DETERMINATION OF INULIN IN DOUGH PRODUCTS

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A simple, rapid and sensitive spectrophotometric method for the qualitative determination of inulin in dough products (salty sticks with inulin) was successfully developed. The method includes sample preparation steps - pretreatment with petroleum ether, ultrasonic extraction of inulin with hot water and its determination by resorcinol assay. The proposed spectrophotometric method has been based on the formation of colored compound by interaction of inulin with resorcinol and thiourea in the hydrochloric acid medium, as described by the familiar Seliwanoff test for ketoses. The presence of aldose did not show any interference during the inulin analysis. Satisfactory linearity (R^2=0,997) was obtained in the concentration range of fructose 0,5-20 μg/ml. The results showed good method precision with average RSDs of 5 % for repeatability and 7 % for reproducibility. The developed spectrophotometric method was compared with test analysis of the salty sticks by HPLC with refractive index detection. The results demonstrated that the spectrophotometric method is accurate, reproducible, cheap and less time consuming.

Key words: inulin analysis, salty sticks, ultrasonic extraction, Seliwanoff test, HPLC

Introduction

Inulin is a polydisperse linear polysaccharide, member of fructan family, which serves as a reserve carbohydrate in underground part of the Compositae plants such as *Cichorium intybus, Inula helenium and Helianthus tuberosus* [5, 13, 24]. Inulin has been defined as consisting mainly of β-(2→1) fructosyl fructose units (Fm), and usually but not always the chain contains a terminal α-glucopyranose unit (1→2) (GFn) (Figure 1). A small percentage of inulin molecules have a terminal fructoside unit found primarily in the pyranose form in aqueous solution [6, 23]. The degree of polymerization (DP) of inulin varies from 2 to 70 and depends on plant species, harvesting time and post-harvest conditions [6, 24]. Molecules with DP<10 are called oligofructoses or fructooligosaccharides (FOSs) and is a subgroup of inulin [15, 19]. Some of the important physicochemical properties of pure inulin are its good solubility in hot water and its bland neutral taste [8].

Inulin and FOSs are classified as soluble dietary fiber [6, 8]. Due to the absence of enzyme in human and animal organisms, which can hydrolyze the β-glycoside bounds in the chain, inulin and FOSs are not absorbed or metabolized in the stomach and small intestine and reached large intestine unaltered. There they act as prebiotics, because stimulate growth of *Bifidobacteria*, which fermented inulin and FOSs to into short-chain fatty acid (SCFA), mostly acetic, propionic acid, and gases [6, 15, 19]. In recent issues, inulin is presented as immunomodulator and anticancer agent [2].

Inulin has no E-number. It is used in food production as stabilized, texture modifier. FOSs are also sweetener, because of it taste. The improvement of technologically properties of foods and the importance for human health made inulin and FOS commonly used in food industry [8]. In this reason

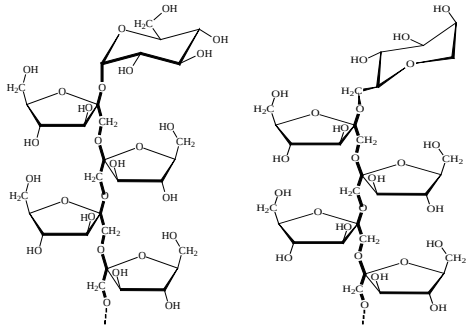


Fig. 1 Chemical structure of inulin

the quantity of inulin and FOSs in food products, have to be defined for the needs of food labeling and to be checked to prevent an adulteration. The increasing interest to inulin and FOSs as prebiotics also evokes the need of modern and routine method for fructan determination.

Determination of inulin can be performed using different approach: spectrophotometric (colorimetric) [1, 16, 17, 21], enzyme [14,18] and HPLC methods [21, 24, 25]. Inulin couldn`t be assessed by standard AOAC methods used in analysis of dietary fibers because of its solubility in 95 % ethanol [14, 18]. From the recent HPLC methods high-performance anion-exchange chromatography with pulsed amperometric detection

(HPAEC-PAD) has been accepted as the most determination of inulin. It provides not only the content of inulin but also the DP profiles [23, 24]. The disadvantages of this method are connected with relatively high cost of the the analytical anion exchange columns and the lack of availability of suitable standards (oligomers). For determination of inulin and FOSs in food products HPLC with refractive index detection RI [12, 25] or high-performance liquid chromatography with evaporative light scattering detection are used [11]. In most of the analysis, the sample has to be hydrolyzed before analysis with enzymes [25]. FOSs can be also analyzed by high-temperature capillary gas chromatography [10]. AOAC offer TLC method for quantitative and qualitative determination of inulin in foods (chocolate, yoghurt, ect.) [22]. Other TLC method for determination of FOSs in feed has also been described [19].

Indirect determination methods are based on hydrolysis of inulin followed by measurement of the released fructose and glucose by different techniques including HPAEC-PAD [18, 21], as well as spectrophotometry using various reagents for derivatization such as dinitrosalicylic acid (DNS) [20] and p-hydroxybenzoic acid hydrazide (PAHBAH) [3]. Many reports using analytical methods based on enzymatic hydrolysis and detection have been published [10, 14, 18]. The enzymes and HPLC methods have big application in analysis of fructan in foods but the need of the high cost equipment, specific and expensive enzymes with high purities and some long-time consuming reactions through the sample preparation are the reason in most of the cases spectrophotometric methods to be preferred.

Therefore, development of a simple analytical method using common chemicals available in laboratories for the determination of inulin is of interest. Some of developed spectrophotometric methods for inulin assay are applied for blood and urine samples [16] or for determination of inulin in plant materials [1, 21]. In our previous article we discussed for the first time the application of spectrophotometric method for determination of inulin in chewing gums on the base of resorcinol assay [17].

Dough products are commonly consumed by people and the addition of inulin in them increase the total dietary fiber. The recent method for inulin and FOSs analysis in these food products are on the base of enzymatic or HPLC analysis [12, 14]. Now in this report we offer a new and innovative ultrasonic extraction of inulin, followed by analysis with resorcinol. We describe the application of this simple powerful method for direct method to the routine analysis of inulin in dough products.

Materials and methods:

Chemicals and reagents

All chemicals and reagents were of analytical reagent grade. All aqueous solutions were prepared in deionized water obtained from Ultrapure Water Systems Arium® 611DI (SartoriusAG , Goettingen, Germany). Sensus (Roosendaal, the Netherlands) supplied fructooligosaccharides - Frutafit CLR, Frutafit HD and inulin - Frutafit TEX extracted from chicory. Frutafit CLR contains a high level of oligofructoses with the average chain length of 7-9 monomers. Frutafit HD contains FOSs with an average chain length 12 monomers. Frutafit TEX was characterized with mean degree of polymerization DP 22, while Raftiline (Beneo) has average DP 25. Sugars standards – glucose, fructose, galactose, sucrose and lactose were supplied by Sigma® (St. Louis, MO, USA).

Instrumentation

The inulin extraction of the salty sticks was carried out in an ultrasonic bath SIEL UST 5.7-150 (Gabrovo, Bulgaria) operating with 35 kHz ultrasonic frequency and power 240 W. The sample was centrifuged on centrifuge MLW T23.

The spectrophotometric experiments were carried out on a Camspec M107 Vis spectrophotometer (UK).

Chromatographic separations were performed on HPLC Shimadzu, coupled with LC-20AD pump, refractive index detector RID-10A, Pb^{2+} cation-exchanger column (pore size 5 μm) and degasser Waters In-Line–IF (Milford, MA, USA). The separations were performed on a Shodex® Sugar SP0810 with Pb^{2+} a guard column(50X 9,2 mm i.d.) and an analytical column (300 mm x 8,0 mm i.d.). The mobile phase used for separation was distilled water with plow rate 0,5 ml/min. The injection volume was 20 μL. The column was placed inside a temperature controlled unit LCO 102 (ECOM spol. s.r.o., Czech Republic). The operating column temperature was 85 °C. The control of the system, data acquisition, and data analysis were under the control of the software program LC solution version 1.24 SP1 (Shimadzu Corporation, Kyoto, Japan).

Sample preparation:

The salty sticks were bought from the local supermarket. Then they were finely ground with pestle and mustard to the powder. The sample was store at room temperature in a plastic vessel with a screw cap.

Ultrasonic extraction of inulin from the salty sticks

Two grams finely ground salty sticks were weighted into 50 ml centrifuge tube on an analytical balance. Petroleum ether 5 ml were added in it and the sample was centrifuged 10 min at 2500 rpm The sample was aspirated and the petroleum ether was weighted into 50 ml centrifuge tube on an analytical discarded without siphoning off the solid material. The extraction was repeated once again. The residue of petroleum ether was evaporated under a gentle stream of nitrogen and the sample was broken up with glass rod. Forty five ml deionized water were added to the defatted sample in the centrifuge tube and the extraction procedure was carried out in a ultrasonic bath at temperature 75°C for 25 min. The water extract was centrifuged for 10 min at 3000 rpm and then it was filtered through 0,45 μm paper filter. The extraction procedure was repeated as all the obtain extracts were collected in 100 volumetric flask. Then the combined extract was diluted to 100 ml with deionized water and it was analyzed by the spectrophotometric method for fructans developed in our lab [17].

Thin layer chromatography (TLC)

The carbohydrate content in the salty sticks water extract was determinate qualitatively by thin layer chromatography (TLC). TLC of the obtained salty stick extracts were performed on silica gel 60 F_{254} plates (Merck, Germany) with *n*-BuOH:*i*-Pro:H_2O:CH_3COOH (7:5:4:2) (v/v/v/v) as eluent; spots were detected by dipping the plates into the solution with detecting reagent – diphenylamine-aniline-H_3PO_4–acetone (1:1:5:50) and heating at 80 °C [13]. As a standards were used 2 μl glucose, fructose, sucrose, galactose, lactose, inulin (Frutafit TEX and Raftiline HP) and fructooligosaccharides (Frutafit CLR and HD) each of them with concentration 2 mg/ml.

Spectrophotometrical method for determination of fructans in foods

Hundred microliters from the obtained water extract of the salty sticks were put into 10 ml glass tube, then 100 μl resorcinol (1 mg/ml), 100 μl thiourea, 800 μl 95% EtOH and 900 μl k. HCl were added. The sample was heated for 8 min at 80 °C, cooled to the room temperature and then diluted to 10 ml with distilled water. The absorbance of pink-colored compound was read at 480 nm against distilled water. The concentration of inulin in the salty sticks extract was calculated using the equation (eq. 1) obtained from the calibration curve of fructose. The calibration curve was linear in the range of 0,5–20 μg mL^{-1} with a correlation coefficient of 0,997 [17].

$$Y= 0,1174x+0,0087 \quad R^2=0,997 \qquad (1)$$

where: y – absorbance at 480 nm;
x – concentration of fructose, μg mL^{-1}

Validation parameters

The proposed spectrophotometric method was tested and validated for various parameters according to the ICH (International Conference on Harmonization) guidelines [9]. Parameters of linearity curve: the equation is characterized with the correlation coefficient R^2 =0,997. To evaluate the repeatability and reproducibility of the proposed method, six replicate determinations on the same day and six determinations of samples on different days by six different persons were done [4, 7, 9]. Intermediate precision was estimated as the same analyst analyzed six samples (one per day) in a period of six different days.

The standard addition method was used to test the accuracy of the analysis. Three levels of standard concentration of fructose 4; 8 и 10 μg.mL^{-1} were added to a sample (salty sticks) with known mass around 2 g. Then they were analyzed as the described extraction procedure and spectrophotometric determination of inulin [4]. The accuracy of the method was calculated on the base of the relative error [4, 17].

HPLC analysis of the sample

Before HPLC analysis and an injection of water extract into the column of the HPLC apparatus sample was precipitated with addition of Carrez I and II solutions. A 0,2 mL volume of Carrez I reagent (distilled water solution of potassium hexacyanoferrate(II), $K_4Fe(CN)_6.3H_2O$, 15 g/100 mL) was added to the water extract and mixed. Subsequently, a 0,2 mL volume of Carrez II reagent (distilled water solution of zinc acetate, $Zn(CH_3COO)_2 \cdot 2H_2O$, 30 g/100 mL) was added to the 100 ml water extract of salty sticks and was mixed. Then the sample was filtered and diluted to 100 ml. Before injection into the HPLC column the sample is filtrated through 0,45 μm filter and then 20 μl sample was injected and analyzed upper under the mention conditions.

Results

The obtained results from TLC screening procedure of the water extract of salty sticks showed absence of sucrose in the samples and presence of inulin with high degree of polymerization about 22 - 25 as the inulin standard Frutafit TEX and Raftiline HD. The analyzed samples also contained monosaccharides galactose and fructose, disaccharides lactose and fructooligosaccharides as

the standards Frutafit CLR and HD (Fig. 2) The carbohydrate profile obtained from TLC quantitative analysis allows salty sticks samples to be analyzed by spectrophotometric method, thus no sucrose can interfere through the resorcinol assay.

The spectrophotometric method developed for the needs of our lab was based on ketose specific reaction with resorcinol in a strong acid medium. Aldohexoses, disaccharides and starch showed any interference through the spectrophotometric measurement of resulting absorbance of formed pink colored compound (Fig.3). To check the interference of other cabohydrates and specificity of method for ketose the standard solutions - fructose, glucose, galactose, sucrose, lactose, maltose, fructooligosaccharides (FOS) all with concentrarion 10 µg/ml and starch (with concentration 100 µg/ml) analysed by spectrophotometric method was scaned between the wavelength range 340 – 620 nm (Fig 3).

Fructose, sucrose and oligofructose formed with resorcinol in acid medium coloured compound (Fig. 4)with maxium absorbance at 480 nm wavelength.

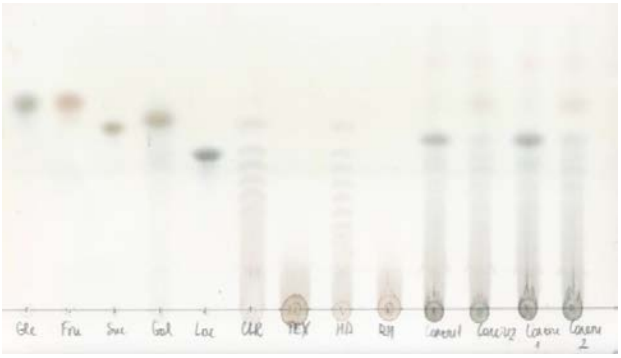


Fig. 2. TLC of salty sticks water extract (1) and (2) and standards Glu - glucose, Fru - fructose, Suc - sucrose, CLR and HD- FOSs Frutafit, TEX and RH – inulin , Gal - galactose and Lac- lactose.

Other invastigated carbohydrates show any interfernece and they do not formed red complex compound with resorcinol.

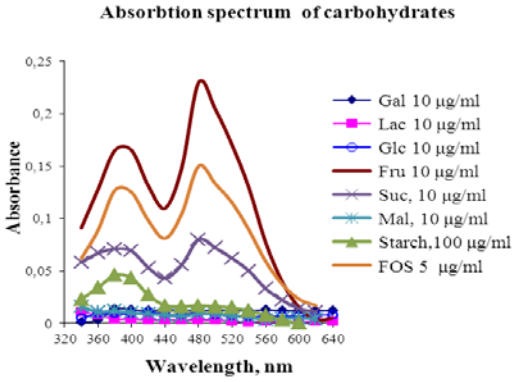


Fig. 3 Absorption spectrum of the complex compounds formed by interaction of fructose, glucose, galactose, sucrose, maltose, lactose and starch all with concentration with resorcinol

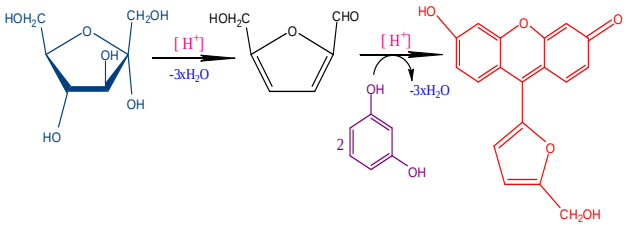


Fig. 4 Scheme of Selivanoff reaction

Validation of the method

The precision of the method was evaluated by repeatability, intermediate precision and reproducibilty. Repeatability is a measure of the ability of the method to generate similar results for multiple preparations of the same homogeneous

Table 1 Evaluation of precision of the proposed method

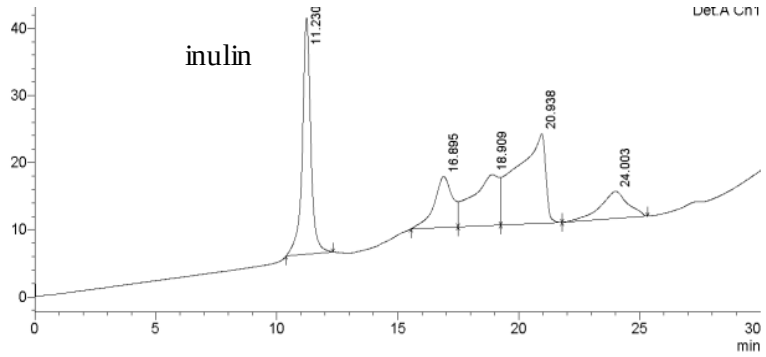
Sample number	Content of fructans in salty sticks, %		
	Repeatability	Intermediate precision	Reproducibility
1	2,5	3,1	2,4
2	2,6	2,9	2,5
3	2,2	2,9	2,7
4	2,5	2,6	2,8
5	2,5	3,2	2,5
6	2,4	2,9	3,0
Mean, %	**2,4**	**2,9**	**2,7**
SD	**0,1**	**0,2**	**0,2**
RSD, %	**5,3**	**6,9**	**7,4**

SD – standard deviation
RSD – relative standard deviation, %

Table 2 Accuracy of the test method

Slope	y-intersept	Vs, g/100g	Vo, g/100g	Relative error, %	Accuracy,%
0,140	0,136	1,1	3,8	2,5	97,5

Vs – the true inulin content in the sample calculated from the curve obtained from standard method addition[4].
Vo – the measured inulin content in the sample



Column: Shodex® Sugar SP0810
Mobile phase: deionized H_2O
Flow rate: 0,5 ml/min
Detector: RID-10A
Column temperature: 85 °C

Fig.5 HPLC –RI chromatogram of water extract of salty sticks with inulin

sample by one analyst using the same instrument in a short time duration. Intermediate precision is a measure of the variability of method results where the same samples are tested and compared using different analysts, different equipment, and on different days, etc. The results of the repeatability test are reported in Table 1 and showed adequate performance of the method for determination of fructan in dough products (salty sticks). The RSDs for impurity methods are around 5 % for repeatability and below 10 % for intermediate precision and reproducibility. The tested spectrophotometric method showed good results for the proposed rules for method validation [7, 9].

The results from standard addition method (table 2) were used to obtain the accuracy of the method. The developed spectrophotometric method for determination of inulin in dough products is characterized with relative error 2,5 % and accuracy 97,5 %.

HPLC analysis of salty sticks

After the salty sticks sample was analyzed by the developed spectrophotometric method for fructan determination the same sample was tested by the HPLC coupled with refractive index detector. The obtained chromatogram (Fig. 4) proved the absence of sucrose in the sample and confirmed the presence of inulin (t_R=11,2 min), fructose(t_R=24 min), lactose (t_R=16,8 min) and galactose (t_R=20,9 min) in it. The HPLC analysis proved the results obtained from the TLC analysis. The sum of quantities of inulin and fructose in the salty sticks was around 3 %, which is the same as the results obtained by the spectrophotometric method. The HPLC method with refractive index detector is very sensitive and suitable for routine analysis of inulin as well as spectrophotometric method. The disadvantages of HPLC method is additional cleaning-up the sample and its expensive instrumentation. The spectrophotometric method for determination fructan in food is perfect when sucrose is absent in samples and the total fructose content have to be defined. The complex sample matrix did not cause such interference through the analysis and that made spectroscopic method to be preferred as a working method. The fact that the limit of detection of fructose at 480 nm was 0,14 μg mL^{-1} revealed that the method can be recommended for the quantitative determination of inulin and FOS in case of cereal products.

Conclusion

It has been developed new spectrophotometric method for routine analysis of inulin in dough products used an ultrasonic extraction of inulin and its further analysis with resorcinol assay. The method based on Seliwanoff test for ketoses is simple, rapid and proper for routine laboratory practice. The method has wider linear range and showed good precision and accuracy. The results of this method was compared with these obtained from HPLC. But the cheaper instrumentation and price of the analysis and information for total fructan made the spectrophotometric method proper for our needs.

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