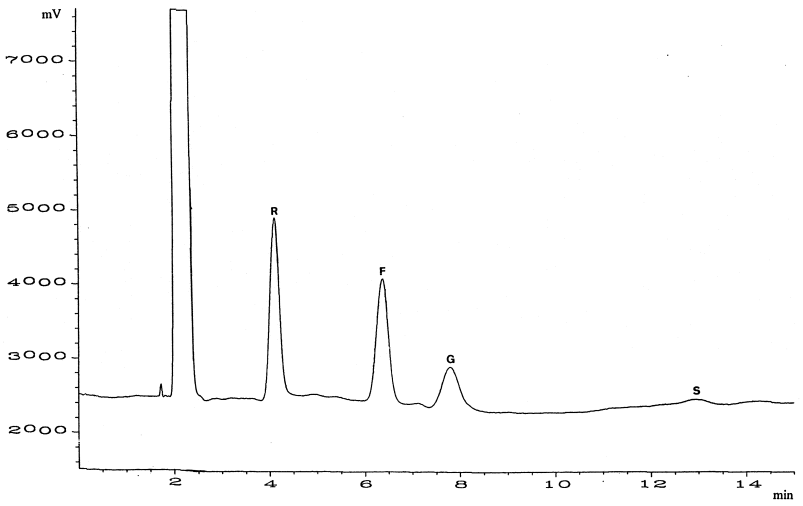

Fig. 1. Chromatogram of hydrolysed food sample (mortadelle). R, rhamnose; F, fructose; g, glucose; and S, sucrose.

Since inulin is a natural product, the ratio of fructose/glucose fluctuates somewhat in practice according to the origin of the raw product from which the inulin is obtained [2].

Therefore, it was necessary to study the amount of fructose obtained from inulin by hydrolysing the sample with Fructozyme enzyme to get the conversion factor of fructose into inulin. Thus, the response of the amount of fructose obtained versus the range 8.12–65.0 μg of inulin showed good linearity ($r=0.9998$) (Table 1). In the same way, the mean conversion factor for fructose to inulin between the five levels was calculated ($A=1.03$). Values of conversion factor very close to 1 stand for high purity of inulin.

For samples containing sucrose, a correction has to be made since the Fructozyme enzyme also splits sucrose, producing glucose and fructose.

This problem can be solved by operating on a duplicate sample of identical weight but without inulinase being added and followed likewise by HPLC analysis. The free fructose (F_2) and the fructose originating from sucrose (F_3) can be thus assessed and subtracted also from the amount of fructose (F_1) obtained by the inulinase digestion.

That is why the plot of the peak area ratios of sucrose to rhamnose versus amount of sucrose, giving a linear response in the range 2.8–60.0 μg of sucrose ($r=0.9995$, $a=0.018$, $b=0.406$), had to be determined. The mean relative response factor of sucrose between the six levels ($f_y=0.734$) was also calculated.

At the same time, it was necessary to settle the amount of fructose obtained from sucrose due to its hydrolysis with the Fructozyme enzyme in order to get the conversion factor of fructose into sucrose. Thus, the response of the amount of fructose obtained versus the range 20.48–61.42 μg of sucrose showed good linearity ($r=0.9998$) (Table 1). In the same way, the mean conversion factor for fructose to inulin between the three levels was calculated ($B=2.13$). Values of conversion factor very close to 2 stand for composition of sucrose of about 50% fructose and 50% glucose.

3.1.2. Precision

To evaluate the repeatability and reproducibility of the method, six replicate determinations on the same day and three determinations on three different days using the same sample were carried out, respectively. The mean relative standard deviation was 2.37% for repeatability and 5.02% for reproducibility (Table 2). This intra-laboratory precision is within the limits of acceptable variability in methods of analysis, proposed by Horwitz [19] for analyte concentrations of the order of 20 mg/g sample.

3.1.3. Recovery

The standard addition method was used to test the recovery of the analysis. Three levels of standard concentrations of fructose were added to a known mass sample, and then they were carried through the entire procedure including enzymatic hydrolysis with Fructozyme enzyme, and then injected in triplicate into the column. The mean recovery obtained was 104.48%. Details are given in Table 3.

Table 1
Linearity of the enzymic hydrolysis

Hydrolysis of inulin		Hydrolysis of sucrose	
Inulin level (μg)	Fructose obtained (μg)	Sucrose level (μg)	Fructose obtained (μg)
8.12	8.08	20.48	10.02
16.24	15.69	40.94	18.86
32.50	32.09	61.42	28.38
40.62	38.31		
65.00	61.32		
$a^a=-0.7338$, $b=1.0685$, $r=0.9998$		$a^a=-1.5945$, $b=2.2288$, $r=0.9998$	

[a] $y=bx+a$.

Table 2
Precision of the method[a]

Analysis	Repeatability $n=3$			Reproducibility $n=3$		
	Mean	SR	RSDr	Mean	SR	RSDR
1	2.25	0.01	0.35	2.25	0.01	0.35
2	2.36	0.10	4.26	2.43	0.04	1.75
3	2.3	0.07	3.15	2.29	0.06	2.45
4	2.38	0.09	3.86	2.18	0.06	2.60
5	2.37	0.05	2.08	2.37	0.05	2.08
6	2.39	0.07	2.82	2.13	0.03	1.61
Mean	2.34±0.06			2.27±0.11		

[a] Sr, repeatability standard deviation; SR, reproducibility standard deviation; RSDr, repeatability relative standard deviation; and RSDR, reproducibility relative standard deviation.

Table 3
Recovery of the method

Fructose level (mg)	Recovery ($n=3$) (%)		
0.01	103.99	103.61	99.82
0.02	106.20	106.83	106.14
0.03	105.83	103.91	104.00
Mean	104.48±2.13		

3.1.4. Sensitivity

To check the sensitivity of the method under the working conditions proposed, the detection limit (DL) and the quantification limit (QL) were studied. The limits were calculated according to the USP criteria [20]. The blank used was water. The results found were 0.82 μg of inulin expressed as free fructose for DL and 2.72 μg for QL. These limits show that the method has excellent sensitivity [21,22].

In conclusion, the HPLC method with refractive index detection is suitable for the routine quantification of inulin and fructoligosaccharides in meat products. The method provides excellent separation, precision, recovery and sensitivity of inulin. This method was tested on pure inulin samples, as well as on industrial food products containing inulin. Table 4 shows the results obtained.

Table 4
Inulin contents of meat products

Meat product	Fructose[a] (mg/ml)	Inulin[a] (g/100 g)	Fiber declared[b] (g/100 g)
Ham ($n=2$)	0.40±0.02	0.54±0.02	1
Mortadelle ($n=6$)	1.71±0.04	2.34±0.06	2.7

[a] Mean values.
[b] Labelling fiber declared.

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