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Table of Contents

List of Figures 4
List of Tables 4
1 Executive Summary 6
2 Introduction 7
3 Methodology 8
3.1 Biomass and Fractionation Process 8
3.2 Characterization Methods 9
3.3 Preparation of Botanical Extracts 10
3.4 Analysis of Fatty Acids, Carotenoids and Phytosterols 11
3.5 Analysis of Polyphenolic Compounds 12
3.6 Statistical Methods 13
4 Results 13
4.1 Fractionation and Dry Matter Content 13
4.2 Composition of *S. europaea* Cultivated in Different Salinities 14
4.3 Composition of *T. pannonicum* Cultivated in Different Salinities 16
4.4 Composition of *C. maritimum* Cultivated in Different Salinities 18
4.5 Composition of Greenhouse Cultivated *S. ramosissima* 20
4.6 Extraction Yields from Fibre Residue Fractions 21
4.7 Fatty Acid Profiles of Halophyte Extracts 22
4.8 Carotenoids and Phytosterols in Halophyte Extracts 22
4.9 Phenolic Compounds in Halophyte Extracts 23
5 Conclusions and Outlook 24
6 Bibliography 26

List of Figures

Figure 1: Simplified schematic of halophyte-based green biorefinery. ... 7
Figure 2: *S. ramosissima* green juice and dried, size-reduced fibre residue ... 7
Figure 3: *Salicornia ramosissima* obtained from Les Douceurs du Marais (left), *Tripolium pannonicum* and *Crithmum maritimum* cultivated in a hydroponic system in optimized conditions in Leibniz Universität Hannover. ... 8
Figure 4: Extraction of *C. maritimum* fibre residue with Soxhlet. ... 10
Figure 5: Lab-scale devices at CEL for the extraction by supercritical CO_2 (SFE 100ml lab station at the left) and subcritical water (Dionex ASE350 at the right). ... 11
Figure 6: GC-MS Thermo ITQ900 (ion trap detector) ... 12
Figure 7: UPLC Xevo-TQ system. ... 13
Figure 8: Composition of *S. europaea* green juice (A), fibre residue (B) and whole biomass (C), and total sugar profile (D). Different letters above the bars denote significantly different ($p < .05$) results calculated individually for each chemical group. ... 15
Figure 9: Composition *of T. pannonicum* green juice (A), fibre residue (B) and whole biomass (C), and total sugar profile (D). Different letters above the bars denote significantly different ($p < .05$) results calculated individually for each chemical group. *) Not possible to give the standard deviation for the sample. ... 17
Figure 10: Chemical composition of *C. maritimum* green juice (A), fibre residue (B) and whole biomass (C), and total sugar profile (D). Due to the small concentration of organic acids (< 0.1 g/100 g DM), all bars are not visible in the graphs. Different letters above the bars denote significantly different ($p < .05$) results calculated individually for each chemical group. ... 19
Figure 11: Composition of *S. ramosissima* (RSR) green juice. "Other" is the undefined fraction after cumulative mass balance calculations. ... 20
Figure 12: Composition of *S. ramosissima* (RSR) fibre residue. "Other" is the undefined fraction after cumulative mass balance calculations. ... 21
Figure 13: Mass spectrum difference observed for the peak of beta-sitosterol standard at RT: 22.63 min (left) and in *T. pannonicum* extract at RT: 22.63 min (right) ... 23

List of Tables

Table 1: Biomass batches considered in the studies. ... 8
Table 2: Solvent system gradient of elution for carotenoids method. ... 11
Table 3: Solvent system gradient of elution for polyphenols method. ... 12
Table 4: Green juice and fibre residue fractions from biomass with corresponding dry matter (DM) contents. Presented values are means with (± st. dev.) and different letters denote significantly different ($p < .05$) results calculated individually for each plant species and its biomass fractions. *) Oven dry weight, biomass dired overnight at 60 °C fan oven. ... 13
Table 5: Minerals present in the ash fraction of *S. europaea* biomass. n.d.: amounts > 100 ppm not detected. ... 16
Table 6: Minerals present in the ash fraction of *T. pannonicum* biomass. n.d.: amounts > 100 ppm not detected. . 18
Table 7: Minerals present in the ash fraction of *C. maritimum* biomass. n.d.: amounts > 100 ppm not detected. ... 20
Table 8: Yield of extractive material from halophyte biomass. Presented values are means with (± st. dev.) ... 21
Table 9: Fatty acid (FA) profile of non-polar halophyte extracts. Presented values are means with (± st. dev.) ... 22
Table 10: Main phytosterols [mg/g extract] quantified from the supercritical CO_2 extracts from fibre residue fractions. n.d.: not detected. **) See text about the identification issue. ... 22

Table 11: Carotenoids quantified in the supercritical extracts by HLPC-DAD. n.d.: not detected. 23
Table 12: Polyphenols quantified in the subcritical water extracts of the fibre residue materials of *S. ramosissima* (RSR), *T. pannonicum* (10 g/l NaCl) and *C. maritimum* (0 g/l NaCl) by ULPC-DAD/MS [mg/kg dry extract]. <QL: detected but lower than quantification limit, n.d.: not detected. 24
Table 13: Polyphenols quantified in the subcritical water extracts of the fibre residue materials of *S. ramosissima* (RSR), *T. pannonicum* (10 g/l NaCl) and *C. maritimum* (0 g/l NaCl) by ULPC-DAD/MS [mg/kg raw material]. <QL: detected but lower than quantification limit, n.d.: not detected. 24

1 Executive Summary

This report summarizes the chemical characterisation results achieved in the following AQUACOMBINE project tasks:

- Task 5.1 - Green fractionation
- Task 5.2 - Extraction and analysis of lipids and non-polar compounds from fresh *Salicornia europaea* biomass
- Task 3.2 - Extraction of targeted HCA
- Task 6.1 - Chemical characterization of the botanical extracts

Fresh but non-food grade (partly lignified) *Salicornia europaea, Tripolium pannonicum* syn. *Aster tripolium, Crithmum maritimum* and *Salicornia ramosissima* were fractionated to green juice and fibre residue fractions and characterized for the contents of crude protein, carbohydrates, lipids, organic acids, and minerals. Soxhlet extraction with water, ethanol, and hexane, as well as pressurized fluid extraction with water and CO_2, were performed for the fibre residue fractions. Botanical extracts from *S. ramosissima*, *T. pannonicum* and *C. maritimum* were analysed for fatty acids, carotenoids, phytosterols, and polyphenolic compounds.

The cultivation salinity affects the chemical composition of *S. europaea* biomass. For *T. pannonicum* and *C. maritimum*, the cultivation salinity affects rather the biomass yield than the composition. Out of the studied fractions and extracts, *T. pannonicum* has exhibited the highest protein content and bioactive secondary metabolites. Water extract from fresh *S. ramosissima* has a similar concentration of simple hydroxycinnamic acids as the extracts obtained from lignified fraction, but it lacks the more high-value compounds such as chlorogenic acids.

2 Introduction

Due to high concentration of health-beneficial compounds, halophytes have been used as medicinal herbs for centuries and nowadays, fresh shoots are harvested for culinary use[1]. However, the food production period is seasonal, as plants turn woodier as they mature, making them unpleasant to eat. Lignified shrubs are considered as agricultural waste, even if they could provide a feedstock for multi-product biorefinery. In green biorefinery, biomass is harvested fresh but non-food grade and fractionated into liquid and solid phases, which are processed separately. This approach for halophyte processing has been previously tested only for a few *Salicornia* spp.[2,3]. The fractionation process is illustrated in **Figure 1**.

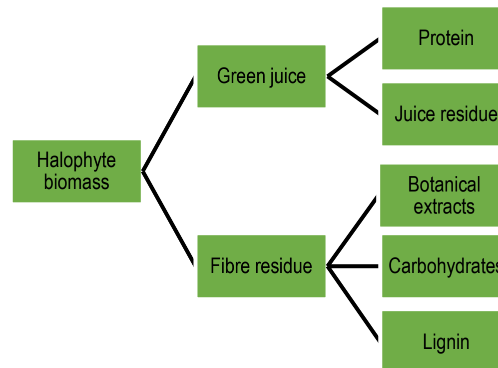

Figure 1: Simplified schematic of halophyte-based green biorefinery.

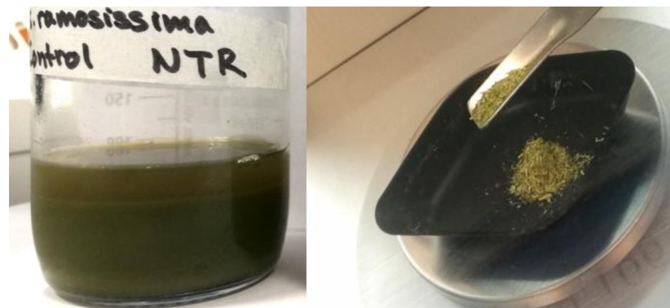

Figure 2: S. ramosissima green juice and dried, size-reduced fibre residue.

The key compound to be valorised in halophyte-based green biorefinery is protein. The demand for high-quality plant-derived protein has increased, and for livestock, farmers are currently strongly dependent on imported sources, such as soybean[4]. As an answer to the increasing demand, fresh halophytes could provide a source for locally produced protein. Other possible value-added products from halophyte-based green biorefinery healthy fatty acids, prebiotics, and bioactive botanical extracts[5–7]. The high content and variety of possible value-added compounds make the biomass desirable for biorefinery, as the production of high-value compounds can improve the feasibility of producing bioenergy and bulk products, such as lignocellulose-derived biochemicals.

AQUACOMBINE WP5 focuses on the green, partly lignified halophyte biomass. This report considers the outcomings of following project tasks:

- Task 5.1 - Green fractionation (**Section 3.1 – 3.2**, **Section 4.1 – 4.5**)
- Task 5.2 - Extraction and analysis of lipids and non-polar compounds from fresh *Salicornia europaea* biomass (**Section 3.3 – 3.4**, **Section 4.6 – 4.8**)
- Task 3.2 - Extraction of targeted HCA (**Section 3.3**)
- Task 6.1 - Chemical characterization of the botanical extracts (**Section 3.4**, **Section 4.9**)

Figure 3: Salicornia ramosissima obtained from Les Douceurs du Marais (left), Tripolium pannonicum and Crithmum maritimum cultivated in a hydroponic system in optimized conditions in Leibniz Universität Hannover.

3 Methodology

3.1 Biomass and Fractionation Process

At the beginning of the project period, it was decided to add *Salicornia ramosissima* to the list of considered halophyte species. *Salicornia europaea*, *Tripolium pannonicum* and *Crithmum maritimum* cultivated in hydroponic systems were obtained from Leibniz Universität Hannover (LUH). *Salicornia ramosissima* was obtained from Riasearch (RSR) and Les Douceurs du Marais (LDM). At the beginning of the AQUACOMBINE project period, biomass was fractionated to green juice and fibre residue factions using lab-scale single auger juicer. When biomass batches grown in optimized cultivation conditions were considered, as well as when processing larger amount of biomass, a pilot-scale twin auger juicer was used for the fractionation process. Biomass batches and respective cultivation conditions and fractionation methods are summarized in **Table 1**.

Table 1: Biomass batches considered in the studies.

Species	Source	Cultivation salinity	Received	Fractionation
S. europaea	LUH	0 g/l NaCl	02/2020	Laboratory-scale single-auger juicer
		10 g/l NaCl		
		20 g/l NaCl		
		30 g/l NaCl		
		40 g/l NaCl		
T. pannonicum	LUH	0 g/l NaCl	11/2020	Laboratory-scale single-auger juicer
		10 g/l NaCl		
		20 g/l NaCl		
		30 g/l NaCl		
		40 g/l NaCl		
T. pannonicum	LUH	7.5 g/l NaCl	07/2021	Pilot-scale twin-auger juicer
C. maritimum	LUH	0 g/l NaCl	04/2021	Laboratory-scale single-auger juicer
		5 g/l NaCl		
		10 g/l NaCl		
		15 g/l NaCl		
		20 g/l NaCl		
C. maritimum	LUH	0 g/l NaCl	09/2021	Pilot-scale twin-auger juicer
S. ramosissima	RSR	approx. 20 g/l NaCl	05/2020	Laboratory-scale single-auger juicer
S. ramosissima	LDM	seawater irrigation	07/2021	Pilot-scale twin-auger juicer

3.2 Characterization Methods

At AAU, a detailed chemical characterization was performed for *S. europaea*, *T. pannonicum,* and *C. maritimum* biomasses cultivated under different salinities to study how changes in the cultivation salinity affect the composition of biomass fractions. Halophyte fractions were analysed for their contents of dry matter (DM) and ash, carbohydrates, crude protein, lipids, and organic acids. Characterization was also performed for *S. ramosissima* biomass obtained from RSR.

The DM and ash content of the fractions were determined according to the protocols by National Renewable Energy Laboratory (NREL)[8,9]. These protocols are commonly used in studies considering the processing of lignocellulosic biomass, hence allowing easy comparison to the existing literature.

Minerals present in the ash were analysed in CEL for *S. europaea*, *T. pannonicum* and *C. maritimum* biomasses. Ash samples were digested in acidic conditions (240 °C, 40 bar) using a microwave system (Ultrawave ECR, Milestone) in compliance with EN 13805:2014 standard. Inductively coupled plasma atomic emission spectrometry (8300 DV ICP-AES, Perkin Elmer) with a method adapted from EN 11885 standard was used to determine the concentrations of the following mineral: aluminium, antimony, arsenic, barium, cadmium, calcium, chromium, cobalt, copper, iron, lead, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, selenium, silver, sodium, strontium, titanium, vanadium, and zinc. Inductively coupled plasma with mass spectrometry (820 ICP-MS CRI, Varian) with a method adapted from EN 15763 standard was used to determine the concentrations of rubidium, scandium and yttrium. Due to the lack of material, mineral analysis was run only once on each sample, and detected minerals with a concentration higher than 100 ppm in DM were reported.

The crude protein content was determined by measuring the total nitrogen using an elemental analyser and applying Jones conversion factor 6.00[10].

Lipids were extracted from fibre residue samples (sample size 5 g) with n-hexane using the Soxhlet apparatus (extraction time 6 h), and the excess solvent was evaporated using a rotary evaporator. For green juice, liquid-liquid extraction was performed by mixing juice sample and n-hexane in a 1:2 ratio, mixing samples for 1 hour, centrifuging samples to get the phase separation, and recovering the non-polar phase.

Structural carbohydrates and Klason lignin were determined for fibre residue samples using the protocols by NREL, using a sample size of 0.16 g[11,12]. Weak acid hydrolysis was performed to release the sugars present in the juice fraction, as previously described by Alassali et al.[2]. Volume of 10 ml of green juice was mixed with 10 ml of 8 % sulphuric acid in an aqueous solution and autoclaved at 121 °C for 10 mins. Strong acid hydrolysis, weak acid hydrolysis, and following sugar analysis were run as duplicates for samples and recovery standards. Separated sugar monomers were glucose, xylose, and arabinose. Organic acids and free sugar monomers were detected directly from fresh juice samples by applying protocol by NREL[13], and separated acids were lactic acid, acetic acid, malic acid, succinic acid, and glycolic acid. Hydrolysates and fresh juice samples were analysed with high-performance liquid chromatography (HPLC) (1260 Infinity II, Agilent Technologies) using organic acid column (Aminex HPX-87H), 0.005 M sulphuric acid mobile phase, and refractive index detector. The operation temperature of the column was 63 °C and the flow rate inside the system was 0.6 ml/min. Refractive index detector operated at 35 °C.

3.3 Preparation of Botanical Extracts

At AAU, fibre residue fractions from *S. ramosissima* (LDM), *T. pannonicum* (7.5 g/l NaCl) and *C. maritimum* (0 g/l NaCl) were extracted with water and 70 % ethanol to analyse the bioactivity of food-grade extracts. Extractions were run as parallel experiments using a Soxhlet apparatus (**Figure 4**) with 100 ml extraction chamber, and approximately 5 g of biomass was used for each cellulose thimble. Extraction times were 10 h and 8 h for water and ethanol extractions, respectively. Non-polar compounds were extracted as described earlier in **Section 3.2**.

Figure 4: Extraction of C. maritimum fibre residue with Soxhlet.

At CEL, the fibre residue from *S. ramosissima* (RSR), *T. pannonicum* (10 g/l NaCl) and *C. maritimum* (0 g/l NaCl) were extracted for the determination of the content of non-polar active compounds (phytosterols and carotenoids) and hydroxycinnamic acids and polyphenols in the fibre residue fractions. Samples were successively extracted by supercritical CO_2 and subcritical water at the lab scale. Briefly, 20 g of dried fibre residues fractions were extracted on SFE 100ml lab-station by supercritical CO_2 at 65 °C and 350 bar for 30 mins at a flow of 40 g CO_2/ min. The lipidic extracts were collected and analysed for carotenoids content and phytosterol content. Then, 2 g of the residual materials after supercritical CO_2 extraction were further extracted by supercritical water. These materials were transferred in an 11ml-cell of a Dionex ASE350 extractor and were extracted by water at 140 °C and 100 bar for two cycles of 15 mins. The resulting aqueous extracts were freeze-dried before analysing the hydroxycinnamic acid/polyphenol content. Those extraction conditions were based on the optimization experiments performed for lignified *S. ramosissima* in WP3 using an experimental design plan.

Figure 5: Lab-scale devices at CEL for the extraction by supercritical CO_2 (SFE 100ml lab station at the left) and subcritical water (Dionex ASE350 at the right).

3.4 Analysis of Fatty Acids, Carotenoids and Phytosterols

Lipid samples extracted with Soxhlet from *S. ramosissima* (LDM), *T. pannonicum* (7.5 g/l NaCl) and *C. maritimum* (0 g/l NaCl) fibre residues were derived to fatty acid methyl esters by transesterification. A small amount of lipids was dissolved to 1 ml of 0.5 M methanolic sodium hydroxide at 90 °C. To the cooled samples, 1 ml of boron trifluoride and 0.5 ml of hydroquinone solution was added, and samples were kept at 90 °C for 5 mins. Phase separation was achieved using 4 ml of saturated sodium chloride solution and 3 ml of n-heptane. After separation, the n-heptane fraction was recovered. The fatty acid (FA) profile was determined with gas chromatography (GC) coupled with mass spectrometry (MS) (Clarus 500, Perkin Elmer), capillary column (Elite-WAX, 30 m x 0.25 mm ID x 0.25 µm, Perkin Elmer) and helium as carrier gas. The temperature programme was 1 min at 150 °C, heating 10 °C/min until reaching 240 °C, and 10 mins at 240 °C.

For the analysis of carotenoids, supercritical extracts were dissolved with a ratio of 50 mg in 10 ml in a mixture of methyl tert-butyl ether (MtBe) and methanol (50:50). The samples were then quantified by calibration curves with concentrations between 1 and 10 mg/L. The quantification of carotenoids was carried out using high-performance liquid chromatography with a diode array detector (Thermo-Fischer). For this purpose, a C30 carotenoid bonded silica-based reversed-phase column (YMC) was used to separate the targeted carotenoids and their isomeric structures: lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene, and lycopene. The column was kept at 30 °C, and the solvent gradient (**Table 2**) used a constant flow rate of 1 ml/min. All peaks were analysed at 450 nm.

Table 2: Solvent system gradient of elution for carotenoids method.

Time (min)	Solvent A (%) MeOH:MtBe:H_2O (81:15:4)	Solvent B (%) MeOH:MtBe:H_2O (6:90:4)
0	100	0
20	40	60
24	0	100
31	0	100
35	100	0
40	100	0

Three of the main phytosterols (β-sitosterol, stigmasterol and campesterol) were quantified by GC-MS (ion trap Thermo ITQ900) using a Rxi5SilMS column (Restek 30mx0.25mmID – 0.25µm particles). As pretreatment, the lipid extracts were saponified using a 7.5 % potassium hydroxide solution in methanol in the presence of 2-tert-butylhydroquinone as an antioxidant. The saponification was carried out under nitrogen at 65 °C in a water bath under vigorous agitation for 1 h. Unsaponifiable compounds were then extracted by a 1:1 mixture of petroleum ether/hexane. After washing, the resulting organic solution was concentrated before injection in GC-MS. 5-alpha-cholestane was used as internal standard, and phytosterols were quantified by calibration curves made with pure standards with concentrations between 10 and 500 mg/l.

Figure 6: GC-MS Thermo ITQ900 (ion trap detector).

3.5 Analysis of Polyphenolic Compounds

Extracts from supercritical water extraction were dissolved with a ratio of 50 mg in 10 ml of a 50:50 v/v% methanol-water mixture containing 0.05 % of formic acid. An ultrasonic bath was used to facilitate the solubilization. Polyphenols in the samples were identified by their m/z values and fragment patterns after comparing with the CEL database, which contains more than 100 compounds and metabolites. Taking into account the previous results obtained from *Salicornia,* following 16 molecules were quantified using calibration curves made with pure standards of the chosen molecules with concentrations between 0.25 and 10 mg/L: caffeic acid, coumaric acid, chlorogenic acid, crytpochlorogenic acid, ferulic acid, protocatechuic acid, hyperoside, isorhamnetin, isorhamnetin-3-rutenoside, isoquercitrin, kaempferol-3-glucoside, kaempferol-7-rutenoside, neochlorogenic acid, quercetin, rutin, and vanillic acid.

The identification and quantification of specific polyphenol compounds were carried out at CEL by ultra-pressure liquid chromatography (UPLC) coupled to MS (Xevo-TQ system, Waters) using an Acquity BEH Shield RP 18 column and the solvent gradient (**Table 3**) with a constant debit of 0.5 ml/min. The column temperature was fixed at 40 °C, and the vials containing the samples were maintained at 13 °C. An injection volume of 3.5 µL was used in this method. The analyses were monitored by Masslynx Software (Waters).

Table 3: Solvent system gradient of elution for polyphenols method.

Time (min)	Solvent A (%) Ammonium Formate Buffer	Solvent B (%) Acetonitrile
0.0	95	5
7.2	80	20
10.0	75	25
11.5	50	50
12.0	0	100
12.5	0	100
14.0	95	5
16.5	95	5

Figure 7: UPLC Xevo-TQ system.

3.6 Statistical Methods

At AAU, all analyses were run in triplicates unless stated otherwise, and results are given as mean values with standard deviation (n = 3). One-way analysis of variance (ANOVA) combined with Turkey honest significant difference (HSD) test were run to evaluate significance of the differences between the results.

4 Results

4.1 Fractionation and Dry Matter Content

As presented in **Table 4**, the green juice fraction covers up to 90.2 w/w% of the total biomass after fractionation. Due to low biomass yields, it was not possible to perform the fractionation for *T. pannonicum* and *C. maritimum.* Therefore, these biomass batches were considered as a whole. In the case of *S. europaea* and *T. pannonicum*, the higher cultivation salinity has a positive correlation with the increased juice fraction. The pilot-scale twin-auger juicer provided more efficient fractionation compared to lab-scale single-auger one, and the juice fraction of C. maritimum grown in non-saline conditions increased from 70.4 w/w% to 82.2 w/w%, and the dry matter of fibre residue fraction increased from 27.2 w/w% to 35.7 w/w%.

Table 4: Green juice and fibre residue fractions from biomass with corresponding dry matter (DM) contents. Presented values are means with (± st. dev.) and different letters denote significantly different (*p* < .05) results calculated individually for each plant species and its biomass fractions. *) Oven dry weight, biomass dried overnight at 60 °C fan oven.

Species	Salinity [g/l NaCl]	Juice [w/w%]	Fibres [w/w%]	DM juice [w/w%]	DM fibres [w/w%]	DM whole [w/w%]
S. europaea	0	84.61	15.39	4.71 (0.03) [c]	27.24 (1.18) [a]	9.15 (0.65) [a]
	10	85.71	14.28	4.71 (0.04) [c]	23.00 (0.28) [b]	n/a
	20	85.29	14.71	5.10 (0.00) [bc]	21.80 (1.16) [b]	8.02 (0.38) [a]
	30	86.42	13.58	5.26 (0.39) [ab]	21.11 (0.54) [b]	7.85 (0.74) [a]
	40	90.23	9.77	5.82 (0.12) [a]	21.54 (1.32) [b]	8.05 (0.04) [a]
T. pannonicum	0	74.97	25.03	3.87 (0.03) [b]	23.78 (0.20) [b]	8.07 (0.19) [c]
	10	82.51	17.49	4.17 (0.04) [a]	26.30 (1.23) [a]	7.96 (0.67) [c]
	20	n/a	n/a	n/a	n/a	11.45 (0.39) [b]

	30	n/a	n/a	n/a	n/a	11.47 (0.47) ^{b}
	40	n/a	n/a	n/a	n/a	14.65 (0.22) ^{a}
C. maritimum	0	70.35	29.65	5.58 (0.02) ^{b}	27.22 (0.68) ^{a}	11.06 (0.22) ^{b}
	5	70.46	29.54	5.39 (0.00) ^{c}	27.15 (0.35) ^{a}	11.77 (0.53) ^{b}
	10	66.97	33.03	7.44 (0.00) ^{a}	26.06 (0.61) ^{a}	13.56 (0.54) ^{a}
	15	n/a	n/a	n/a	n/a	13.40 (0.14) ^{a}
	20	n/a	n/a	n/a	n/a	14.79 (0.98) ^{a}
C. maritimum	0	82.15	17.85	7.26 (0.01)	35.67 (0.84)	16.18 (3.94) *
***S. ramosissima* (RSR)**	20	67.57	32.43	6.74 (0.21)	29.89 (2.84)	13.71 (0.45)

4.2 *Composition of* S. europaea *Cultivated in Different Salinities*

The crude protein content of *S. europaea* is relatively high, 24.3 g/100gDM and 20.0 g/100gDM in biomass cultivated in 0 and 10 g/l NaCl salinities, respectively. Considering the fractions separately, the changes in the crude protein content by cultivation salinity were more significant in the juice fraction ($p < .001$) than in the fibre residue ($p > .011$). Lipid content was low, 1.7 - 3.7 g/100gDM and based on results, no clear relationship can be observed between cultivation salinity and lipid content. The share of lignocellulose was the highest, 26.1 g/100gDM, in the plants cultivated in 20 g/l NaCl (cultivation condition with highest biomass production yield). The average composition of lignocellulose was 43.5 % cellulose (glucose), 41.3 % hemicellulose (xylose and arabinose, and 15.2 % Klason lignin. Low concentrations of free sugar monomers were detected from the juices, and glucose concentration varied from 0.91 (0.79) g/100 gDM (30 g/l NaCl) to 2.15 (0.01) g/100 g DM (0 g/l NaCl). Analysis of organic acids showed low amount (< 1.00 g/100gDM) of lactic acids in the samples analysed. Other acids were not detected. The composition of the biomass fractions is presented in **Figure 8**.

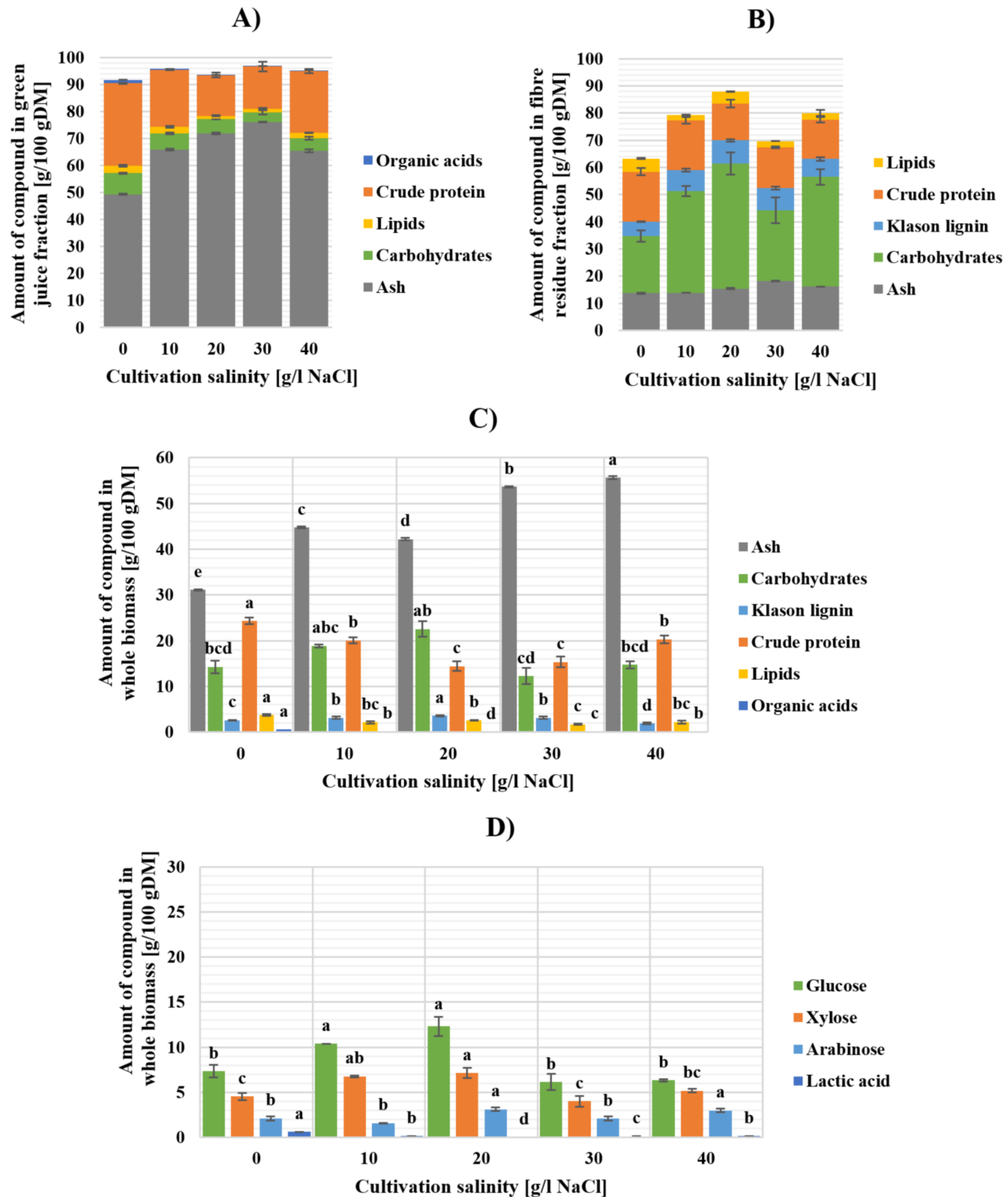

Figure 8: Composition of S. europaea green juice (A), fibre residue (B) and whole biomass (C), and total sugar profile (D). Different letters above the bars denote significantly different (p < .05) results calculated individually for each chemical group.

The ash content of *S. europaea* increased as the cultivation salinity increased due to the high amount of water-soluble salts in the juice fraction. This can be observed when fractions are considered separately: ash content of fibre residue fraction varied only between 13.7 - 18.2 g/100 g DM, but the ash content of the juice increased from 49.31 (0.19) g/100 g DM (0 g/l NaCl) up to 76.02 (0.11) g/100 g DM (30 g/l NaCl). The mineral composition is determined on the basis of fresh weight (FW). Aluminium was detected from the ash from *S. europaea* juice, but as the plants were cultivated in a hydroponic system without contamination, a plausible source for aluminium is contamination from the dish where the juice was dried. Minerals present in *S. europaea* ash are summarised in **Table 5**.

Table 5: Minerals present in the ash fraction of *S. europaea* biomass. n.d.: amounts > 100 ppm not detected.

Element [mg/ 100 gFW]	*S. europaea* cultivation salinity				
	0 g/l NaCl	10 g/l NaCl	20 g/l NaCl	30 g/l NaCl	40 g/l NaCl
Aluminum	0.48	3.26	< 2.94	< 4.32	5.01
Barium	0.11	< 0.03	n.d.	n.d.	n.d.
Calcium	219.45	46.30	28.26	21.31	24.48
Copper	< 0.07	< 0.09	< 0.09	< 0.10	< 0.09
Iron	0.34	0.37	0.29	0.23	0.31
Potassium	478.05	238.18	158.29	115.04	112.38
Magnesium	49.47	30.87	15.85	10.12	8.87
Manganese	0.15	0.09	0.07	0.09	0.16
Molybdenum	n.d.	n.d.	n.d.	n.d.	n.d.
Sodium	494.12	1191.11	1328.44	1463.83	1492.54
Phosphorus	30.93	22.48	19.33	18.23	16.73
Rubidium	0.03	n.d.	n.d.	n.d.	n.d.
Strontium	0.27	0.06	< 0.04	n.d.	n.d.
Vanadium	0.03	n.d.	n.d.	n.d.	n.d.
Zinc	0.20	0.13	0.09	0.08	0.11

4.3 Composition of T. pannonicum *Cultivated in Different Salinities*

Increased cultivation salinity affected the biomass yield and size of fractions rather than the chemical composition of *T. pannonicum* plants. An inverse relationship was observed between cultivation salinity and the total crude protein content. The crude protein content of *T. pannonicum* DM was comparable to widely used legumes, such as chickpea (24.0 g/100 g), lentil (26.1 g/100g) or green pea (24.9 g/100g), which makes it the most interesting species for protein production[14]. However, the presence of non-protein free amino acids may cause an overestimation in protein content[15]. Based on the obtained results, it was not possible to observe a clear relationship between cultivation salinity and biomass lipid content. Considering the analysis of sugars, it has to be noted that only the amount of structural carbohydrates was determined for the samples considered as a whole (non-fractionated). The lignocellulose consists of 30.1 % cellulose, 27.9 % hemicellulose and 42.0 % Klason lignin. High concentrations of free sugar monomers were detected from fresh *T. pannonicum* juice, xylose being the most abundant sugar monomer with concentrations of 16.43 (0.32) g/100gDM (0 g/l NaCl) and 7.99 (0.08) g/100gDM (10 g/l NaCl). Green juice was also rich in glucose, but arabinose was not detected from the samples. During the composition analysis, the cumulative mass balance of samples exceeded 100 %, which is suggested to be caused by the overestimated amount

of crude protein and large standard deviations in the ash content results. Composition of *T. pannonicum* is presented in **Figure 9**.

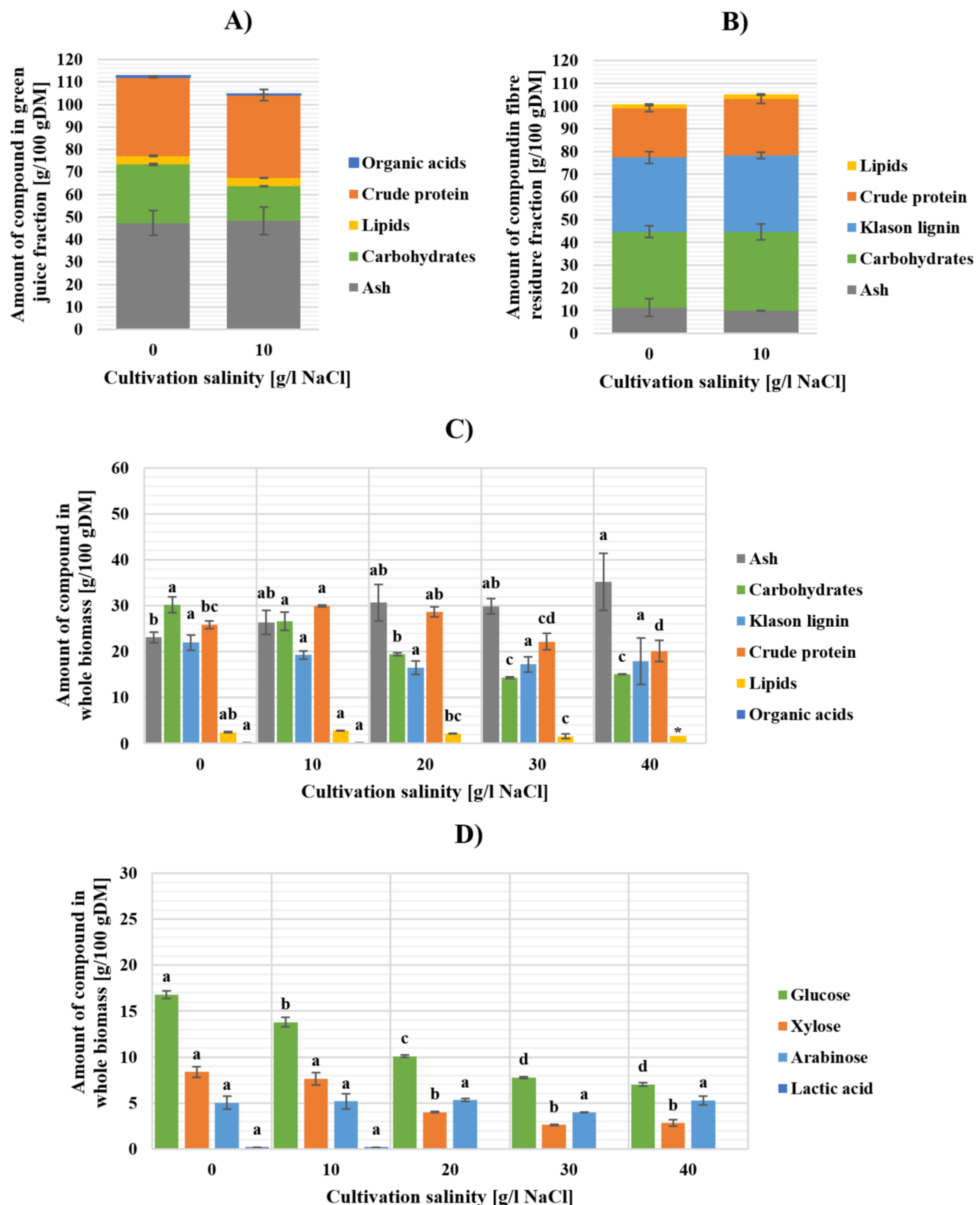

*Figure 9: Composition of T. pannonicum green juice (A), fibre residue (B) and whole biomass (C), and total sugar profile (D). Different letters above the bars denote significantly different ($p < .05$) results calculated individually for each chemical group. *) Not possible to give the standard deviation for the sample.*

The significant difference in the total ash content was only observed in the samples cultivated in the lowest and the highest salinity ($p = .020$), whereas changes between other samples were non-significant. The mineral analysis was

run using the ash fractions of whole biomass samples. Due to low biomass yield and the lack of material, it was not possible to run the mineral analysis for these samples from high salinities. Besides the increase in the sodium concentration, the higher cultivation salinity also increased the amount of phosphorus in biomass (**Table 6**).

Table 6: Minerals present in the ash fraction of *T. pannonicum* biomass. n.d.: amounts > 100 ppm not detected.

Element [mg/ 100 gFW]	*T. pannonicum* cultivation salinity				
	0 g/l NaCl	10 g/l NaCl	20 g/l NaCl	30 g/l NaCl	40 g/l NaCl
Aluminum	0.08	0.07	0.09	n/a	n/a
Barium	0.06	0.03	0.04	n/a	n/a
Calcium	112.17	54.76	62.62	n/a	n/a
Copper	0.08	0.07	0.11	n/a	n/a
Iron	0.35	0.27	0.49	n/a	n/a
Potassium	743.29	496.30	506.00	n/a	n/a
Magnesium	31.65	10.38	12.03	n/a	n/a
Manganese	0.16	0.13	0.32	n/a	n/a
Molybdenum	0.02	0.02	0.06	n/a	n/a
Sodium	145.22	502.85	1078.87	n/a	n/a
Phosphorus	52.15	54.90	77.52	n/a	n/a
Rubidium	0.04	0.03	n.d.	n/a	n/a
Strontium	0.11	0.05	0.06	n/a	n/a
Zinc	0.25	0.16	0.35	n/a	n/a

4.4 Composition of C. maritimum *Cultivated in Different Salinities*

Cultivation salinity affected mainly the biomass yield and less to the chemical composition of *C. maritimum* (**Figure 10**). Total crude protein content varied between 21.3 - 23.1g/100gDM. The lignocellulose, which covered 52.98 (2.16) g/100 gDM of total dry matter of sample grown in non-saline conditions, consisted of 31.5 % glucose, 19.3 % hemicellulose and 49.2 % Klason lignin. Fresh *C. maritimum* juice was rich in free sugars, and glucose was the most abundant sugar monomer. The largest amount of lactic acid was detected from the 5 g/l NaCl cultivated sample (3.77 g/100gDM), but in other juice samples, lactic acid concentrations were low (< 0.1g/100gDM). The cumulative mass balance exceeded 100 % during the composition analysis of all fibre residue fractions and the juice fraction from biomass grown with 0 g/l NaCl. The reason for this could be the overestimated amount of crude protein or relatively large standard deviations for the results considering the Klason lignin content.

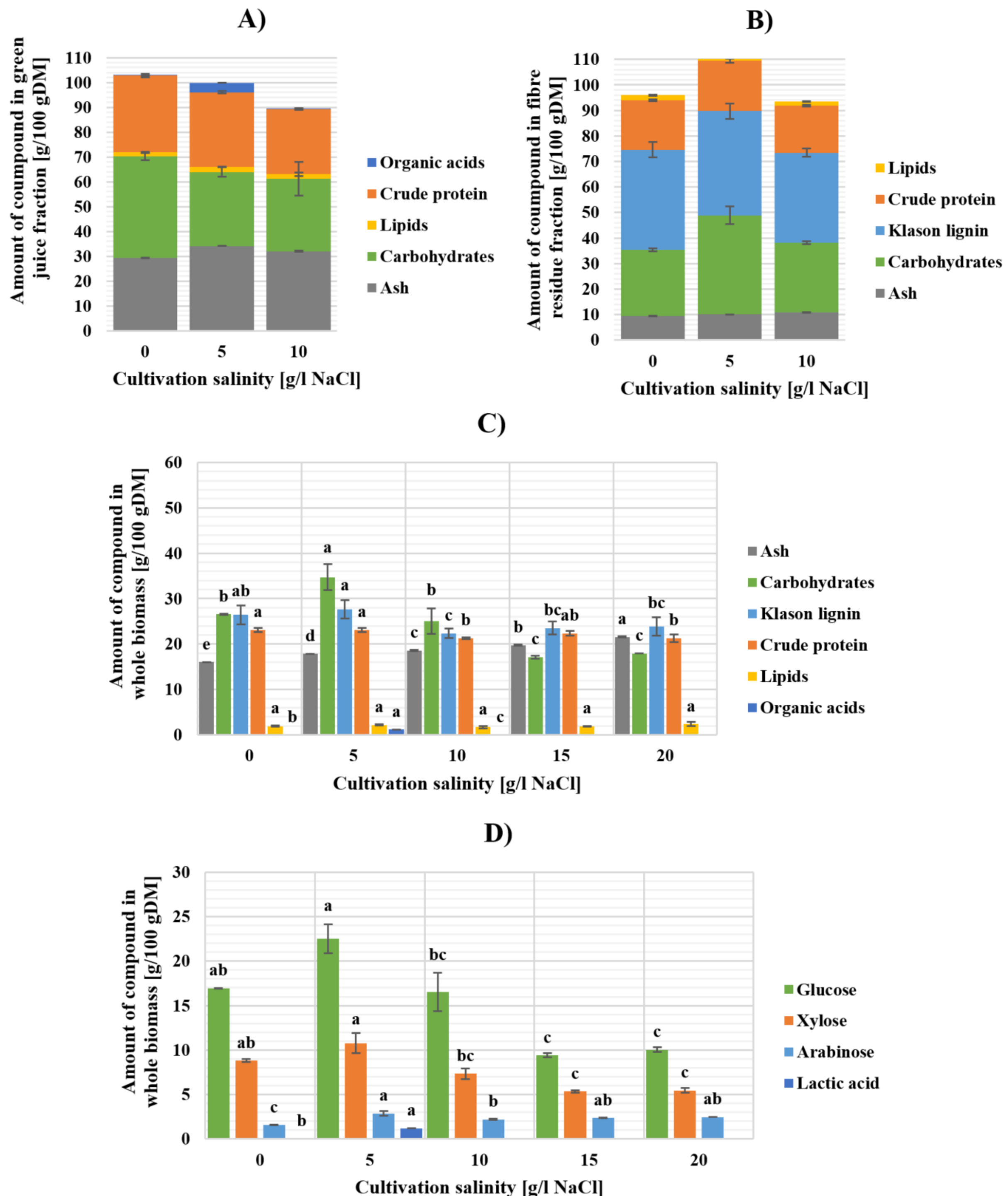

Figure 10: Chemical composition of C. maritimum green juice (A), fibre residue (B) and whole biomass (C), and total sugar profile (D). Due to the small concentration of organic acids (< 0.1 g/100 g DM), all bars are not visible in the graphs. Different letters above the bars denote significantly different ($p < .05$) results calculated individually for each chemical group.

Compared to the two other species cultivated in the same salinities, *C. maritimum* exhibited lower salt accumulation. The sodium concentrations in 10 g/l NaCl and 20 g/l NaCl cultivated samples were 42.3 % and 32.2 % lower, respectively, compared to *T. pannonicum* cultivated in respective salinities. Compared to *S. europaea*, sodium concentration was 64.7 % lower in 20 g/l NaCl cultivated samples and more than three times lower in 10 g/l NaCl cultivated samples. The mineral analysis showed that increased cultivation salinity decreased the uptake of calcium, potassium and magnesium, whereas the concentrations of phosphorus and iron increased with cultivation salinity.

Table 7: Minerals present in the ash fraction of C. maritimum biomass. n.d.: amounts > 100 ppm not detected.

Element [mg/ 100 gFW]	*C. maritimum* cultivation salinity				
	0 g/l NaCl	5 g/l NaCl	10 g/l NaCl	15 g/l NaCl	20 g/l NaCl
Aluminum	0.37	0.40	0.62	0.47	0.69
Barium	0.13	0.11	0.11	0.08	0.07
Calcium	228.81	179.08	152.16	139.42	115.42
Copper	0.06	0.07	0.07	0.06	0.05
Iron	0.37	0.35	0.45	0.48	0.52
Potassium	528.93	515.19	434.36	279.37	220.58
Magnesium	14.51	11.76	12.33	9.57	8.99
Manganese	0.20	0.18	0.20	0.20	0.12
Molybdenum	0.02	0.02	0.02	0.02	0.03
Sodium	11.47	193.25	352.43	555.99	816.29
Phosphorus	82.93	96.32	98.86	116.89	106.72
Rubidium	0.03	0.03	0.03	0.02	0.02
Strontium	0.20	0.15	0.15	0.11	0.10
Zinc	0.11	0.13	0.20	0.23	0.18

4.5 Composition of Greenhouse Cultivated S. ramosissima

The composition of *S. ramosissima* (RSR) green juice and fibre residue fractions are presented in **Figure 11** and **Figure 12**, respectively. Compared to *S. europaea*, *S. ramosissima* has lower crude protein and lipid content and higher lignocellulose content. Cellulose and hemicellulose cover approximately 28 % and 21 % of *S. ramosissima* fibre residue, respectively. The Klason lignin content of 11.78 g/100gDM is higher compared to previously reported for *Salicornia* spp. The green juice consists of 2.59 (0.12) g/100gDM glucose, 2.62 (0.12) g/100gDM xylose and 0.82 (0.13) g/100gDM arabinose. In fibre residue fraction, the share of undefined material is large (26.16 g/100gDM), which gives an approximation about the content of extractive material, such as phenolic compounds, present in the biomass. Similar amounts of extractive material have been determined for *S. ramosissima* biomass (see **Section 4.6**).

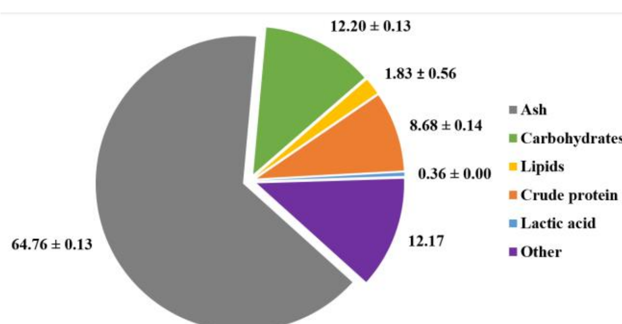

Figure 11: Composition of S. ramosissima (RSR) green juice. "Other" is the undefined fraction after cumulative mass balance calculations.

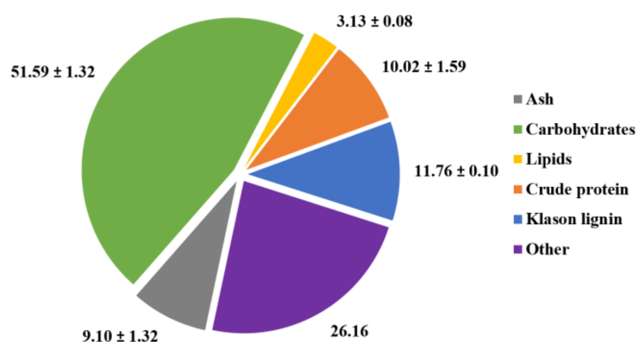

Figure 12: Composition of S. ramosissima (RSR) fibre residue. "Other" is the undefined fraction after cumulative mass balance calculations.

4.6 Extraction Yields from Fibre Residue Fractions

The yields obtained with lab-scale Soxhlet at AAU can be compared to those obtained with pressurized fluid by CEL. For the non-polar extracts, the yields obtained with n-hexane are 30 - 40 % lower than those obtained with supercritical CO_2, meaning this technology seems to be more efficient. With both methods, the lowest yield of non-polar compounds was obtained with *S. ramosissima*.

The comparison of subcritical water with Soxhlet is not so easy. If we compare the polarity of the medium, subcritical water is somewhere between boiling water and boiling ethanol 70 %. Interestingly, the recovery yield for *S. ramosissima* is quite similar for these three extraction techniques, where subcritical water leads to a much higher yield for *T. pannonicum* and *C. maritimum*. This is probably linked to other properties of subcritical water, which is its acidity and its possibility to hydrolyse more recalcitrant lignocellulosic material. This is probably due to a difference in the composition of *S. ramosissima* in comparison to the two other species, where the hemicellulose fraction, which can be hydrolysed by subcritical water, is lower.

Table 8: Yield of extractive material from halophyte biomass. Presented values are means with (± st. dev.)

Biomass	Extraction method	Solvent	Yield [w/w%]	Partner
***S. ramosissima* (LDM)**	Lab-scale Soxhlet	Water	33.68 (2.31)	AAU
		Ethanol 70 %	31.45 (0.59)	
		n-Hexane	1.13 (0.06)	
***S. ramosissima* (RSR)**	Lab-scale pressurized fluid	Water	30.0	CEL
		CO_2	1.9	
***T. pannonicum* (7.5 g/l NaCl)**	Lab-scale Soxhlet	Water	18.73 (0.50)	AAU
		Ethanol 70 %	16.54 (0.33)	
		n-Hexane	2.87 (0.04)	
***T. pannonicum* (10 g/l NaCl)**	Lab-scale pressurized fluid	Water	38.9	CEL
		CO_2	3.9	
***C. maritimum* (0 g/l NaCl)**	Lab-scale Soxhlet	Water	25.88 (2.47)	AAU
		Ethanol 70 %	13.59	
		n-Hexane	2.20 (0.10)	
	Lab-scale pressurized fluid	Water	37.6	CEL
		CO_2	3.4	

4.7 Fatty Acid Profiles of Halophyte Extracts

The FA profile of the halophyte lipids is presented in **Table 9**, including the calculated total amounts of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). The content of health-beneficial PUFA was highest in *T. pannonicum*, covering 78.2 % of total FA. *T. pannonicum* lipids are also high in ω-3 fatty acids, and the low ratio of ω-6 and ω-3 could make the species interesting for nutritional use. Three FAs (arachidic acid, behenic acid and lignoceric acid) were only detected in *S. ramosissima* lipids, and palmitoleic acid was only present in *C. maritimum* lipids.

Table 9: Fatty acid (FA) profile of non-polar halophyte extracts. Presented values are means with (± st. dev.).

Fatty acid [% of total FA]	*S. ramosissima*	*T. pannonicum*	*C. maritimum*
Myristic acid	0.4 (0.4)	n.d.	1.5 (2.2)
Palmitic acid	30.9 (2.3)	19.0 (0.2)	28.9 (1.8)
Palmitoleic acid	n.d.	n.d.	1.9 (2.7)
Stearic acid	2.7 (0.1)	1.6 (0.0)	6.3 (0.3)
Oleic acid	1.3 (0.1)	1.2 (0.5)	11.5 (0.2)
Linoleic acid	34.5 (0.7)	24.9 (0.2)	34.4 (1.8)
α-Linolenic acid	23.7 (1.4)	53.2 (0.3)	15.5 (0.7)
Arachidic acid	1.0 (0.9)	n.d.	n.d.
Behenic acid	3.9 (0.1)	n.d.	n.d.
Lignoceric acid	2.1 (1.8)	n.d.	n.d.
Σ SFA	41.0 (0.9)	20.6 (0.2)	36.7 (4.3)
Σ MUFA	1.3 (0.1)	1.2 (0.5)	13.4 (2.9)
Σ PUFA	58.2 (2.0)	78.2 (0.5)	49.9 (1.2)
ω-6 / ω-3	1.5 (0.1)	0.5 (0.0)	2.2 (0.2)

4.8 Carotenoids and Phytosterols in Halophyte Extracts

Three phytosterol compounds were quantified in the extracts, and the results are presented in **Table 10**. It appeared that *C. maritimum* has a lower amount of phytosterols than *S. ramosissima* or *T. pannonicum*.

Table 10: Main phytosterols [mg/g extract] quantified from the supercritical CO_2 extracts from fibre residue fractions. n.d.: not detected. **) See text about the identification issue.

Compound [mg/g extract]	*S. ramosissima* (RSR)	*C. maritimum* 0 g/l NaCl	*T. pannonicum* 10 g/l NaCl
Campesterol	0.38	n.d.	0.25
Stigmasterol	5.42	0.54	10.06
β-Sitosterol	9.82	1.83	3.91 **

Phytosterol yields are interesting (more than 1% in the lipid extract). However, when taking a closer look at the results, we observed that the mass spectrum of the peak identified as beta-sitosterol in *T. pannonicum* does not correspond to the one of β-sitosterol standard as the parent peak (m/z = 414 in the standard and m/z = 412 in the sample). Therefore, it is clear that this peak is not beta-sitosterol but another phytosterol with a molecular mass two units lower. It is not clearly identified yet, but it could be avenasterol, fucosterol, norcycloartenol, spinastero,l or maybe

vitamin D5. Considering vitamins, a peak of α-tocopherol (vitamin E) was observed in the extracts during this analysis, but not quantified.

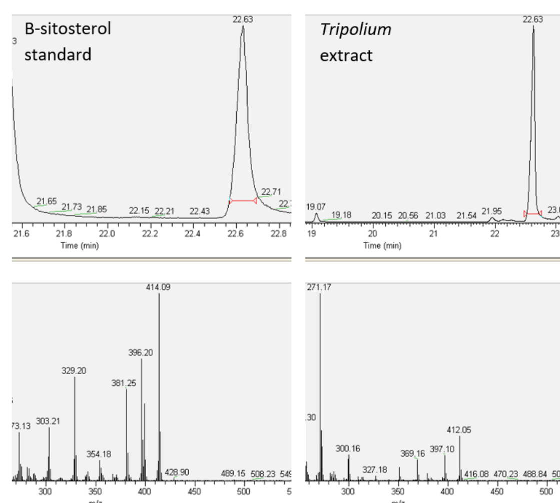

Figure 13: Mass spectrum difference observed for the peak of beta-sitosterol standard at RT: 22.63 min (left) and in T. pannonicum extract at RT: 22.63 min (right)

Considering carotenoids, none of the carotenoids mentioned in **Section 3.4** were detected, except lutein and β-carotene in *C. maritimum* and *T. pannonicum* samples (**Table 11**). *T. pannonicum* residue seems to be the most promising material with high amount of lutein in the extract. When reported to the amount of raw material treated, we observed that almost 200 mg of lutein and 100 mg of β-carotene could be extracted by kg of dried residue. However, it has to be mentioned that the stability of those molecules in these extracts are quite reduced, and they have to be stored at -80 °C protected from the light, otherwise, the content of carotenoids rapidly decreases.

Table 11: Carotenoids quantified in the supercritical extracts by HLPC-DAD. n.d.: not detected.

Carotenoid [mg/kg extract]	*S. ramosissima*	*T. pannonicum*	*C. maritimum*
Lutein	n.d.	5335	3520
β-Carotene	n.d.	5335	1450
Carotenoid [mg/kg raw material]	***S. ramosissima***	***T. pannonicum***	***C. maritimum***
Lutein	n.d.	208.1	119.7
β-Carotene	n.d.	208.1	49.3

4.9 Phenolic Compounds in Halophyte Extracts

Phenolic compounds were quantified by UPLC-MS on the basis of a selection of standards (HCAs + some flavonoids) done in WP3 for the characterization of lignified *S. ramosissima* extracts. **Table 12** and **Table 13** summarize the results expressed as observed in the dry extract (after solvent recovery) and the amount per kg raw material (dried fibre residue), respectively. Compared with the results reported in D3.1, it is interesting to note that the amount of simple hydroxycinnamic acids obtained in *S. ramosissima* is quite similar to that obtained from a lignified plant. The main difference in a significantly lower amount of more valuable compounds, such as chlorogenic acids, hyperoside, or isoguercitrin. If comparing three species, the amount of polyphenols is twice as high in *T. pannonicum* than in *C. maritimum*, which is twice as high as in *S. ramosissima*. Those differences are linked to the presence of the higher

amount of chlorogenic derivatives. In *T. pannonicum* residue, more than 1 g of chlorogenic acids per kg of raw material can be recovered.

Table 12: Polyphenols quantified in the subcritical water extracts of the fibre residue materials of *S. ramosissima* (RSR), *T. pannonicum* (10 g/l NaCl) and *C. maritimum* (0 g/l NaCl) by ULPC-DAD/MS [mg/kg dry extract]. <QL: detected but lower than quantification limit, n.d.: not detected.

Unit : mg/kg dry extract	*S. ramosissima*	*C. maritimum*	*T. pannonicum*
Protocatechuic acid	87.2	80.0	92.4
Coumaric acid	N.D.	N.D.	N.D.
Vanilic acid	121.5	N.D.	N.D.
Caffeic acid	N.D.	<QL	104.4
Ferulic acid	255.5	<QL	N.D.
Quercetin	<QL	<QL	<QL
Isorhamnetin	<QL	N.D.	N.D.
Neochlorogenic acid	116.9	351.7	801.2
Cryptochlorogenic acid	92.1	320.4	708.8
Chlorogenic acid	98.0	788.4	1174.5
Kaempferol-3-Glucoside	<QL	N.D.	N.D.
Hyperoside	<QL	<QL	N.D.
Isoquercitrin	146.1	<QL	<QL
Kaempferol-7-Rutinoside	N.D.	N.D.	<QL
Rutin	N.D.	<QL	<QL
Isorhamnetin 3-rutinoside	<QL	N.D.	<QL
TOTAL	**917.3**	**1540.5**	**2881.4**

Table 13: Polyphenols quantified in the subcritical water extracts of the fibre residue materials of *S. ramosissima* (RSR), *T. pannonicum* (10 g/l NaCl) and *C. maritimum* (0 g/l NaCl) by ULPC-DAD/MS [mg/kg raw material]. <QL: detected but lower than quantification limit, n.d.: not detected.

Unit : mg/kg Raw Mat	*S. ramosissima*	*C. maritimum*	*T. pannonicum*
Protocatechuic acid	26.2	30.1	35.9
Coumaric acid	N.D.	N.D.	N.D.
Vanilic acid	36.5	N.D.	N.D.
Caffeic acid	N.D.	<QL	40.6
Ferulic acid	76.6	<QL	N.D.
Quercetin	<QL	<QL	<QL
Isorhamnetin	<QL	N.D.	N.D.
Neochlorogenic acid	35.1	132.2	311.7
Cryptochlorogenic acid	27.6	120.5	275.7
Chlorogenic acid	29.4	296.4	456.9
Kaempferol-3-Glucoside	<QL	N.D.	N.D.
Hyperoside	<QL	<QL	N.D.
Isoquercitrin	43.8	<QL	<QL
Kaempferol-7-Rutinoside	N.D.	N.D.	<QL
Rutin	N.D.	<QL	<QL
Isorhamnetin 3-rutinoside	<QL	N.D.	<QL
TOTAL	**275.2**	**579.2**	**1120.9**

5 Conclusions and Outlook

The results of the chemical characterization of halophyte biomass fractions and obtained botanical extracts exhibit the following key findings:

- Cultivation salinity significantly affects the composition of *S. europaea,* as well as the composition of facultative halophytes (*T. pannonicum* and *C. maritimum*), but to a less extent.

- *T. pannonicum* has the highest crude protein content, and amino acid analysis would be useful to determine the amount of true protein in the biomass fractions.
- *S. ramosissima* has a higher lignocellulose content compared to *S. europaea* and may be a more suitable feedstock for processes targeting lignocellulose-derived products.
- Pressurized fluid extraction led to a higher yield of non-polar and polar extracts compared to Soxhlet (excluding the water extract from *S. ramosissima*).
- Extracts from *T. pannonicum* were found to contain the highest concentration content of healthy polyunsaturated fatty acids, stigmasterol and carotenoids.
- Out of the studied species, botanical extract from *T. pannonicum* exhibited the highest concentration of polyphenolic compounds, followed by *C. maritimum* and *S. ramosissima* extracts, respectively.

The obtained composition data can be utilized in the planning of upcoming experiments, and possible future design of biorefinery processes and target compounds. At AAU, the composition analysis is continuing for biomass batches fractionated with pilot-scale equipment: *T. pannonicum* (7.5 g/l NaCl), *C. maritimum* (0 g/l NaCl) and *S. ramosissima* (LDM), and the bioactivity of the extracts are being analysed. Biomass delivery of *S. europaea* grown at optimized conditions (15 g/l NaCl) has been received (03/2022) from Alpha-Aqua (ALA), and the composition analyses will be carried out during summer 2022. At UCL, a time-dependent compositional analysis study is carried out by following the composition of *Salicornia europaea* over a growing season, checking the biomass composition every week for 15 weeks.

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