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METHODS PAPER

Optimization of the bicinchoninic acid assay for quantifying carbohydrates of soil extracellular polymeric substances

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Abstract

Background and aims The bicinchoninic acid (BCA) method was not yet applied on soil extracts of extracellular polymeric substances (EPS) to quantify polysaccharides, although this might be possible by introducing a cleavage step to produce monosaccharides. A pre-extraction with $CaCl_2$ to remove interfering substances is usually performed before extracting EPS. The main objective of this study was to optimize the BCA assay for total carbohydrates quantification by applying a hydrolysis step to the EPS extracts, while also testing carbohydrate contents of $CaCl_2$ pre-extracts.

Methods Total carbohydrates were quantified with BCA in EPS extracts of three soils, after hydrolysis with H_2SO_4, using two acid concentrations (0.75 and 1.0 M), three hydrolysis temperatures (100, 120 and 130 °C), and five hydrolysis times (10, 30, 50, 70, and 90 min). EPS were extracted with the cation exchange resin (CER) method adapted to soils. Two versions of pre-extraction with $CaCl_2$ were tested twice consecutively.

Results More carbohydrates were measured after hydrolysis with 0.75 M H_2SO_4 at below 100 °C and after 10 min for all soils. Decreasing values were seen after longer reaction times and higher temperatures. $CaCl_2$ extracted no or negligible amounts of carbohydrates from the soil.

Conclusion The pre-extraction step can be done without in most cases. The BCA assay is free of toxicity and easily performed, while also tolerant to interferences from most compounds in EPS extracts. These characteristics highlight the potential of this method for a rapid quantification of carbohydrates in studies of extractable polymers in several areas of soil biogeochemistry.

Keywords Bicinchoninic acid · BCA · Carbohydrates · Polysaccharides · Extracellular polymeric substances · EPS

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Introduction

Extracellular polymeric substances (EPS) are an important component of microbial residues that embed microorganisms (Wingender et al. 1999). They can improve soil aggregate stability (Cania et al. 2020; Chenu and Plante 2006; Guhra et al. 2022) and protect microbial cells and extracellular enzymes against drought (Bhattacharjee et al. 2020; Kakumanu et al.

2013, 2019). EPS can also trap and store nutrients (Costa et al. 2018; Flemming and Wingender 2010; Or et al. 2007).

The presence of EPS in soils is usually investigated by quantification of their components, i.e., polysaccharides (Costa et al. 2018), after extraction. Currently, a variety of methods are available for such quantifications. The bicinchoninic acid (BCA) method has been extensively used for total carbohydrate quantification in a wide range of areas, from automated high-performance liquid chromatography (Joergensen and Meyer 1990; Mopper and Gindler 1973; Sinner and Puls 1978) to manual spectrophotometry (Arnal et al. 2017; Hussain et al. 2003; McFeeters 1980; Nadour et al. 2015; Utsumi et al. 2009; Waffenschmidt and Jaenicke 1987). The reaction of Cu^{2+} with reducing sugars of mono- and oligosaccharides, and the consequent chelation of the reduced Cu^{+} with BCA produces a purple colour (Mopper and Gindler 1973; Sinner and Puls 1978), which can be read colorimetrically at an absorbance of 562 nm, similarly to protein assays (Huang et al. 2010). The BCA assay has been used to quantify carbohydrates in litter (Joergensen and Meyer 1990; Khan et al. 2012; Rottmann et al. 2011), in K_2SO_4 soil extracts Joergensen et al. 1990, 1994), but also in the soil microbial biomass (Joergensen et al. 1996).

However, the BCA assay does not appear to have been applied to soil EPS extracts to date. Since polysaccharides mostly do not exhibit reducing ends (BeMiller 2019), using the BCA assay to quantify total carbohydrates in EPS extracts might require the addition of a hydrolysis step. In cellulose and hemicellulose extracts from litter, hydrolysis has already been applied as an additional step before the BCA method (Rottmann et al. 2011). The frequently used phenol sulphuric method (DuBois et al. 1956) applies a concentrated sulphuric acid to break down polysaccharides, oligosaccharides and disaccharides to monosaccharides, before they can react with phenol to produce colour (Nielsen 2010). This method, however, has been shown to suffer interference from glycoproteins (DuBois et al. 1956) and phenol is known to be highly toxic and carcinogenic (Velamakanni et al. 2021). The high sensitivity of the BCA assay for reducing ends and its higher tolerance to interference from most compounds (Walker 1996) highlight its advantages. The optimization of conditions for the application of the BCA assay on EPS extracts has the potential to yield reliable and consistent values from EPS extracts free of toxicity.

The main objective of the present work is to optimize the BCA assay for quantification of total carbohydrates in EPS extracts after introducing a hydrolysis step. We investigate carbohydrates present in the EPS extracts of three distinct soils to cover a range of EPS carbohydrate contents, after hydrolysis with sulphuric acid (H_2SO_4), using two different acid concentrations, three hydrolysis temperatures, and five hydrolysis times. Another objective was to check the carbohydrate and SOM content in the 0.01 M $CaCl_2$ pre-extract of the current three soils. This pre-extraction is usually conducted as a mean to reduce interfering effects of extractable non-EPS SOM (Bérard et al. 2020; Redmile-Gordon et al. 2014; Zhang et al. 2023), although it is not always performed (Sher et al. 2020).

Materials and methods

Soils

Three arable soils under conventional winter rye cultivation were sampled at 0–20 cm depth in March 2022. Soil 1 was sampled at Herberhausen (51°32′47.3”N 9°59′49.3″E) and soil 2 at Gleichen (51°27′43.9”N 9°58′59.0″E), both near Göttingen, Lower Saxony, Germany. Soil 3 was sampled at Neu Eichenberg (51°22′35.0”N 9°53′52.0″E), Hessia, Germany (Table 1). Soil 1 was a Cambisol developed from Loess, soil 2 was a Eutric Cambisol developed

Table 1 Information on sampling sites, soil type and average soil organic carbon (SOC), total nitrogen (N) and extracellular polymeric substances (EPS) protein content

No.	Soil type	pH (H_2O)	SOC (mg g^{-1} soil)	Total N	EPS-proteins (µg g^{-1} soil)
1	Cambisol (Loess)	6,22	19.7	1.95	122
2	Cambisol (New Red Sandstone)	7,46	13.3	1.29	42
3	Stagnic Luvisol (Loess)	7,44	13.7	1.31	60

from New Red Sandstone, and soil 3 was a Stagnic Luvisol again developed from Loess according to the WRB-FAO classification system (IUSS Working Group WRB 2022). All field moist soils were sieved (< 2 mm) and stored at 4 °C. Soil samples were analysed for total C and N, using a Vario MAX (Elementar, Hanau, Germany) elemental analyzer after being dried for 24 h at 105 °C and ball milled. Soil organic C (SOC) was determined as total C minus carbonate C, which was gas-volumetrically determined after the addition of 10% HCl to the soil using a Scheibler apparatus (Blume et al. 2011). Soil pH was measured potentiometrically using a soil to water ratio of 1 to 2.5 (w/v).

Pre-extraction

In their original method description, Redmile-Gordon et al. (2014) suggested an initial pre-extraction with 0.01 M $CaCl_2$ to reduce interfering effects of extractable non-EPS SOM. To check the relevance of this step for the current soils, this pre-extraction was performed twice consecutively in two different versions: (I) Following the original method proposed by Redmile-Gordon et al. (2014), three replicates of 2.5 g (on an oven-dry basis) moist soil were extracted with 25 ml 0.01 M $CaCl_2$.at 120 rev min^{-1} for 30 min at 4 °C in the dark. The soil slurry was centrifuged at 4000 g for 10 min and the supernatant was decanted. To examine the effect of multiple extractions, we added a step to the method, wherethe remaining soil was extracted again with 25 ml 0.01 M $CaCl_2$ and centrifuged. (II) To increase the yield of extractable SOM, the shaking force was increased to 200 rev min^{-1} and the temperature from 4 °C to room temperature. Organic C was determined immediately after both tests using a Multi N/C 2100S analyser (Analytik Jena, Germany). Carbohydrates were measured as described below with and without the final hydrolysis step the next day after storage at 4 °C.

EPS extraction

EPS extraction followed the cation exchange resin (CER) method by Frølund et al. (1996), adapted to soils by Redmile-Gordon et al. (2014), except that no pre-extraction with $CaCl_2$ was performed.

For extracting EPS, first CER (Dowex 'Marathon C' Na form, strongly acidic, 20–50 mesh) was washed with phosphate buffered saline (PBS) solution (2 mM $Na_3PO_4 \cdot \times 12\ H_2O$ [0.760 g L^{-1}], 4 mM $NaH_2PO_4 \cdot \times H_2O$, [0.552 g L^{-1}], 9 mM NaCl [0.526 g L^{-1}], 1 mM KCl [0.0746 g L^{-1}]) for 1 h at 4 °C in the dark. Three replicates of each field-moist soil, equivalent to 2.5 g dry weight, were weighed into centrifuge tubes, washed CER was added together with 25 ml chilled PBS and tubes were shaken for 2 h at 120 rev min^{-1} in the dark. The amount of CER used for each soil was calculated based on the soil organic carbon (SOC) content according to Redmile-Gordon et al. (2014), i.e. 177.8 g CER g^{-1} SOC. After shaking, tubes were centrifuged at 4200 *g* for 20 min and supernatant containing EPS was frozen at −20 °C. A temperature of 4 °C was maintained throughout the extraction process.

EPS-proteins were estimated using a modified Lowry assay (Lowry et al. 1951) adapted to soil extracts for evading potentially confounding polyphenolic compounds (Redmile-Gordon et al. 2013, 2020). Protein was colourimetrically quantified on a microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany).

EPS polysaccharide hydrolysis was performed by adding H_2SO_4 to EPS extracts in reagent tubes, tightly closed to avoid evaporation and placed in an autoclave. The autoclave was chosen for the test because of its high temperature capacity. A reflux system might also be used for hydrolysis. Two different final H_2SO_4 concentrations were evaluated (0.75 and 1.0 M), together with three temperatures (100, 120 and 130 °C), and five durations (10, 30, 50, 70, and 90 min.). A final 0.75 M acid concentration was achieved by adding one ml 1.5 M H_2SO_4 to one ml EPS extract, whereas 1 M acid concentration was prepared adding 0.75 ml H_2SO_4 to 1.25 ml EPS extract. The EPS extract from each soil was examined in triplicate, resulting in nine samples for each acid concentration, and this was repeated for each temperature level and hydrolysis time, yielding a total of 270 samples. Total carbohydrates were determined according to Mopper and Gindler (1973), adapted by Joergensen et al. (1996), by the reduction of Cu^{2+} to Cu^{+} in the ends of mono- and disaccharides. The BCA reagent was prepared by combining 50 ml of an aqueous solution of 4% Na_2CO_3, 4% $(NaPO_3)_6$ and 0.2% aspartic acid with 6 ml of a 4% bicinchoninic acid disodium salt solution and 0.9 ml of a 4% $CuSO_4$ solution. D(+)-glucose was used as standard. Hydrolysed

extracts were then neutralized by adding 0.3 ml 5 M NaOH ml^{-1} hydrolysate for extracts with a 0.75 M final H_2SO_4 concentration, and 0.4 ml 5 M NaOH ml^{-1} hydrolysate for extracts with a 1.0 M final H_2SO_4 concentration. Carbohydrates were quantified by adding 2 ml BCA reagent to 0.5 ml neutralized hydrolysates in a test tube, placing it into a heating cabinet at 60 °C for 120 min and reading it colourimetrically at 562 nm, using a microplate reader.

Statistical analysis

Data were evaluated using R version 4.3.1 (R Core Team 2023). Mean values of EPS-carbohydrate contents were examined using a two-factor ANOVA for the effect of soil, acid concentration, temperature, and hydrolysis time on the data, followed by the Tukey HSD (honestly significant difference) post-hoc test to check differences between groups. Variance and homogeneity were checked using a Levene's test, and the normal distribution of the residuals was checked with a Shapiro-Wilk-test. To check for a possible random effect of soil, a mixed linear model was performed using the "lme4" package in R, with restricted maximum likelihood (REML), using hydrolysis time, temperature and acid concentration as fixed effects, and soil as random variable. Significance of fixed effects was verified using the Kenward-Roger approximation for degrees of freedom (Kenward and Roger 1997). Total SOC and carbohydrates in pre-extracts were analysed with a one-way ANOVA, using soil as factor and extraction version as repeated measures.

Results

Pre-extraction

No organic C or total carbohydrates were observed in the pre-extracts of version I (the method originally proposed by Redmile-Gordon et al. (2014)) at both steps (Table 2), indicating that carbohydrates in the extracts were below the limit of detection (LOD, 0.15 µg carbohydrates ml^{-1} extract). Version II, applying higher shaking force, rendered an average of 8.5 µg organic C g^{-1} soil extractable with 0.01 M $CaCl_2$ in the pre-extracts of the first step. This mean was reduced by 50% in the pre-extracts of the second step. In contrast, extractable carbohydrates using version II, varied around a mean of 0.30 µg g^{-1} soil without any effect of soil and pre-extraction step (Table 2). A 10 min hydrolysis of the extract with 0.75 M H_2SO_4 at 100 °C did not increase the carbohydrate content (results not shown).

Table 2 Results from tests version I and II: Extractable SOC and total carbohydrates in two consecutive extractions with 0.01 M $CaCl_2$; probability values of a one-way ANOVA, using soil as factor and extraction as repeated measures

	Organic C		Total carbohydrates	
	Version I	Version II	Version I	Version II
	(µg g^{-1} soil)			
Extraction 1				
Soil 1	0	9.4 a	0	0.35
Soil 2	0	7.0 b	0	0.28
Soil 3	0	9.0 a	0	0.35
Extraction 2				
Soil 1	0	5.0 a	0	0.44
Soil 2	0	2.8 b	0	0.25
Soil 3	0	2.8 b	0	0.19
Probability values				
Soil	–	0.01	–	NS
Extraction	–	<0.01	–	NS
Extraction × soil	–	NS	–	NS
CV (± %)	–	13	–	24

CV mean coefficient of variation between replicates ($n=3$); different letters within a column indicate an extraction-specific significant difference ($P<0.05$; Tukey-HSD)

EPS extracts

Loess-originated soils 1 and 3 had significantly greater carbohydrate yields than sand-originated soil 2 (Table 3), which was in accordance with the higher mean protein content (Table 1). ANOVA results show a non-significant effect of acid concentration on carbohydrates, but the mixed linear model shows a significant effect, highlighting the random effect of soil type (Table 3). Temperature and hydrolysis time presented significant effects on carbohydrates in both statistical tests. There were no significant interactions between soil type and the abovementioned parameters. Greater carbohydrate values are visible after hydrolysis with 0.75 M H_2SO_4 (Fig. 1a), under 100 °C (Fig. 1b) and after 10 min (Fig. 1c) for all soils. The hydrolysis time

Table 3 Mean EPS-carbohydrates; probability values of two-way ANOVAs using soil and acid concentration, soil and temperature, or soil and hydrolysis time as factors, and a mixed linear model using soil as random variable

	EPS-carbohydrates (μg g^{-1} soil)
Soil 1	398 a
Soil 2	239 c
Soil 3	264 b
Probability values of three two-way ANOVAs	
Soil	<0.01
Acid concentration	NS
Soil × acid concentration	NS
Soil	<0.01
Temperature	<0.01
Soil × temperature	NS
Soil	<0.01
Hydrolysis time	<0.01
Soil × hydrolysis time	NS
Probability values of mixed linear model	
Acid concentration	<0.01
Temperature	<0.01
Hydrolysis time	<0.01
Hydrolysis time × acid concentration	NS
Hydrolysis time × temperature	<0.01
Acid concentration × temperature	<0.01
CV (± %)	5.0

CV mean coefficient of variation between replicates ($n = 3$); letters represent differences between soils ($P < 0.05$, Tukey HSD)

× temperature as well as acid concentration × temperature interactions were both significant (Table 3). Carbohydrates hydrolysed at 120 °C and 130 °C already reached their maximum value after 10 min, whereas maximum carbohydrate values occurred after 50 min when hydrolysing at 100 °C (Fig. 2).

Discussion

Pre-extraction

The current three arable soils contained negligible amounts of extractable SOM and carbohydrates that might interfere with the CER method for extracting soil microbial EPS. The organic compounds observed in pre-extracts (Zhang et al. 2023) have sometimes been named soluble microbial products (Wang et al. 2019) or loosely bound EPS (Bérard et al. 2020). However, this is misleading as soil extracts always contain soluble SOM from various origins, including non-EPS, non-microbial residues, and a range of other compounds (Zhang et al. 2023) even after one pre-extraction. In soils, the extractable fraction simply reflects the cation- and anion-specific equilibrium between the liquid and solid phase of SOM (Joergensen 1995a, b; van Erp et al. 1998), which slowly declines only after repeated extraction steps (Mueller et al. 1992), as also demonstrated for the current soils.

The omission of the pre-extraction step in low-organic matter soils facilitates the use of the EPS-extraction method proposed by Redmile-Gordon et al. (2014). However, this might be different in experiments where easily decomposable material has been added, such as glycerol (Redmile-Gordon et al. 2014) or bio-diesel coproducts (Redmile-Gordon et al. 2015). The same should be true for high-organic matter soils, developed on peat or forest land (Wang et al. 2019).

EPS extracts

Carbohydrate yields in the current study were closely connected to the soil origin, whereas the influence of hydrolysis parameters is applicable to all soils in a similar trend. In other words, temperature, time, and acid concentration affected all soils equally. Our results indicate that hydrolysing carbohydrates with 0.75 M H_2SO_4 resulted in a generally higher carbohydrate yield than with 1.0 M (Fig. 1). Mild sugar hydrolysis (0.5–1.0 M H_2SO_4) without pre-treatment has already been successfully performed in soil (Cheshire and Mundie 1966) and marine particulate organic matter (Hanisch et al. 1996; Mopper 1977; Tanoue and Handa 1987), with 1.0 M being reported as the best concentration, also when compared to other acids (Mopper 1977).

Carbohydrates in soil are known to show resistance to hydrolysis due to polysaccharide bonding to soil components and trace metals like iron and copper, resulting in aggregation and salt formation (Martin 1971; Martin et al. 1972; Parfitt and Greenland 1970), whereas such conditions do not apply in liquid EPS extracts. Melton et al. (1976) performed partial hydrolysis of industrial EPS from *Xanthomonas campestris* using 0.5 M H_2SO_4, whereas Goo et al.

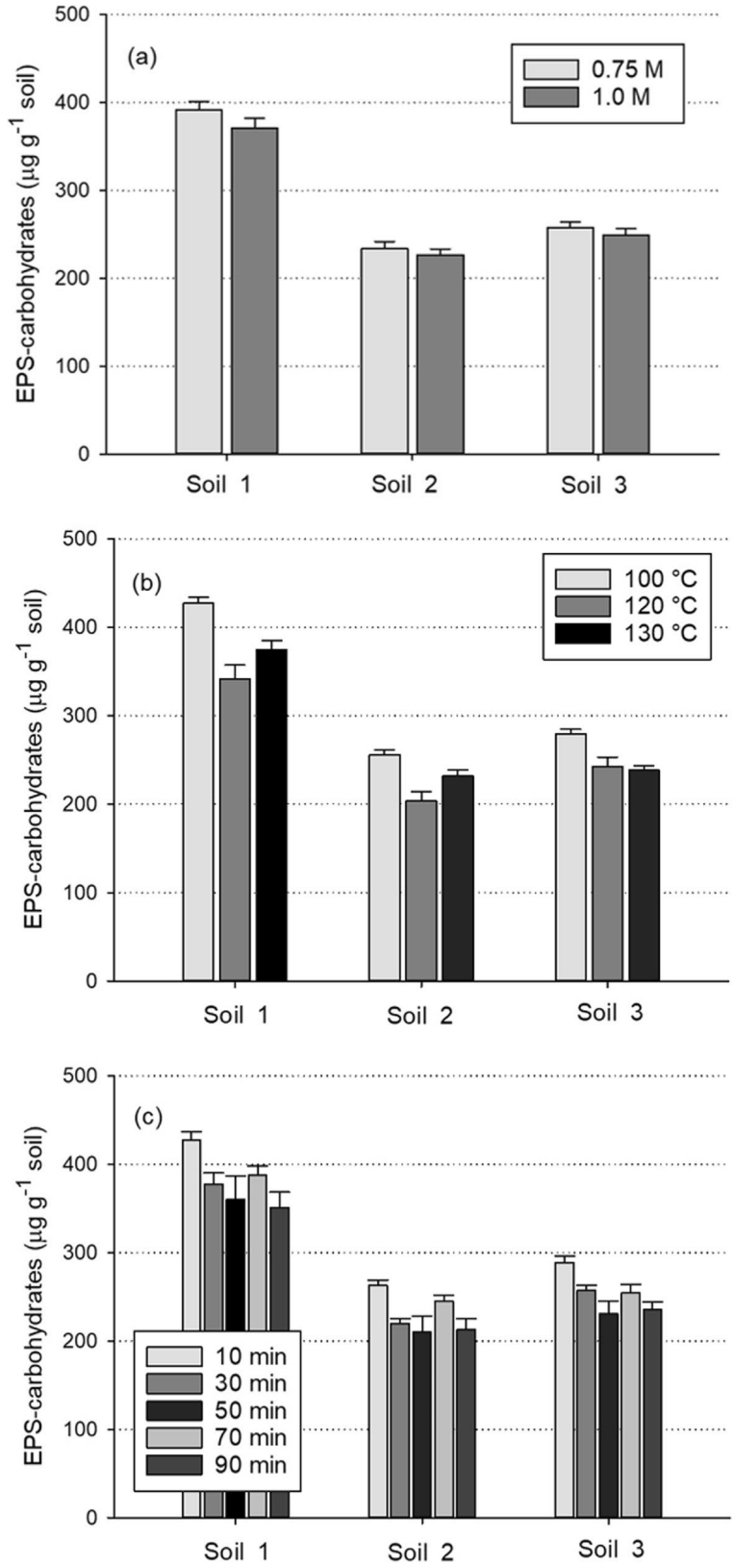

Fig. 1 EPS-carbohydrates in three soils (**a**) after hydrolysis with 0.75 M and 1.0 M H_2SO_4 (error bars represent one standard error (SE), $n = 45$), (**b**) after hydrolysis at 100, 120, and 130 °C (error bars represent one SE, $n = 30$), and (**c**) after a hydrolysis time of 10, 30, 50, 70 and 90 min (error bars represent one SE, $n = 18$)

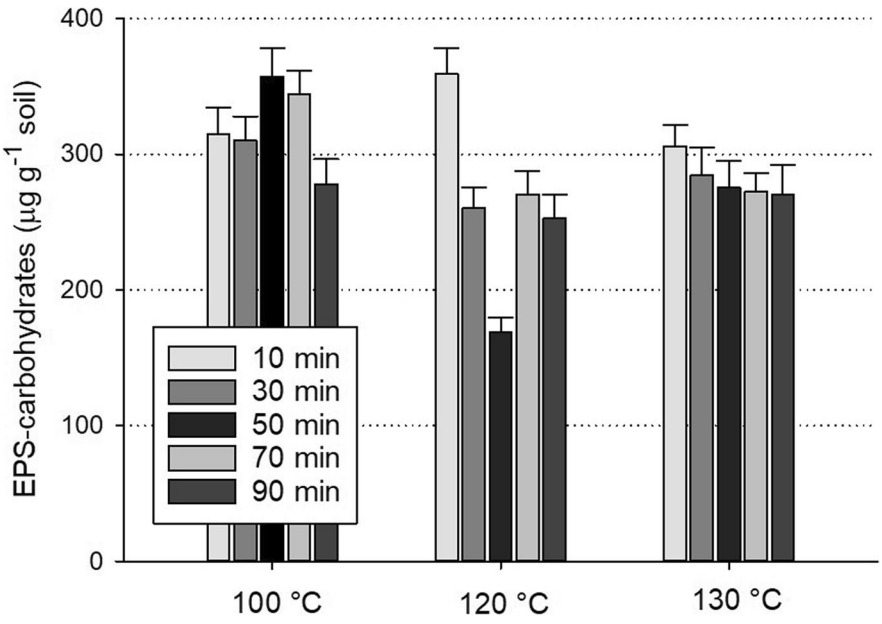

Fig. 2 EPS-carbohydrates at three temperatures after a hydrolysis time of 10, 30, 50, 70, and 90 min (error bars represent one SE, $n = 18$)

(2013) used 0.5–1.5% H_2SO_4 solutions for hydrolysis of dry EPS.

Polysaccharide hydrolysis in aqueous EPS extracts are not common, except when the cleavage of glycosidic bonds of polysaccharides is already included in EPS-carbohydrates quantification methods, such as the phenol-sulphuric acid method (DuBois et al. 1956). This method, however, was found to overestimate EPS-carbohydrate quantities, since it presents variable absorbances to different sugar structures, and therefore is not suitable for complex samples (Pierce and Nerland 1988). The use of 0.75 M H_2SO_4 in EPS extracts might avoid overestimation and prevent unnecessary sugar degradation if, otherwise, a more concentrated acid were be used (Josefsson 1970).

Carbohydrate yield in the present study was found to be generally highest when hydrolysing extracts at 100 °C. Hydrolysis temperatures of 100 °C with H_2SO_4 are widely reported for detecting carbohydrates in marine particular organic matter (POM) (Hedges et al. 1994; Sigleo 1996; Tanoue and Handa 1987), marine dissolved organic matter (Cauwet et al. 2002; Kirchman et al. 2001; Mopper 1977; Sweet and Perdue 1982), marine extracellular polymer particles (Mopper et al. 1995; Zhou et al. 1998), and soil (Cheshire and Mundie 1966). The application of higher temperatures and acid concentrations on polysaccharides may not only hydrolyse, but may further degrade their monosaccharides into furan derivatives, leading to inaccurate underestimation of carbohydrates (Antonetti et al. 2016; Bajpai 2018; Li et al. 2007).

A detailed temperature × time analysis showed that 50 min would be the most suitable hydrolysis time at 100 °C, however, with small differences compared to 10 and 30 min. The absence of an increasing trend visible over time indicates that the peak at 50 min may be caused by the natural variability between soils. Acid hydrolysis comes with intrinsic side reactions and mass losses are always prone to occur, so hydrolysis conditions need to be selected carefully (Uçar and Balaban 2003). When looking at the general data (Fig. 2c), lower carbohydrate yields are visible after longer periods of hydrolysis. This might be a consequence of degradation processes from the heating times (Teh et al. 2017), decreasing the amount of reducing sugars and increasing that of by-products.

Hang et al. (2020), when using H_2SO_4 on EPS of *Ophiocordyceps sinensis*, employed a reaction time of 2 h at 55 °C, using concentrated sulphuric acid, while Yan et al. (2010) hydrolysed EPS from the same fungus for 8 h at 100 °C using 2.0 M H_2SO_4. Both quantified monosaccharide constituents by gas-chromatography and the results showed mannose, galactose and glucose as the three main monomers in EPS extracts, similarly to other findings (Soltani et al. 2013; Zhang et al. 2011).

The reaction time and acid concentration selected vary greatly among authors. The traditional colourimetric method of DuBois et al. (1956) also uses concentrated acid (95.5%), resulting in a final concentration in sample of approximately 7.0 M. In this method, however, the objective is to obtain furfural derivatives, resulting from a further monosaccharide degradation, which then form yellow-coloured complexes when in contact with phenol (Kurzyna-Szklarek et al. 2022). Such derivatives are of no use when in contact with bicinchoninic acid, so a reaction of this magnitude is not needed in the current assay.

It is important to note that the BCA method for carbohydrate quantification might present some background information in the measurements due to the interference from other reducing substances such as proteins, which are also commonly quantified using BCA (Eklöf et al. 2012). A detailed compositional analysis employing more specific methods, as in high-performance liquid chromatography might be necessary to determine the degree of overestimation in such samples. Utilizing the same EPS extraction method, coupled with the phenol-sulphuric technique, Redmile-Gordon et al. (2020) reported an average of 376 μg g^{-1} soil for an arable land with high silt and clay content, similar to the currently analysed soil 1, with 398 μg g^{-1} EPS carbohydrates, and, in another study, showed a mean of 401 μg carbohydrates g^{-1} soil for grasslands with high SOC content (Redmile-Gordon et al. 2014). Similar studies on agricultural soils are not frequent, and isolated EPS are often applied to soils for the beneficial effects of such substances instead of field characterizations. The present paper represents a relevant attempt at simplifying field analysis for the detection of the reaction of EPS to environmental conditions.

Conclusion

The low carbohydrate contents obtained after pre-extraction with 0.01 M $CaCl_2$ indicate that this step can be done without in most cases. Carbohydrate hydrolysis at 100 °C for 10 min using a final H_2SO_4 concentration in extract produced optimal carbohydrate yield, with decreasing amounts after longer reaction times and higher temperatures. The BCA assay is easy to perform, non-toxic, sensitive, and tolerant to interferences from most compounds. The ability to rapidly quantify carbohydrates in EPS extracts has ample potential for improving and simplifying processes in studies of extractable polymers in several areas of soil biogeochemistry.

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Declarations

Conflict of interest The authors declare no conflicts of interest.

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