

# SELECTIVE FRACTIONATION OF PREBIOTIC CARBOHYDRATES FROM COMPLEX MIXTURES BY SUPERCRITICAL CO<sub>2</sub> WITH DIFFERENT COSOLVENTS

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## ABSTRACT

In this work, we study the use of supercritical fluid technology based on carbon dioxide to fractionate a complex carbohydrate mixture, which is a dairy-based prebiotic ingredient containing galacto-oligosaccharides (GOS) along with monosaccharides and disaccharides. The beneficial health effects of this commercial mixture, such as the improvement of the immune system, have been associated to its GOS content; therefore, a purification and/or removal of other sugars can be of great importance.

The goal of this study was focused in the fractionation of the complex carbohydrate mixture according to the degree of polymerization of the different carbohydrates. Supercritical carbon dioxide (SC-CO<sub>2</sub>) with different ethanol/water mixtures as co-solvents has been tested for the selective recovery of the prebiotic carbohydrates. The appropriate selection of the co-solvent employed, together with the most suitable extraction conditions (including temperature, pressure and co-solvent flow rate) allowed selective fractionation of the mixture with satisfactory yield and purity.

## INTRODUCTION

Health conscious consumers seek simple solutions to wellness issues and manage their health through products that can provide added healthful benefits, the so called functional foods. One of these functional food groups are prebiotic carbohydrates, defined as *non digestible carbohydrates that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health* [1]. Therefore, in order to be effective, a prebiotic must escape digestion in the upper gastrointestinal tract so that it can be released in the lower tract and used by beneficial microorganisms in the colon, mainly bifidobacteria and lactobacilli.

Nowadays a growing population has realized many benefits of prebiotics, and is seeking out products that contain prebiotics. This demand will continue to grow due to an ageing population, increased health costs, changes in food regulation, etc. One of these prebiotics carbohydrates are oligosaccharides. Two general classes of oligosaccharides preparations are widely used in Europe. These are fructans and  $\beta$ -galacto-oligosaccharides (GOS). GOS, which also occur naturally, are manufactured from lactose by glycosyl transfer catalyzed by  $\beta$ -galactosidase and occur as complex mixtures with various glycosidic linkages [2]. The prebiotic properties of GOS have been established in several clinical studies, including increased production of beneficial short-chain fatty acids such as butyrate, increased absorption of calcium and magnesium, and improved elimination of toxic compounds. GOS are also incorporated in a wide range of products such as fermented milk products, breads, jams, confectionery, and beverages.

Commercially available GOS are mixtures of several molecular species of oligosaccharides (more than 55%), lactose (20%), glucose (20%), and a small amount of galactose. GOS are

available in liquid and powder forms. For example, our sample from commercial GOS is a dairy-based product rich in galacto-oligosaccharides, which are soluble, non-digestible carbohydrates. It contributes to increased calcium absorption, fiber enrichment, gut health and is widely used for a wide range of products that include infant nutrition, yoghurt, fruit juices, breakfast drinks, slimming drinks. It is soluble, heat and acid stable. Besides commercial GOS mixtures are GRAS (Generally Recognized As Safe). Purification and/or removal of prebiotics carbohydrates from this complex mixture would increase its product added value.

On the other side, the use of environmentally friendly technologies has been gaining increasing interest. Among them, supercritical fluid extraction technology is particularly attractive as an environmentally benign replacement for traditional solvents. Although pure SC-CO<sub>2</sub> as a solvent is not suitable to extract sugars, previous studies have shown that using polar co-solvents the solubility of carbohydrates in SC-CO<sub>2</sub> may be considerably enhanced [3].

In previous works carried out in our laboratory [4-5] we demonstrated the usefulness of supercritical fluid technology to fractionate solid binary carbohydrate mixtures (lactulose-lactose and tagatose-galactose) achieving a selective recovery of the ketosugar from the binary ketose-aldose mixture. Based on these results, it seemed possible to reach a compromise among the extraction conditions and type of co-solvent, in order to selectively purify carbohydrates from complex mixtures [6]. Different alcohols and alcohol/water mixtures previously studied, shown that ethanol/water mixtures are suitable modifiers to be employed in carbohydrate SC-CO<sub>2</sub> technology. Additionally, by increasing the water content in the ethanol/water co-solvent the amount of carbohydrates extracted can be considerably increased.

The manufacture of prebiotic carbohydrates of high purity, obtained from a commercial mixture and using a clean technology, is of great industrial interest considering their addition in a wide range of food products to confer them prebiotic properties. Thus, the aim of the present research was to carry out the necessary studies to select appropriate co-solvents and extraction conditions to fractionate complex carbohydrate mixtures. Commercial GOS mixture was selected as raw material. Solubility studies of all type of carbohydrates comprising the complex mixture were conducted in ethanol/water (varying water content) liquid mixtures, to explore the selectivity of these solvents towards the different carbohydrates. Then, appropriate ethanol/water ratios were selected and applied as modifier in the supercritical fractionation assays.

## **MATERIALS AND METHODS**

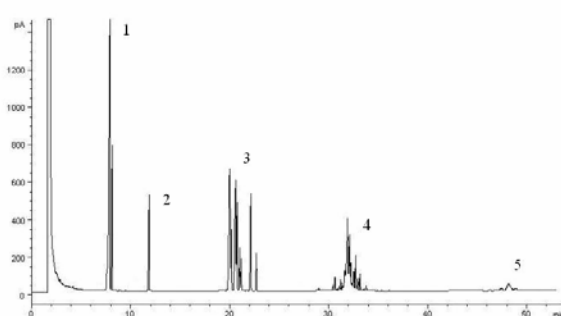
**Materials:** Commercial GOS mixture was obtained from a store in aqueous solution containing 57% wt GOS, 23% wt lactose, 19% wt glucose and 0.9% wt galactose. Composition data were taken from the label of commercially available GOS mixture syrup. Internal standard (phenyl-β-D-glucoside) and three derivatizing reagents (trifluoroacetic acid, 99+%, spectrophotometric grade, hexamethyldisilazane and hydroxylamine hydrochloride ACS Reagents) were obtained from Sigma (St. Louis, MO, USA). The other derivatizing agent, pyridine dried, was supplied by Merck (Darmstadt, Germany). Sea sand and glass wool washed chemically pure were acquired from Panreac Química S.A (Barcelona, Spain). Ethanol absolute was from Prolabo (Fontenay sous Bois, France). 18.2 MΩcm Ultrapure water quality with 1–5 ppb TOC and < 0.001 EU/mL pyrogen levels (Milli-Q) was produced in-house using a Laboratory water purification Milli-Q Synthesis A10 system (Millipore, Bellerica, MA, USA) and was used throughout. The carbon dioxide liquefied at high pressure used in supercritical extraction was supplied by Praxair Inc. (Danbury, CT, USA).

**Supercritical fluid extraction system:** The scheme of the supercritical fluid extraction device employed to carry out all the experiments can be found elsewhere [4]. Extraction processes were performed on samples placed into the extraction cell (8 cm<sup>3</sup>), consisting of 100, 200, 500 or 1000 mg of freeze-dried commercially available GOS mixture (one part) mixed with nine parts of sea sand in a laboratory mill (Janke and Kunkel IKA A-10, Labortechnik, Staufen, Germany), Fenske rings or glass beads (2 mm diameter). Samples were packed with glass wool into the extraction cell. A continuous flow rate (1.2 g/min) of CO<sub>2</sub> mixed with different amounts

of the polar cosolvent employed in the particular assay was maintained through the extraction cell for six hours.

The effects of different factors, such as extraction pressure, extraction temperature and cosolvent flow rate, on the amount of carbohydrates extracted was first studied in previous assays in order to select the optimal conditions to approach the purification of trisaccharides from a complex mixture of carbohydrates (commercial GOS mixture).

**GC analysis of supercritical extracts:** Prior to analyze the collected extracts, we analyzed commercially available GOS mixture without any treatment by gas chromatography. GC commercial GOS mixture composition (Figure 1) was: monosaccharides (1) 18.4 %, disaccharides (3) 52.8 %, trisaccharides (4) 28.3 % and tetrasaccharides (5) 0.5 %. (2) Internal standard. Samples as well as collected extracts were prepared for gas chromatography. 0.1 milliliter of the sample was added to 0.5 mL of a solution of 0.01% phenyl- $\beta$ -D-glucoside in methanol/water (70/30, v/v) as internal standard (w/v). Previous to derivatization, samples were dried at 38-40 °C in a rotary evaporator (from Büchi Labortechnik AG, Flawil, Switzerland).



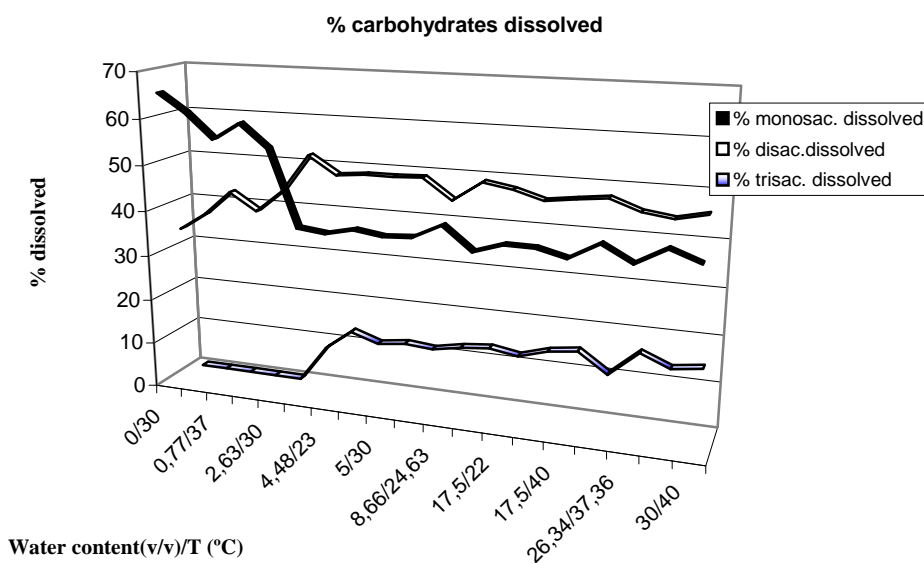
**Figure 1.** GC composition of commercial GOS mixture.

Sugar oximes were formed by adding 350  $\mu$ L hydroxylamine chloride (2.5 %) in pyridine and heating the mixture at 70°C for 30 min. Subsequently, the oximes obtained in this step were silylated with hexamethyldisilazane (350 $\mu$ L) and trifluoroacetic acid (35 $\mu$ L) and kept at 50°C for 30 min. Reaction mixtures were centrifuged at 8600  $\times$  g for 10 min (MiniSpin Eppendorf AG, Hamburg, Germany). Supernatants were injected in the GC or stored at 4 °C prior to analysis.

GC was performed with a Hewlett-Packard gas chromatograph (HP6890) equipped with a flame ionisation detector (FID). The trimethylsilyl oximes were separated using a 30 m  $\times$  0.32 mm inside diameter and 0.5  $\mu$ m film fused silica capillary column SPB<sup>TM</sup>-17, bonded, crosslinked phase poly (50% diphenyl / 50% dimethylsiloxane) (Supelco, 595 North Harrison Road, Bellefonte, PA, USA). Data was acquired by means of HP ChemStations (Agilent Technologies Inc., Wilmington, DE., USA).

## RESULTS

In order to study the effect of different ethanol/water modifiers in the SC-CO<sub>2</sub> extraction of commercial GOS mixture, the solubility behaviour of the commercial carbohydrate mixture in ethanol/water solutions was previously analysed. The effect of temperature and water content was studied using a central composite rotatable experimental design. A total of 21 experiments were carried out in randomized run order. Variables tested at the different experimental levels were temperature (ranging between 22 and 40 °C) and water content (between 0 and 30 % v/v) in the ethanolic solvent. The response variables selected were mg/mL of monosaccharides, disaccharides, trisaccharides and tetrasaccharides dissolved in the liquid ethanol/water solvent. Range limits were selected following results obtained in our previous studies [4-5] and bibliography available [7]. Results obtained are shown in Figure 2.



**Figure 2.** % Carbohydrates dissolved in ethanol/water solvent after 30 minutes.

As can be deduced from Figure 2, no selective dissolution of the different type of carbohydrates in the ethanol/water solvent is achieved if water content is greater than 5%; for a water content lower than 5% a monosaccharides versus disaccharides enrichment is observed (maximum enrichment at 2.4% water content) while no trisaccharides or higher carbohydrates are dissolved in the liquid ethanol/water solvent.

#### **Commercial GOS mixture Supercritical Extraction using CO<sub>2</sub> + ethanol/water co-solvents**

Considering the differences in molecular size between mono, di, tri and tetrasaccharides, it is expected that the solubility of carbohydrates in SC-CO<sub>2</sub> decrease according molecular weight increase. Additionally, the preliminary solubility studies of commercial GOS mixture in liquid ethanol/water solvent indicated a maximum selectivity of monosaccharides versus disaccharides (and higher carbohydrates) if the water content in the ethanolic solvent is 2.4%. Thus, ethanol/water 97.6:2.4 v/v mixture was initially used as co-solvent in the supercritical extraction assays.

Table 1 shows the results obtained for 200 mg of commercial GOS mixture placed in the extraction cell, 1.2 g/min of CO<sub>2</sub> flow, and different extraction conditions (temperature, pressure, amount of modifier and inert matrix employed to disperse the carbohydrate mixture). A two-step extraction process to fractionate commercial GOS mixture was employed (see Table 1). Conditions selected for each step were according to the results obtained in previous works [4-5] to favor the extraction of monosaccharides (Step 1 in Table 1) and disaccharides (Step 2 in Table 1).

As can be observed in Table 1, a first extraction step carried out at 150 bar, 80°C and using 0.6 mL/min of ethanol/water 97.6:2.4 v/v co-solvent, produce extracts with a 3-fold increase of monosaccharide concentration; extracts containing 60-70% wt of monosaccharides (except for Fenske rings matrix) were achieved while the content of monosaccharides in commercial GOS mixture is 18.4%. Nevertheless, the amount of monosaccharides recovered is very low (ca. 20%) and thus, purification of disaccharides can not be achieved in the second fractionation step. In order to increase monosaccharides yield, water content in the ethanol/water modifier was increased up to 5%.

Table 2 shows the results obtained for different two-step supercritical extraction processes when ethanol/water 95:5 v/v is employed as co-solvent. Different extraction conditions and also different amounts of carbohydrate mixture were tested. In this case, when the same conditions

employed before (150 bar, 80°C and 0.6 mL/min co-solvent) were applied in the first fractionation step to 200 mg of commercial GOS mixture, an extract with 68% wt of monosaccharides purity and 90% yield was obtained. That is, monosaccharides recovery was considerably increased (from 20% to 90%) by increasing water content in the ethanol/water co-solvent from 2.4 to 5%. Additionally, the second step (100 bar, 100 °C and 0.4 mL/min co-solvent) produced an extract with 89% wt disaccharides and 65% yield.

Summarizing, the two-step extraction procedure shown in the first experimental assay of Table 2 allowed almost complete removal of monosaccharides and disaccharides from commercial GOS mixture mixture and thus, the material not extracted and collected in the extraction cell were mainly tri- and tetrasaccharides with ca. 75% wt of purity and 94% recovery.

Additional experimental assays were carried out varying extraction conditions and increasing the amount of carbohydrates placed in the extraction cell. As can be observed in Table 2, extraction yield considerable decrease when the amount of commercial GOS mixture is increased; this result verify that the optimal relation among solvent flow and amount of mixture processed is 1.2 g/min CO<sub>2</sub> per 200 mg of carbohydrate mixture.

**Table 1.** Commercially available GOS mixture (CA GOS) Supercritical Extraction using CO<sub>2</sub> + Ethanol/Water 97.6:2.4 (v/v) co-solvent.

M: monosaccharides; D: disaccharides; T: tri- and tetrasaccharides.

Step	supercritical extraction conditions pressure (bar) / temperature (°C) / modifier flow (mLmin <sup>-1</sup> )	amount of CA GOS (mg)	matrix	% extracted (yield)			extract composition (% weight)		
				M	D	T	M	D	T
1	150/80/0.6 (0-3 hours)	208.6	sea	17.48	3.23	0.00	65.34	34.66	0.00
2	100/100/0.4 (3-6 hours)		sand	25.85	31.57	7.30	20.24	70.96	8.80
1	150/80/0.6 (0-3 hours)	199.6	sea	23.83	2.88	0.00	74.22	25.78	0.00
2	100/100/0.6 (3-6 hours)		sand	37.00	14.49	20.89	33.42	37.56	29.02
1	150/100/0.6 (0-3 hours)	202.0	sea	67.55	38.16	0.00	38.16	61.84	0.00
2	100/100/0.6 (3-6 hours)		sand	34.63	13.56	21.06	32.69	36.73	30.58
1	150/80/0.6 (0-3 hours)	203.4	sea	19.45	4.71	0.00	59.00	41.00	0.00
2	100/80/0.6 (3-6 hours)		sand	12.93	4.81	0.00	48.35	51.65	0.00
1	150/80/0.6 (0-3 hours)	211.3	sea	16.64	3.00	0.00	65.89	34.11	0.00
2	100/80/0.4 (3-6 hours)		sand	7.72	2.49	0.00	51.90	48.10	0.00
1	150/80/0.6 (0-3 hours)	206.7	Fenske	18.93	9.43	3.71	36.62	52.34	11.04
2	100/100/0.4 (3-6 hours)		rings	76.49	54.28	37.92	26.32	53.61	20.07
1	150/80/0.6 (0-3 hours)	204.9	Glass	20.98	2.99	0.00	70.94	29.06	0.00
2	100/100/0.4 (3-6 hours)		beads	15.68	7.79	0.00	41.21	58.79	0.00
1	150/80/0.6 (0-3 hours)	205.2	no	22.78	5.27	0.00	60.10	39.90	0.00
2	100/100/0.4 (3-6 hours)		matrix	52.63	43.50	19.46	25.38	60.19	14.43

## CONCLUSION

In this work we present a first stage to achieve the fractionation of carbohydrates from commercially available complex mixtures using SC-CO<sub>2</sub> as solvent plus ethanol/water co-solvent. Extractions conditions (temperature, pressure, type and amount of modifier) which are in agreement with previous works [4-6] have shown that is possible to isolate monosaccharides, disaccharides and higher carbohydrates from the complex carbohydrate mixture with satisfactory purity and yield. Some extra assays must be done to achieve complete fractionation

of monosaccharides, disaccharides, trisaccharides and tetrasaccharides and for better comprehension about matrix influence in supercritical extraction in disaccharides and trisaccharides. Next steps will consist in the separation of different carbohydrates with the same degree of polymerization.

**Table 2.** Commercially available GOS mixture (CA GOS) Supercritical Extraction using CO<sub>2</sub> + Ethanol/Water 95:5 (v/v) co-solvent.

M: monosaccharides; D: disaccharides; T: tri- and tetrasaccharides.

Step	supercritical extraction conditions pressure (bar) / temperature (°C) / modifier flow (mLmin <sup>-1</sup> )	amount of CA GOS (mg)	matrix	% extracted (yield)			extract composition (% weight)		
				M	D	T	M	D	T
1	150/80/0.6 (0-3 hours)	208.0	sea	90.23	16.99	0.00	67.97	32.03	0.00
2	100/100/0.4 (3-6 hours)		sand	9.77	65.1	5.69	6.59	89.23	4.18
1	150/80/0.6 (0-3 hours)	499.5	sea	41.95	12.20	0.00	54.52	45.48	0.00
2	100/100/0.4 (3-6 hours)		sand	49.90	32.94	2.39	33.69	63.82	2.48
1	150/80/0.6 (0-3 hours)	499.8	sea	39.67	10.04	0.00	57.92	42.08	0.00
2	100/100/0.6 (3-6 hours)		sand	64.59	89.52	21.06	18.25	72.59	9.15
1	150/100/0.6 (0-3 hours)	992.8	sea	67.52	24.07	2.48	48.08	49.20	2.72
2	100/100/0.6 (3-6 hours)		sand	12.73	23.25	11.80	13.04	68.36	18.60
1	150/80/0.6 (0-3 hours)	997.0	Fenske	17.93	2.72	0.36	68.23	29.66	2.12
2	100/100/0.4 (3-6 hours)		rings	21.77	15.68	6.67	28.27	58.42	13.31
1	150/80/0.6 (0-3 hours)	1012.1	no	20.91	7.87	0.00	48.08	51.92	0.00
2	100/100/0.4 (3-6 hours)		matrix	24.10	19.88	3.33	27.93	66.13	5.94
1	150/80/0.6 (0-3 hours)	993.4	Glass	19.61	3.57	0.00	65.68	34.32	0.00
2	100/100/0.4 (3-6 hours)		beads	20.53	15.45	5.23	28.17	60.80	11.03

## ACKNOWLEDGMENTS

*This work has been financed under a R+D program of the Spanish Ministry of Education and Science (AGL2004-07227-CO2-02) and of the Comunidad Autónoma de Madrid (S-0505/AGR/000153). F.M. thanks MEC for a FPI grant. T. F. would like to acknowledge the financial support of the Ramon y Cajal Program from the Ministry of Education and Science.*

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