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KB Development of techniques for analysis of (modified) carbohydrates.
Deliverable 1.2

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PUBLIC

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Preface

This research project has been subsidised by the Dutch Ministry of Agriculture, Fisheries, Food Security and Nature. Under the Wageningen University & Research Knowledge Base Programme on Biobased and Circular Society (KB-51-000-006). To obtain a successful shift towards sustainability one of the KB-themes at Wageningen University and Research is the primary production and processing of biobased raw materials and products. Carbohydrates are a component of biomass and are essential in our daily lives, found in food as well as in materials and chemicals. However, their complexity and variability make it challenging to fully understand and predict their functionality. This project aims to develop improved and advanced characterization technologies to obtain more knowledge of carbohydrate structures and their potential applications. By doing so, we can make better use of (underutilized) biomass, as we gain insights into carbohydrate quantities from diverse biomass sources and their functional potential of these carbohydrates in various applications. This will increase the number of biobased products instead of using fossil-based products.

1 Introduction

Carbohydrates play an important role in our daily lives like in food or as component in materials or chemicals. However, their complexity and variability can make it challenging to understand and predict their functionality in applications and consequently limit their use. In addition, for some types of carbohydrates, current methods for characterizing their structures and properties are also limited. In the Knowledge Base Programme: Biobased & Circular Society at Wageningen University and Research the aim is to have a transition from a linear fossil-based society to a biobased circular society. To ensure a successful shift towards sustainability one of the themes is the primary production & processing of biobased raw materials and products. Here, the processing of sustainable biobased raw materials for high value-added products and applications following the total use and cascading principle is important. Therefore, understanding the structure-properties relation of carbohydrates for biobased applications but also for food is crucial for utilization of biomass sources.

In this project we focus on carbohydrates. In some cases, the characterization and/or identification of these carbohydrates is limited, thereby limiting their potential utilisation. Here, we develop improved characterization technologies to gain insight into these carbohydrate structures, enabling better predictions of their functionality in food and biobased chemicals/materials. Also, through combining different analytical techniques, we can be able to leverage important characterization and corresponding functionality data. For example, chitosan is one the most abundant polysaccharides in the world. However, it is still not possible to determine its composition (*N*-acetyl glucosamine and glucosamine ratio) in a proper way. This is a question that is often asked by our clients/market. This also appears to depend on the biological source, such as crustaceans, fungi, or insects. In this study, we conducted experimental work aimed at developing and improving the characterization of the structural building blocks of chitosan.

2 Experimental work

The Seaman hydrolysis is often used for the characterisation of plant cell walls. This method was used to determine the glucosamine content of isolated chitosan. Hydrolysis was performed with 12 M H₂SO₄, for 1 h at 35°C and subsequently sulphuric acid was diluted to 2 M and incubated for 1 h at 100°C. The carbohydrate composition, determined by High Performance Anions Exchange Chromatography (HPAEC), is shown in Table 1. Three commercial chitosan (A, B and C) samples were analysed. Sample A showed a high glucosamine content whereas for both other samples (B and C) a low amount of glucosamine was detected. During the acid treatment *N*-acetyl-glucosamine is converted into glucosamine. Thus, only glucosamine will be identified in the analysis. In commercial chitosan samples it is assumed that a high amount of glucosamine will be present, however, under the conditions analysed for sample B and C only very low glucosamine content was found. For sample A higher amount was obtained, although still less than 50% (w/w).

Table 1 Carbohydrate content of commercial chitosan samples after hydrolysis with 12M/2M sulphuric acid.

Content (% w/w)	Fucose	Arabinose	Rhamnose	Galactose	Glucose	Glucosamine	Xylose	Mannose	Total
Chitosan A	0.00	0.00	0.00	0.00	0.00	44.04	0.00	0.00	44.04
Chitosan A	0.00	0.00	0.00	0.00	0.00	40.29	0.00	0.00	40.29
Average	0.00	0.00	0.00	0.00	0.00	42.16	0.00	0.00	42.16
RSD	0.00	0.00	0.00	0.00	0.00	6.28	0.00	0.00	6.28
Chitosan B	0.00	0.00	0.00	0.00	0.00	4.60	0.00	0.00	4.60
Chitosan B	0.00	0.00	0.00	0.00	0.00	4.50	0.00	0.00	4.50
Average	0.00	0.00	0.00	0.00	0.00	4.55	0.00	0.00	4.55
RSD	0.00	0.00	0.00	0.00	0.00	1.62	0.00	0.00	1.62
Chitosan C	0.00	0.00	0.00	0.00	0.00	3.56	0.00	0.00	3.56
Chitosan C	0.00	0.00	0.00	0.00	0.00	3.53	0.00	0.00	3.53
Average	0.00	0.00	0.00	0.00	0.00	3.55	0.00	0.00	3.55
RSD	0.00	0.00	0.00	0.00	0.00	0.58	0.00	0.00	0.58

In some studies, hydrolysis of chitosan with HCl is reported using 6 N HCl for 6 h at 100 °C. The results are shown in Table 2. A large deviation in the results was observed for the repetition of all the duplicate samples. In comparison with the 12M/2M sulphuric acid treatment, the amount of glucosamine was lower for sample A and higher for sample B and C. So, the treatment using different acids can have an influence on the amount of glucosamine measured. However, still the obtained values are too low.

Table 2 Carbohydrate content of commercial chitosan samples after hydrolysis with 6M HCl.

Content (% w/w)	Fucose	Arabinose	Rhamnose	Galactose	Glucose	Glucosamine	Xylose	Mannose	Total
Chitosan A	0.00	0.07	0.00	0.00	0.00	6.76	0.00	0.00	6.83
Chitosan A	0.00	0.06	0.00	0.00	0.00	0.29	0.00	0.00	0.35
Average	0.00	0.06	0.00	0.00	0.00	3.53	0.00	0.00	3.59
RSD	0.00	8.87	0.00	0.00	0.00	129.66	0.00	0.00	
Chitosan B	0.00	0.12	0.00	0.00	0.00	14.40	0.00	0.00	14.52
Chitosan B	0.00	0.04	0.00	0.00	0.00	51.68	0.00	0.00	51.72
Average	0.00	0.08	0.00	0.00	0.00	33.04	0.00	0.00	33.12
RSD	0.00	75.27	0.00	0.00	0.00	79.78	0.00	0.00	79.41
Chitosan C	0.00	0.10	0.00	0.00	0.00	13.59	0.00	0.00	13.69
Chitosan C	0.00	0.07	0.00	0.00	0.00	17.19	0.00	0.00	17.26
Average	0.00	0.09	0.00	0.00	0.00	15.39	0.00	0.00	15.47
RSD	0.00	25.55	0.00	0.00	0.00	16.54	0.00	0.00	16.30

For two samples a higher glucosamine content was found whereas for another sample the glucosamine content was lower as found after 12/2M sulphuric hydrolysis (Table 1 and 2, respectively). It was further investigated if a treatment with 0.1N HCl overnight, at room temperature, followed with a 6 N HCl treatment at 100 °C resulted in higher glucosamine content and less variation in the duplicate samples. The results are shown in Table 3. Sample A showed a lower glucosamine content using a HCl hydrolysis, whereas for Sample B and C higher glucosamine values were obtained.

Table 3 Carbohydrate content of commercial chitosan samples after overnight incubation/hydrolysis with 0.1 N HCl followed by 6M HCl.

Content (% w/w)	Fucose	Arabinose	Rhamnose	Galactose	Glucose	Glucosamine	Xylose	Mannose	Total
Chitosan A	0.00	0.00	0.00	0.00	0.00	11.79	0.00	0.00	11.79
Chitosan A	0.00	0.14	0.00	0.00	0.00	22.35	0.00	0.00	22.49
Average	0.00	0.07	0.00	0.00	0.00	17.07	0.00	0.00	17.14
RSD	0.00	141.42	0.00	0.00	0.00	43.74	0.00	0.00	44.15
Chitosan B	0.00	0.09	0.00	0.00	0.05	47.66	0.00	0.00	47.81
Chitosan B	0.00	0.20	0.00	0.00	0.11	42.75	0.00	0.00	43.05
Average	0.00	0.15	0.00	0.00	0.08	45.21	0.00	0.00	45.43
RSD	0.00	46.69	0.00	0.00	55.29	7.69	0.00	0.00	7.40
Chitosan C	0.00	0.04	0.00	0.00	0.02	40.82	0.00	0.00	40.88
Chitosan C	0.00	0.05	0.00	0.00	0.02	44.56	0.00	0.00	44.63
Average	0.00	0.05	0.00	0.00	0.02	42.69	0.00	0.00	42.75
RSD	0.00	16.51	0.00	0.00	10.25	6.19	0.00	0.00	6.20

For Chitosan A with 6 N HCl treatment (Table 2) resulted in a lower value for glucosamine when using sulphuric acid (Table 1). Various acid treatments and combinations were tested to increase the yield of glucosamine. Method A consisted of overnight treatment with 0.1 N HCl at room temperature followed by 12M H₂SO₄/1M H₂SO₄_4M HCl. Method B samples were treated with 0.1M HCl overnight followed with 12M/2M H₂SO₄. Methode C consisted of 72h 0.1M HCl overnight, followed with 12M H₂SO₄/1M H₂SO₄_4M HCl. Method D samples were treated with 72h 0.1M HCl - 12M/2M H₂SO₄. Results are shown in Table 4. Pretreatment with HCl, under all conditions investigated, did not increase the amount of glucosamine compared with solely 12/2M sulphuric acid treatment (Table 1).

Table 4 Glucosamine content after hydrolysis (A) overnight 0.1M HCl - 12M H₂SO₄/1M H₂SO₄_4M HCl, (B) overnight 0.1M HCl - 12M/2M H₂SO₄, (C) 72h 0.1M HCl - 12M H₂SO₄/1M H₂SO₄_4M HCl and (D) 72h 0.1M HCl - 12M/2M H₂SO₄.

Glucosamine content (% w/w)	A	B	C	D
Treatment				
Chitosan A	6.72	5.09	7.11	3.59
Chitosan A	7.09	5.65	7.01	3.24
Average	5.37	5.37	7.06	3.41
RSD	3.84	7.25	1.05	7.21

This indicates that the type and/or treatment of isolated chitosan plays a role in the determination of glucosamine. In addition, also the type and/or combination of acid hydrolyses plays a role. It is not clear how this is related to each other. However, the amounts detected are too low for glucosamine as expected.

It is assumed that crystallinity plays a role in the accessibility for the acid to hydrolyse chitosan into its building blocks. Hence, to investigate the role of crystallinity, chitosan was milled using a planetary mill. This will decrease the crystallinity without destroying the chains. Both milled and untreated chitosan A was hydrolysed with 12/2 M sulphuric acid. However, this hydrolysis was preceded by an overnight incubation with 0.1 M HCl.

The results are shown in Table 5. Both milled and untreated chitosan A gave similar glucosamine content although the milled chitosan A showed a much higher variation between the samples. Furthermore, the amount of glucosamine was much higher as observed before (Table 4, column B) where the same condition was applied for the untreated sample. It is not clear what the reason is for this difference.

Table 5 *Glucosamine content after hydrolysis of untreated and planetary milled chitosan A with 0.1 N HCl overnight followed by 12M H₂SO₄/2M H₂SO₄.*

Untreated	Glucosamine (% w/w)	Milled	Glucosamine (% w/w)
Chitosan A	28.44	Chitosan A	33.50
Chitosan A	31.32	Chitosan A	20.49
Chitosan A	33.21	Chitosan A	35.92
Chitosan A	33.66	Chitosan A	24.66
Average	31.66	Average	28.64
RSD	7.49	RSD	25.42

3 Conclusions

To determine the amount of glucosamine and/or *N*-acetylglucosamine in chitosan is challenging. The most common methods using acids such as HCl or sulphuric acid or combination hereof. The released carbohydrates are quantified by high performance anion exchange chromatography or colour assay. However, often an underestimation is found, as shown in the experimental part above. Acid hydrolysis depends on the cleavage breaking of the glycosidic linkages, but can also lead to decomposition into other molecules, thereby decreasing the yield of carbohydrates after the hydrolysis reaction. Also, the accessibility of the acid can be hindered by for example crystallinity that result in a lower release of monosugars.

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