

Borates and Aluminates Reduction of Complex Prebiotic Carbohydrates Mixtures By Supercritical Extraction With CO₂ and Ethanol/Water as a Cosolvent

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ABSTRACT

Nutritional authorities around the world are strongly emphasizing the need to include more healthful foods in consumers' diet. The variety of functional foods provide many opportunities to reach this more healthful diet goal. Functional foods include a wide variety of foods and food components capable to improve health and well-being, reduce the risk of specific diseases, or minimize the effects of other health concerns. Our work deals with one of these functional foods: prebiotic carbohydrates. Complex prebiotic carbohydrates mixtures are components present in foods, or that can be incorporated into foods, which yield health benefits related to their interactions with the gastrointestinal tract. According to Gibson et al 1995 definition are "nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth of one or a limited number of bacterial species in the colon, such as *Bifidobacteria* and *Lactobacilli*, which have the potential to improve host health". Food-grade complex carbohydrate mixtures manufactured commercially via transglycosylase reactions using disaccharides such as sucrose or lactose as the raw material are, in general, not pure products. In the present work, a new process based on the extraction with supercritical CO₂ plus a cosolvent has been developed to purify reaction mixtures leading to pure prebiotic oligosaccharides.

INTRODUCTION

Prebiotic carbohydrates are non-digestible functional foods unaffected by the digestion process and reach the colon where stimulating growth of health-promoting species belonging to the genera *Bifidobacterium* and *Lactobacillus* in colonic microbiota. The possible benefits of use of prebiotic carbohydrates are supported by the scientific data available. These benefits include improvement of bowel habit and calcium bioavailability [1, 2]. Extensive research has been conducted with lactose-derived oligosaccharides (GOS) [3, 4] and, it has been observed that the chemical structure of the oligosaccharide (glycosidic linkages, degree of polymerisation and monosaccharide composition) may affect their physiological effects [5]. Prebiotic GOS are usually produced from lactose from different sources [6]. Two of these sources are aluminates [7-10] and borates [7, 11-14] which facilitate the reaction with a minimum of secondary reactions, resulting in a process with a high yield of lactulose (75% and higher) through elimination of lactulose from the reaction equilibrium mixture in the form of a complex. However, they are unsatisfactory from the industrial aspect because of the difficulty of eliminating the aluminate and borate and those have to be removed for safety reasons. In this work we use supercritical CO₂ extraction with a cosolvent to remove aluminates or borates from the carbohydrate mixture. At the same time, we can separate carbohydrate mixture according to its degree of polymerization.

MATERIALS AND METHODS

Reagents

Lactose was acquired from Scharlau (Barcelona, Spain). Sodium aluminate was purchased from Merck (Divisione Chimica Industriale, Milano). Boric acid was obtained from 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). The commercial enzyme Lactozym 3000L HP G, a soluble preparation of β -galactosidase from *Kluyveromyces lactis* gift from Novozymes (Bagsvaerd, Denmark). Sea sand washed was acquired from EMD (Gibbstown, NJ, USA). 18.2 M Ω cm 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 CO₂ liquefied at high pressure used in supercritical extraction was supplied by Praxair Inc. (Danbury, CT, USA).

Synthesis of GOS from Lactozym 3000L HP G.

The synthesis of GOS was carried out in the optimal conditions previously reported by Martinez-Villaluenga et al. [15]. Thus, a concentration of 3 U/mL of enzyme was added to a concentration of 250 g/L of lactose. Two different conditions were assayed: 1) incubation during 2 hours at 40°C in a buffer phosphate 50 mM and 1 mM MgCl₂ at pH 7.5; 2) incubation during 5 hours at 50°C in a buffer phosphate 50 mM and 1 mM MgCl₂ at pH 6.5. After this period the mixture was immediately immersed in boiling water during 10 min to inactivate the enzyme.

Isomerization reactions

The assays of isomerization of carbohydrate mixture with aluminates and borates were based on the method of Zokaee et al [7]. In all the experiments, total carbohydrate concentration was 10% (w/v) in deionised water. Thus, 8 g of total carbohydrates present in hydrolyzed mixtures was dissolved in deionised water and mixed with sodium aluminate in different sodium aluminate/carbohydrate (lactose) molar ratios, 1:1 and 2:1. Same molar ratios were used for experiments with boric acid. The mixture was immersed into a water bath adjusted to the required temperature (40°C for aluminates experiments and 70°C for borates experiments) and heated for a specific time period. Isomerization reactions were taken at 2, 4, 6, 8 and 10 hours for aluminates experiments and 60, 90, 120, 150 and 180 minutes for experiments with borates. Reactions were stopped by placing the tube in an ice-bath and then adding a few drops of sulphuric acid to neutralize the pH. Afterwards, samples centrifuged at 10,000 rpm during 10 min. The supernatant was collected and freeze dried.

Extractions at supercritical conditions

The equipment employed to carry out solubility measurements is based on a Suprex Prep Master (Suprex Corporation, Pittsburg, PA, USA) with several modifications [16]. It has a thermostatic oven heated by air convection where a cell (8 mL) containing the solid sample is placed. A computer-controlled mass flow meter was used to adjust the supercritical solvent (CO₂ + 95:5 ethanol:water) flow rate at the values selected for each experiment.

Measurements were performed on samples comprising one part of carbohydrate and nine parts of sea sand weighted on an analytical balance with a 0.1 mg precision. The sea sand was selected as inert material to hold the sample. 1g of the carbohydrate-sea sand mixture was introduced into the cell and packed with glass wool. Once the experimental conditions were

reached the supercritical solvent passed through the cell for two hours. After depressurization, the material dissolved was collected in a collection vessel [17].

Sample analysis

Extracted samples were analysed in two different ways: 1) for carbohydrates quantification via gas chromatography [18]; 2) for aluminium quantification with inductively coupled plasma mass spectrometry (ICP-MS); for boron quantification via spectrophotometry technique [19].

RESULTS

As explained in materials and methods, firstly lactose isomerization was carried out with Lactozym 3000L HP G at two different conditions. Results obtained are shown in Table 1. After the isomerization reaction, samples were freeze dried. Isomerization reaction produces not only GOS, also unreacted lactose as well as small amounts of by-products such as galactose and glucose [16]. The presence of glucose and lactose are not recommended for people with diabetes or lactose intolerance. That is the reason why we did an isomerization of GOS with sodium aluminate and boric acid.

Table 1. Lactose enzymatic isomerization.

Enzymatic reaction time (minutes)	Lactose employed (g)	pH	Temperature(C)	% M	% D	% T
120	60	7.5	40	36.88	47.12	16.00
300	60	6.5	50	61.19	25.94	12.87

% M: % monosaccharides in isomerization; % D: % disaccharides in isomerization; % T: % trisaccharides in isomerization.

During the reaction of isomerization of GOS, lactose, glucose and galactose were isomerized to lactulose, fructose and tagatose, respectively, and allolactose, 6 galactobiose and 6'galactosyl-lactose were also converted to the corresponding keto-sugars. Results of these isomerizations are summarized in Table 2, where only those experiments producing higher amount of trisaccharides (GOS) are presented. After isomerization, aluminates and borates were precipitated with sulphuric acid. In some samples, aluminates and borates were not removed.

Table 2. GOS isomerization with sodium aluminate and boric acid.

Enzymatic reaction time (minutes)	Molar ratio sodium aluminate/carbohydrate	Isomerization time GOS (hours)	% M	% D	% T
Lactose isomerization reaction			36.88	47.12	16
120	1:1	8	35.91	45.23	18.86
120	2:1	8	28.74	53.07	18.19
Lactose isomerization reaction			61.19	25.94	12.87
300	1:1	8	58.1	24.77	17.13
300	2:1	8	51.64	32.78	15.58
Enzymatic reaction time (minutes)	Molar ratio boric acid/carbohydrate	Isomerization time GOS (minutes)	% M	% D	% T
Lactose isomerization reaction			36.88	47.12	16

120	1:1	90	38.01	44.06	17.93
120	2:1	90	40.22	42.22	17.56
Lactose isomerization reaction			61.19	25.94	12.87
300	1:1	90	58.5	27.69	13.81
300	2:1	90	56.33	30.22	13.45

After GOS isomerization, samples were freeze dried again, and we proceeded to supercritical CO₂ extraction with ethanol/water 95:5 (v/v) as a cosolvent. Experiment pool was designed in two steps as explained in previous research [20]. Both steps lasted 3 hours. Conditions of first step were 150 atm., 80°C and 0.6 mL/min⁻¹ of cosolvent. Second step were 100 atm., 100°C and 0.4 mL/min⁻¹ of cosolvent (Table 3 for extraction with aluminates and Table 4 with borates). In all supercritical extraction experiments 200 mg of carbohydrates were placed in the extraction cell.

Table 3. Supercritical extraction GOS isomerization with aluminates.

Sample	Extraction conditions	% M mixture	% D mixture	% T mixture	% M extracted	% D extracted	% T extracted	Al (µg/L)
120' 1:1 8 P	initial							676.8
	150/80/0.6 (0-3 h)	94.30	5.70	0.00	92.10	3.70	0.00	17.5
	100/100/0.4 (3-6 h)	67.20	32.80	0.00	7.90	2.50	0.00	9.0
	Total extracted	91.39	8.61	0.00	100.00	6.19	0.00	
120' 2:1 8 P	initial							249.6
	150/80/0.6 (0-3 h)	96.60	3.40	0.00	86.90	2.20	0.00	14.8
	100/100/0.4 (3-6 h)	30.30	69.70	0.00	13.10	22.30	0.00	8.0
	Total extracted	75.07	24.93	0.00	100.00	24.51	0.00	
300' 1:1 8 P	initial							7472.5
	150/80/0.6 (0-3 h)	97.99	2.01	0.00	43.11	2.07	0.00	17.8
	100/100/0.4 (3-6 h)	38.06	61.94	0.00	21.26	81.19	0.00	14.5
	Total extracted	64.46	35.54	0.00	64.37	83.26	0.00	
300' 2:1 8 P	initial							431.3
	150/80/0.6 (0-3 h)	85.79	14.20	1.20	85.05	11.33	0.00	62.7
	100/100/0.4 (3-6 h)	12.38	87.60	0.00	14.95	85.09	0.00	4.7
	Total extracted	45.47	54.50	0.00	100.00	96.40	0.00	
120' 1:1 8 NP	initial							296522
	150/80/0.6 (0-3 h)	100.00	0.00	0.00	50.02	0.00	0.00	74.4
	100/100/0.4 (3-6 h)	100.00	0.00	0.00	54.09	0.00	0.00	39.7
	Total extracted	100.00	0.00	0.00	104.11	0.00	0.00	

120'/300'=lactose isomerization minutes; 1:1/2:1=molar ratio; 8=GOS isomerization hours; P=aluminates precipitated with sulphuric acid; NP=aluminates no precipitated; Al (µg/L)= µg aluminium per litre of water.

During extractions of GOS isomerization with aluminates, we achieved a complete extraction of monosaccharides in all experimental conditions. Disaccharides extraction is near to be complete for isomerization times of lactose of 300 minutes. But in all experimental conditions we reduced significantly aluminium concentration below safe limits for consumers. There is not a specific European Union legislation relating to limits of boron and aluminium in food. The European Food Safety Agency (EFSA) has published various statements/opinions on boron and aluminium. For aluminium the panel of experts established based on the combined evidence a TWI (total week intake) for an adult 1 mg aluminium/kg bw (body weight)/week [21]. According to our results, the quantity of aluminium extracted with carbohydrates varies

around 0.1-1 mg Al/Kg of carbohydrates. Must be said that extraction conditions affect quantity of carbohydrates extracted and this ratio can be decreased or increased according with requirements. Environmental Protection Agency (EPA) recommends aluminium concentration in water till 200 µg Al/L.

When aluminates are not removed after GOS isomerization, only monosaccharides can be eliminated. But aluminium level decrease as in previous extractions.

Table 4. Supercritical extraction GOS isomerization with borates.

Sample	Extraction conditions	% M mixture	% D mixture	% T mixture	% M extracted	% D extracted	% T extracted	B (µg/L)
120' 1:1 90' P	initial							458.8
	150/80/0.6 (0-3 h)	94.99	5.01	0.00	74.79	3.40	0.00	142.3
	100/100/0.4 (3-6 h)	82.67	17.33	0.00	17.86	3.23	0.00	31.6
	Total extracted	92.34	7.66	0.00	92.65	6.63	0.00	
120' 2:1 90' P	initial							1017.3
	150/80/0.6 (0-3 h)	98.96	1.04	0.00	82.96	0.83	0.00	298.4
	100/100/0.4 (3-6 h)	29.18	70.82	0.00	12.80	29.59	0.00	48.8
	Total extracted	74.99	25.01	0.00	95.76	30.42	0.00	
300' 1:1 90' P	initial							390.3
	150/80/0.6 (0-3 h)	98.89	1.11	0.00	47.90	1.14	0.00	177.3
	100/100/0.4 (3-6 h)	29.16	70.84	0.00	17.55	90.08	0.00	37.9
	Total extracted	60.25	39.75	0.00	65.45	91.22	0.00	
300' 2:1 90' P	initial							678.2
	150/80/0.6 (0-3 h)	95.16	4.84	0.00	86.62	8.22	0.00	268.9
	100/100/0.4 (3-6 h)	24.60	75.40	0.00	15.91	90.92	0.00	39.5
	Total extracted	65.84	34.16	0.00	102.53	99.14	0.00	
120' 1:1 90' NP	initial							87520.2
	150/80/0.6 (0-3 h)	100.00	0.00	0.00	52.90	0.00	0.00	9012.3
	100/100/0.4 (3-6 h)	100.00	0.00	0.00	45.75	0.00	0.00	1223.5
	Total extracted	100.00	0.00	0.00	98.65	0.00	0.00	

120'/300'=lactose isomerization minutes; 1:1/2:1=molar ratio; 90'=GOS isomerization minutes; P=borates precipitated with sulphuric acid; NP=borates no precipitated; B (µg/L)= µg boron per litre of water.

During extractions of GOS isomerization with borates, we also achieved a complete extraction of monosaccharides in all experimental conditions. Disaccharide extraction is complete for isomerization times of lactose of 300 minutes. As in experiments with aluminates, there is a significantly decrease in boron concentration below safe limits for consumers. As abovementioned, there is not a specific European Union legislation, but EFSA has published various statements/opinions on boron. Experts have concluded a tolerable upper intake level of 10 mg/day for adults. The level recommended by the US Food Nutrition Board was 20 mg/day and the UK's expert working group on vitamins and minerals (EVM) recommended 6mg [22]. According with our data quantity of boron extracted with carbohydrates varies around 0.3-0.6 mg B/Kg of carbohydrates.

When borates are not removed after GOS isomerization, only monosaccharides can be eliminated, but boron level decrease less than in previous extractions (~16 mg B/Kg of carbohydrates extracted). This quantity is still below limits recommended, assuming eating one kilogram of carbohydrates every day. Environmental Protection Agency (EPA) regulations and recommendations establish 1 mg B/L of water as upper level for boron concentration.

CONCLUSIONS

Monosaccharides present in GOS isomerization could be removed in all supercritical extractions. Thus, non prebiotic carbohydrates (most of monosaccharides), especially glucose, can be eliminated from the original mixture. Also, in some conditions, disaccharides could be removed, that means that lactose can be eliminated thus favouring the use of such products for lactose intolerants. Anyway, there is a dramatic reduction of boron and aluminium concentration in all experimental conditions, demonstrating that supercritical fluid extraction technology can be suitable to obtain quasi-free carbohydrates from GOS isomerization.

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