Revealing the diversity of algal monosaccharides: Fast carbohydrate fingerprinting of microalgae using crude biomass and showcasing sugar distribution in *Chlorella vulgaris* by biomass fractionation

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ABSTRACT

The quantitative and qualitative analysis of carbohydrates from algae is essential for the optimal utilization of algal biomass. A previously established high throughput method for the identification of complex carbohydrates was applied to microalgae. It is based on the selective derivatization of monosaccharides by 1-phenyl-3-methyl-5-pyrazolone (PMP), UHPLC separation and MS analysis of the derivatives. The crude biomass of six different representative microalgae was analyzed and the monosaccharide composition after trifluoroacetic acid (TFA) hydrolysis is reported here. In addition to the usually found neutral sugars, uronic acids, and amino sugars, with this method methylated and phosphorylated or sulfated monosaccharides were also identified. The PMP derivatization enabled a monosaccharide recovery in the hydrolysates between 90% and 105% in five strains. Furthermore, a fractionation process with an overall recovery of 82% was applied to the microalga *Chlorella vulgaris* to investigate the carbohydrate distribution in the cell. The relative amounts of starch, soluble sugars, carbohydrates associated with glycoproteins and glycolipids, as well as the structural polysaccharides were determined. Due to its advantages, this method can help in strain screening and designing of proper strategies for maximal biomass development, formation of rare sugars as well as general utilization in the emerging field of algal biorefinery.

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1. Introduction

In the search of renewable energy and fuel production systems apart from edible sources, micro and macro algae have been increasingly investigated in the last decades. Their use as sustainable raw material is not limited to the production of biofuels, but also for fine chemicals, human nutrition and animal feed. Thus, the concept of algal biorefinery has risen in which all their components can be converted into value-added products [1]. To achieve this, the starting algal biomass must be precisely characterized so that suitable processing strategies can be applied. Since the composition of algal biomass varies according to the specific strain used, different culture strategies and growth phases, fast and reliable determination methods for algal proteins, lipids and carbohydrates are needed to accelerate the screening for new strains, as well as to optimize the production conditions [2,3]. In the recent past, efforts have been focused towards the assessment of existing analytical procedures for terrestrial plant biomass upon their applicability to algae. So the need for analytical improvement has been identified especially regarding carbohydrate analysis focused on algal-specific monosaccharides, such as uronic acids, amino sugars and sulfated sugars [4]. A precise determination of the monosaccharide composition

of crude algae is decisive for the comprehensive utilization of algal biomass in the concept of biorefinery, especially when designing strategies for fermentations on algal hydrolysates. Carbohydrates from algae can be found in different forms depending on the biological task they fulfill, such as storage (starch), structural (cell wall, e.g., cellulose) or signaling (glycolipids or glycoproteins) [5], and the identification of their origin provides valuable information for developing adequate processing strategies, e.g., using hydrolytic enzymes [6,7]. Different chromatographic approaches have been extensively assessed and optimized for resolving the broad palette of microalgal monosaccharides. Conventional high performance liquid chromatography (HPLC) coupled to refractive index detection (RID) or charged aerosol detection (CAD) displays a poor separation on complex monosaccharide mixtures. Gas chromatography (GC) with flame ionization detector (FID) preceded by trimethyl silylation (TMS) or alditol acetate derivatization yields very good chromatographic resolution, is highly sensitive and also compatible with mass spectrometry (MS). However, the quantification of uronic acids and amino sugars is challenging, and the sample preparation is time consuming [8]. High performance anion exchange chromatography (HPAEC) combined with pulsed amperometric detection (PAD) is a very robust method for carbohydrate analysis without the need for sugar derivatization, and configurations have been developed for resolving up to 13 different sugars present in algae. Still, the use of strong ionic eluents impedes the direct application of MS for identifying the

commonly occurring unknown carbohydrates in algal hydrolysates [8]. A comparative overview of relevant existing methods for the analysis of complex carbohydrate mixtures (as those found in algal biomass) is presented in Table 1.

It is evident that standardized, fast and reliable analytical methods for algal carbohydrates are necessary. Addressing this need, we present a procedure for fast fingerprinting of monosaccharides from crude and processed algal biomass. It is based on a previously described method for the fast chromatographic analysis of carbohydrates after TFA-hydrolysis and derivatization with 1-phenyl-3-methyl-5-pyrazolone (PMP) in 96-well format [10]. Often, the derivatization of compounds for the analysis of large sets of samples is undesirable, since sample preparation is time consuming. However, the parallelized derivatization procedure enables a very fast sample processing and can potentially be used for automated platforms and higher throughput, as demonstrated previously for the screening of bacterial exopolysaccharides [11]. During the selective chemical derivatization of aldoses, two PMP molecules react by condensation with the aldehyde group of the saccharides, yielding the corresponding *bis*-PMP derivative [12]. This allows their separation by reverse phase UHPLC. At the same time, the sugars are provided with UV-absorbance, and become ionizable in positive mode for MS analysis. Thus, their identification and quantification via UV- and MS-detection is possible in similar ranges. This is a relevant feature for complex saccharide mixtures, such as biomass hydrolysates, in which potentially interfering compounds (e.g., amino acids, lipids, pigments or degradation products) are present. The two detection systems provide a cross-verification of each compound found, and co-eluting or unknown compounds can be easily identified. Although the procedure has been validated for the quantification of 18 different derivatized mono- and disaccharides in pure solution [10], the effect of potentially interfering aldehyde compounds present in algal hydrolysates (such as acetaldehyde or retinal [13]) needs to be considered. While a similar sugar quantification procedure has been employed for the compositional determination of carbohydrates from *Dunaliella salina*, the conditions were not optimized for a rapid analysis in 96-well format and were MS incompatible [9]. The remarkably short LC running time (12 min), low limit of detection (LOD), and the possibility of MS determination

of unknown or co-eluting peaks as well as derivatization and sample processing in microtiter plates (Table 1) renders the optimized method a highly reliable candidate for rapid fingerprinting of algal carbohydrates. This was demonstrated by analyzing six different representative algae species. The application of this method to algal biomass was validated by spiking the algal hydrolysates and calculating the sugar recoveries. Plus, a lab-scale fractionation process was developed and applied to one of the most commonly investigated microalga, *Chlorella vulgaris*. The monosaccharide composition of the process fractions was analyzed to get an insight on the composition and distribution of the carbohydrates in the cell.

2. Materials and methods

2.1. Chemicals and algal strains

All chemicals were, unless otherwise stated, purchased in analytical grade from Sigma Aldrich (Germany), Merck KGaA (Germany) and Carl Roth GmbH (Germany). Substituted monosaccharide standards were purchased from Carbosynth Ltd. (United Kingdom). Lyophilized whole cells from *C. vulgaris* were purchased from Algomed (Klötze, Germany). All other algal strains were cultivated, lyophilized and kindly provided by Dr. Sabine Mundt (Institut für Pharmazie, University Greifswald). A standard cultivation procedure was applied under no nutrient limitation and without light stress conditions for 14 d at 25 °C. Following culture media were employed: SWES for *N. salina* and *Phaeodactylum tricornutum*, f/2 supplemented with trace elements for *Porphyridium purpureum*, DUN supplemented for *D. salina* and standard BG-11 for *Scenedesmus ovalternus*, as described previously [14].

2.2. Hydrolysis of algal biomass

Analytical chemical hydrolysis was performed by adding 6 mL of 2 M TFA to 6 mg of algal biomass (process fraction or whole lyophilized algae) into 15 mL glass tubes. The tubes were incubated in a heating block (VLM GmbH, EC-Model) for 90 min at 121 °C (referred as standard hydrolysis). After cooling to room temperature, the hydrolysates were

Table 1
Published chromatographic methods for the quantification of complex monosaccharide mixtures.

	HPLC-CAD [8]	GC-FID [8]	HPAEC-PAD [8]	HPLC-UV [9]	HPLC-UV-MS [10]
Column type; phase	Prevail ES carbohydrate; normal phase	DB-225MS	PA-1	ZORBAX Eclipse XDB-C18; reverse phase	Gravity C18; reverse phase
Column temperature (°C)	30	70 to 240 in gradient, 8 °C min^{-1}	37.5	30	50
Eluents	A: water B: ACN gradient elution	Carrier gas: helium	A: 20 mM NaOH B: 100 mM NaOH/150 mM sodium acetate gradient elution	0.1 M phosphate buffer pH 6.7 and ACN 83:17 (v/v %) isocratic elution	A: 5 mM ammonium acetate buffer pH 5.6 with 15% ACN (w/w %) B: 100% ACN gradient elution
Flow rate (mL min^{-1})	1.0	12.8 total flow (split 1:10)	NP	1	0.6
Monosacharide tagging	None	Alditol acetate-derivatization	None	PMP-derivatization	PMP-derivatization
LC running time (min)	60	35	65 (35 + 30 column regeneration)	55	12
Determination neutral sugars	Yes	Yes	Yes	Yes	Yes
Determination amino sugars	Limited	Limited	Yes	Yes	Yes
Determination uronic acids	Limited	Limited	Yes	Yes	Yes
Limit of detection (mg mL^{-1})	NP	0.5 (neutral sugars) – 3.5 (amino sugars)	6.3–40	0.7–2.0[a]	UV: 0.6–2.0 MS: 0.5–2.9
MS compatibility	No	Yes	No	No	Yes
Monosaccharide recovery (%)	NP	93.0–98.1[b]	90.4–106.2[b]	95.2–103.2[c]	UV: 94.6–101.6[b] MS: 90.6–98.8[b]

Abbreviations: CAD: charged aerosol detection, FID: flame ionization detector, PAD: pulsed amperometric detection, NP: not presented, ACN: acetonitrile, PMP: 1-Phenyl-3-methyl-5-pyrazolone.

[a] Values are published in nmol and were converted to mg mL^{-1} under described assay conditions.
[b] Recovery reported as calibration verification standard without matrix.
[c] Recovery reported from spiked samples with algal hydrolysate matrix.