ACS FOOD SCIENCE & TECHNOLOGY

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Influence of Moderate to High Salinity on the Phytochemical Profiles of Two Salinity-Tolerant Spinach Genotypes

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Cite This: ACS Food Sci. Technol. 2021, 1, 205−214 Read Online

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ABSTRACT: Freshwater is a major concern in terms of meeting the growing food demand for the growing population. Recycled waters are currently seen as an alternative, but their higher salinity may impact the yield of most crops and their phytochemical content. In this study, we investigated the effects of five salinity treatments combined with two levels of potassium (K) (deficient K, T1−T5, and enough K, T6−T10) on the phenolic profiles and soluble sugars of two spinach genotypes, Raccoon and Gazelle. We also compared colorimetric assays with chromatographic assays for phenolic quantification. Principal component analysis showed significant variations in near-infrared fingerprints with salinity treatments T1−T5 or T6−T10 for each genotype. On average, the total phenolic content (TPC) [measured by the Folin−Ciocalteu (FC) assay], antioxidant capacity [determined by the ferric reducing antioxidant power (FRAP) assay], the levels of polyphenols, phenolic acids, and sugars gradually decreased with an increase in salinity. Analysis of different extracts by UHPLC-UV-HRESI-MS^n resulted in the identification of 13 polyphenols, four phenolic acids, and three soluble sugars. While the correlation between the sum of polyphenols identified by HPLC and the FC assay was comparatively strong (r^2 = 0.648), it was weak between HPLC and the FRAP assay (r^2 = 0.357). Also, a moderate correlation (r^2 = 0.534) was observed between the colorimetric FRAP assays and TPC. As the salinity influenced the leaf sugar concentrations and sugars interfere with FC and FRAP assays, caution is advised when interpreting the colorimetric assay results for phenolics quantification. These results are relevant to researchers interested in growing crops with recycled and saline waters as sustainable alternatives to freshwater for the increased demand for global food production.

KEYWORDS: *spinach, Raccoon and Gazelle, phytochemicals, polyphenols, sugars, NIR fingerprinting, chromatography, mass spectrometry*

INTRODUCTION

Phytochemicals are naturally produced by various plants and include phenolics, alkaloids, steroids, flavonoids, terpenes, carotenoids, and other compounds commonly found in flowers, leaves, fruits, roots, and seeds of vegetables, legumes, berries, grains, tubers, etc. Although the benefits of dietary phenolics as antioxidants in human health are debatable, it is currently accepted by nutritionists and medical professionals that a diet rich in grains, fruits, and vegetables (all rich sources of phytochemicals), a low level of intake of saturated fat, and moderate exercise are associated with a reduced risk of cardiovascular and degenerative diseases.[1,2] The purported benefits of phytochemicals may be enhanced by vitamins and other nutrients[3,4] but may also depend on their bioaccessibility and bioavailability, which in turn are key modulators of health and disease.[5]

Multiple studies have reported that the accumulation of bioactive compounds is significantly altered by growing conditions.[6-10] Scarcity of freshwater for irrigation is a major impediment to meeting the food demand for a growing global population. For a variety of irrigation needs, research programs around the world are evaluating alternative water resources. Among alternative water sources, recycled wastewater (e.g., drainage effluents from laundry, showers, and bathtubs, municipal reclaimed water, air conditioning condensate, etc.) has been used extensively in irrigation.[11−13] However, the salinity of these waters is a major concern for crop yields and quality traits. The salinity of irrigation water was reported to decrease the sucrose concentration in leaves by 50% but had no impact on tubers' total phenolics or antioxidant capacity in Jerusalem artichoke.[14] Similarly, the salinity also affects the nutraceutical properties of fruits by altering the accumulation of bioactive metabolites, such as lutein, lycopene, and chlorogenic acid in plants.[15] Besides the nutraceutical qualities, the salinity also decreased the yield per plant and the fruit size of tomatoes irrigated under greenhouse conditions with saline solutions having an electrical conductivity (EC_{iw}) of 2, 4, or 6 dS m^{-1}.[15,16] In contrast, the concentrations of β-carotene, lycopene, and vitamin C increased with EC_{iw}.

Recent studies have shown that arbuscular mycorrhizal fungi can increase plant growth and uptake of nutrients under saline conditions.[17,18]

Spinach (*Spinacia oleracea* L.), a green leafy vegetable that belongs to the family Amaranthaceae (subfamily Chenopodioideae), is widely consumed in both fresh and cooked forms

Received: September 30, 2020
Revised: December 15, 2020
Accepted: December 16, 2020
Published: January 8, 2021

https://dx.doi.org/10.1021/acsfoodscitech.0c00034
ACS Food Sci. Technol. 2021, 1, 205−214

Table 1. Chemical Composition (considering soil mineral composition, in $mmol_c\ L^{-1}$) and Electrical Conductivities (EC_{iw}) of the Saline Irrigation Waters[a]

treatment	K^{+b}	Na^{+b}	Cl^{-b}	$H_2PO_4^-$	Ca^{2+b}	Mg^{2+b}	SO_4^{2-b}	NO_3^{-b}	EC_{iw} (dS m^{-1})
T1	0.25	6.5	1.1	0.5	6.0	2.5	4.5	10.0	1.3
T2	0.25	30.5	25.1	0.5	6.0	2.5	4.5	10.0	4.2
T3	0.25	60.5	55.1	0.5	6.0	2.5	4.5	10.0	7.2
T4	0.25	90.5	85.1	0.5	6.0	2.5	4.5	10.0	10.4
T5	0.25	120.5	115.1	0.5	6.0	2.5	4.5	10.0	13.1
T6	5.00	3.0	2.4	0.5	6.0	2.5	4.5	10.0	1.6
T7	5.00	30.5	29.9	0.5	6.0	2.5	4.5	10.0	4.5
T8	5.00	60.5	59.9	0.5	6.0	2.5	4.5	10.0	7.6
T9	5.00	90.5	89.9	0.5	6.0	2.5	4.5	10.0	10.7
T10	5.00	120.5	119.9	0.5	6.0	2.5	4.5	10.0	13.2

[a]Deionized water (EC_{iw} = 0.05 dS m^{-1}) was used to make all saline waters, and all the treatments received half-strength modified Hoagland's as basic fertigation. No K was added to T1 irrigation treatments. pH 5.5. [b]The ion concentration includes the ion concentration determined in the loamy sand soil (in $mmol_c\ L^{-1}$) wet paste: Na^+, 2.5; K^+, 0.25; Ca^{2+}, 1.5; Mg^{2+}, 0.5; NO_3^-, 2.0; Cl^-, 1.1; SO_4^{2-}, 2.5; $H_2PO_4^-$, 0.015. The soil pH was 8.0, and the pre-experimental soil paste electrical conductivity (EC_e) was 0.5 dS m^{-1}.

and has been recognized as a functional food due to its diverse nutritional composition.[19] Spinach shoots are primarily composed of water (91.4%) and contain small amounts of protein (2.9%), carbohydrate (3.6%), and fat (0.4%). The plant also possesses a considerable amount of fiber, vitamins A, C, and K, carotenoids, folic acid, and minerals such as iron, calcium, and magnesium. Spinach also produces other phytochemicals, including flavones, flavanols, and glucuronides.[19−21]

In this study, the impacts of saline water irrigation on the phytochemicals of two spinach genotypes, Raccoon and Gazelle, were investigated. Both genotypes are erect, vigorous, smooth-leaf varieties with dark color and good texture, suitable for the production of bunch or baby spinach and cultivation in the winter, spring, and fall, and can be acquired from different seed businesses such as Corona Seeds (http://coronaseeds.com), Dave's Garden (http://davegarden.com), Johny Seeds (http://www.johnyseeds.com), etc. A previous study with Raccoon and Gazelle genotypes irrigated with waters of increasing salinity combined with two K^+ doses (0.25 and 5.0 $mmol_c\ L^{-1}$), and of the same composition used in this study, reported that plants maintained their shoot concentrations of N, P, K, Mg, and S, although with a significant decrease in the shoot concentration of Ca observed when the salinity increased from 5.0 to 120 $mmol_c\ L^{-1}$ NaCl.[22] The cultivar, cultivation method, soil type, growing season, leaf size, and storage duration of spinach can influence its nutrient composition and phytochemicals.[23,24] In a recent study, a mild salinity stress of 6.5 dS m^{-1} significantly decreased spinach shoot fresh weight and dry weight, leaf relative water content, and specific leaf area, while the salinity increased chlorophyll content relative to control salinity.[25] The authors also reported that salt stress did not affect the concentrations of total phenolics under control nutrient conditions and further concluded that the content of total phenolics and total flavonoids measured by colorimetric assays did not correlate with the antioxidant capacity. In contrast, Ferreira and coworkers reported that the average total antioxidant capacity [measured by the oxygen radical absorbance capacity (ORAC) assay] and the levels of total phenolics (measured by the FC assay) of spinach (Raccoon) did not change with the highest salinity and EC_{iw} containing 80 mM NaCl (EC_{iw} = 9.4 dS m^{-1}).[26] Both of these studies[25,26] used colorimetric assays to measure antioxidant capacity and total phenolic content. As cited previously, there are several problems associated with the measurement of antioxidant capacity by different colorimetric methods, such as inconsistent results from different methods for a single substrate due to the interference from other phytochemicals, particularly reducing sugars (glucose and fructose), pigments, and ascorbic acid.[27−29] Furthermore, it is well documented that salinity has a significant influence on the accumulation of sugars (glucose, fructose, and sucrose) in different plant tissues.[30,31] Considering that salinity may influence sugar concentrations and sugars interfere with FC and FRAP assays, caution is advised when interpreting the colorimetric assay results for the quantification of phenolic compounds.[29,30,32] Thus, there is a critical need to confirm colorimetric assay results with detailed chromatographic analysis.

This study aimed to evaluate the effect of increasing the levels of Na^+ and Cl^- (5, 30, 60, 90, and 120 $mmol_c\ L^{-1}$) in irrigation water combined with two levels of K^+ (0.25 and 5.0 $mmol_c\ L^{-1}$) on the phenolic and sugar contents of two spinach genotypes, Raccoon and Gazelle. Phenolic contents were determined by the colorimetric and chromatographic methods, and the soluble sugar content was determined using ion chromatography (IC). The correlation coefficient between colorimetric and chromatographic methods was established using regression analysis. Furthermore, we also investigated if near-infrared (NIR) spectral fingerprinting methods can be used for the classification of spinach genotypes grown under different salinity conditions.

MATERIALS AND METHODS

Materials. Analytical grade sugar standards (fructose, glucose, and sucrose) were bought from Sigma-Aldrich Chemical Co. (St. Louis, MO). The 15 mL disposable polypropylene centrifuge tubes were purchased from Fisher Scientific (Pittsburgh, PA) were used for the extractions of phytochemicals. Polyvinylidene difluoride (PVDF) syringe filters (pore size of 0.45 μm) were purchased from National Scientific (Duluth, GA). All LC-MS grade organic solvents were obtained from Fisher Chemicals (Fair Lawn, NJ) and used directly without further purification. Deionized water (DI, 18 Ω) was obtained using a Millipore Milli-Q purification system (Millipore Corp., New Bedford, MA).

Plant Materials. Spinach genotypes were grown in a greenhouse maintained at 25 °C (daytime) and 17 °C (nighttime) under natural illumination with pots arranged in a randomized design with 10 salinity treatments (T1−T10). The 10 treatments were composed of five irrigation water salinities with EC_{iw} values ranging from 1.3 to 13.2 dS m^{-1} (equivalent to nominal NaCl concentrations of 5, 30, 60,

https://dx.doi.org/10.1021/acsfoodscitech.0c00034
ACS Food Sci. Technol. 2021, 1, 205−214

90, and 120 $mmol_c\ L^{-1}$) combined with two K^+ concentrations (0.25 and 5.0 $mmol_c\ L^{-1}$) (Table 1). The detailed procedures of seeding, irrigation, and treatment have been described previously.[26] Irrigation with the different treatment solutions continued for 28 days after plants had developed six to eight true leaves, which were harvested, freeze-dried, and stored at −80 °C until further analysis. For this study, three biological replicates were collected and analyzed in triplicate.

NIR Spectral Analysis. A Nicolet 6700 FT-IR instrument operated by OMNIC software was used to record NIR spectral fingerprints of samples. Freeze-dried shoot powders of Gazelle and Raccoon were placed in 4 mL glass vials, and individual vials were analyzed in triplicate (samples were homogenized between each analysis). Spectra were recorded by integrating the sphere diffuse reflectance from 10000 to 4000 cm^{-1} at a resolution of 4 cm^{-1}. The NIR data were converted to Excel format, and principal component analysis (PCA) was performed using Solo software (release 7.8.2, Metlab version 7.9.0.529, Eigenvector Research, Inc., Manson, WA).

Extraction of Samples. Lyophilized ground spinach shoots (200 ± 0.5 mg) were placed in 5 mL of 80% aqueous MeOH in a 15 mL centrifuge tube and sonicated (Advanced Sonic Processing Systems, Oxford, CT) for 15 min. The extraction was repeated twice. The pooled extracts were centrifuged at 1792*g* for 15 min, and the supernatants were filtered through a PVDF filter. The filtered extract was used for different analyses.

Determination of the Total Phenolic Content (TPC). The TPC was determined using the FC assay as previously reported in the literature using gallic acid as a standard.[33] The assay was carried out by pipetting 20 μL of spinach extract into a 96-well plate followed by the addition of 177 μL of water. This mixture was vortexed gently to mix for 1−2 min, and 12.5 μL of FC reagent was added to each well and incubated in the dark. After 8 min, 37.5 μL of a sodium carbonate solution (20%) was added, and the mixture was carefully mixed and incubated for 2 h in the dark. The absorbance of the colored reaction product was measured at 765 nm by a Spectramax 384 Plus microplate reader from Molecular Devices (Sunnyvale, CA). A calibration curve was created using gallic acid dilutions as standards. The level of TPC in the extract was calculated from the calibration curve (six data points were taken at concentrations of 0, 0.03, 0.06, 0.12, 0.18, and 0.25 mg/mL; r^2 = 0.9967). Results were calculated using Microsoft Excel and expressed in milligrams of gallic acid equivalent per gram (mg of GAE g^{-1}) of dried spinach.

Determination of Antioxidant Capacity. The FRAP assay was performed using our previously reported method with some modifications.[34] The calibration curve was constructed using a 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (TROLOX) standard. A FRAP working solution was prepared by mixing 2,4,6-tris(2 pyridyl)-*s*-triazine (TPTZ) (10 mM in 40 mM HCl), sodium acetate buffer (300 mM, pH 3.6), and ferric chloride hexahydrate (20 mM) in a ratio of 1:10:1 (v/v/v). A 20 μL aliquot from each spinach extract (20 mg/mL) was added to a 96-well plate. The 240 μL FRAP working solutions were added to the well, and the mixture was gently shaken for 1−2 min. The mixture was incubated in the dark for 10 min at ambient temperature. The absorption of the colored product was measured at 595 nm using a Spectramax 384 Plus microplate reader from Molecular Devices.

Analyses of Polyphenols and Conjugated Phenolic Acids. For free polyphenols, filtered crude extracts were transferred to 2 mL HPLC vials and analyzed by LC-MS.

Conjugated phenolic acids were analyzed after base hydrolysis according to a method described previously.[33,35,36] In brief, 100 ± 1 mg of ground leaves was transferred to a 15 mL centrifuge tube. The hydrolysis solution was prepared by mixing 0.375 g of EDTA, 1 g of ascorbic acid, and 100 mL of 2 N NaOH; 5 mL of a freshly prepared hydrolysis solution was added to the reaction tube, and the mixture was vortexed and sonicated at 50 °C for 30 min. The mixture was extracted twice with 5 mL of ethyl acetate. The organic supernatant was recovered after centrifugation at 1792*g* for 15 min. The organic solvent was evaporated under N_2, and the residue was reconstituted in 1 mL of the aqueous MeOH mixture [80:20 (v/v)]. The solution was filtered by a 0.45 μm pore size filter, and the extract was analyzed by LC-MS.

The free polyphenols and conjugated phenolic acids were analyzed by an LC-MS system from Agilent, 1200 LC series, consisting of a diode array detector (DAD) (Agilent Technologies, Santa Clara, CA), and coupled with a single quadrupole mass spectrometer (Thermo Fisher Scientific MSQ Plus, Thermo Fisher Scientific, Waltham, MA) operated using Chromeleon software (version 7.2, Thermo Fisher Scientific, Waltham, MA). The compounds from the extract were separated in a C_{18} Agilent column (ZORBAX, Eclipse Plus, 95 Å, 4.6 mm × 50 mm, 1.8 μm, 600 bar pressure limit) using water and acetonitrile both acidified with 0.1% formic acid as mobile phases A and B, respectively, with the following gradient: 10% B at 0 min to 20% at 2 min, maintained at 20% from 2 to 6 min, the gradient was gradually increased to 50% by 9 min, then increased to 90% by 13 min, held at 90% to 15 min, and reduced to 10% at 16 min. The flow rate and the injection volume were maintained at 0.7 mL/min, and 10 μL, respectively. Masses of compounds ranging from m/z 50 to 1000 were acquired using electrospray ionization in negative mode. The identification of compounds was performed using an ultraviolet (UV) detector at four different wavelengths (264, 280, 310, and 330 nm), and the peak area quantification was performed at the λ_{max} of 330 nm.

High-resolution mass spectra of the extracts were analyzed on a Tribrid Mass Spectrometer (Orbitrap ID-X) coupled with a Vanquish UHPLC$^+$ LC system (Thermo Fisher Scientific, San Jose, CA) operated by the Xcalibur software. The extract was analyzed on the same column with the same flow rate and mobile phase as described above (A as water with 0.1% formic acid and B as acetonitrile with 0.1% formic acid). The gradient flow was 7% B at 0 min, increased to 10% by 2 min, 20% at 5 min, 25% at 9 min, 35% at 13 min, maintained at 35% until 18 min, increased 90% at 20 min, held at 90% until 22 min, and reduced to 7% B at 23 min. The ESI conditions were as follows: sheath gas, auxiliary, and sweep gas at 40, 5, and 1 arbitrary units, respectively, spray voltage at −3.9 kV, and capillary temperature at 300 °C. The full scan mass spectra and three DD-MS^n events were acquired at a resolving power of 60000. An isolation width of 1 amu, a maximum ion injection time of 22 ms, a normalization collision energy at 30%, and an activation time of 10 ms were used for MS^n activation. The identification of each of the compounds (major and minor) in the extract was performed on the basis of comparing their mass and UV spectral data with the literature values, as discussed in Results and Discussion.

Analysis of Soluble Sugars. Ground dried shoot samples (25 ± 0.3 mg) were added to a 15 mL centrifuge tube; 5 mL of DI water was added, and the mixture was sonicated for 15 min at ambient temperature. The supernatant was collected by centrifugation at 1792*g* for 15 min. The extract was filtered through a PVDF filter and subjected to ion chromatography. All extractions and analyses were carried out in triplicate.

Analysis of soluble sugars was performed on an ICS-5000 IC system (Dionex, Sunnyvale, CA), consisting of a Dionex gradient pump and electrochemical detector equipped (PAD) with a gold working electrode amperometric cell (1.0 mm diameter) and operated by Chromeleon software 6.8 (Dionex, Thermo Fisher Scientific, Waltham, MA). A 100 μL sample was analyzed on a Dionex CarboPac PA20 IC column (3 mm × 150 mm), proceeded by a guard column (3 mm × 30 mm). An isocratic eluent 30 mM KOH was used at a flow rate of 0.25 mL/min for 50 min. The peak area of each soluble sugar (glucose, fructose, and sucrose) was recorded and added to calculate the total soluble sugar concentration.

An LC-MS/MS method was used to confirm the identity of soluble sugars on a limited number of spinach samples from control and high-salinity treatments (T1, T5, T6, and T10). For this purpose, an Agilent HPLC 1290 instrument (Agilent Technologies), coupled with a Thermo Scientific TSQ Vantage instrument (Thermo Fisher Scientific), operated by Xcalibur version 2.2 (Thermo Fisher Scientific), was used as described previously.[37] In brief, sugar identification was achieved using both the deprotonated molecule mass (m/z) and product ion masses using both data-dependent (MS^2) scans (DDS) [or data-dependent acquisition (DDA)] and

https://dx.doi.org/10.1021/acsfoodscitech.0c00034
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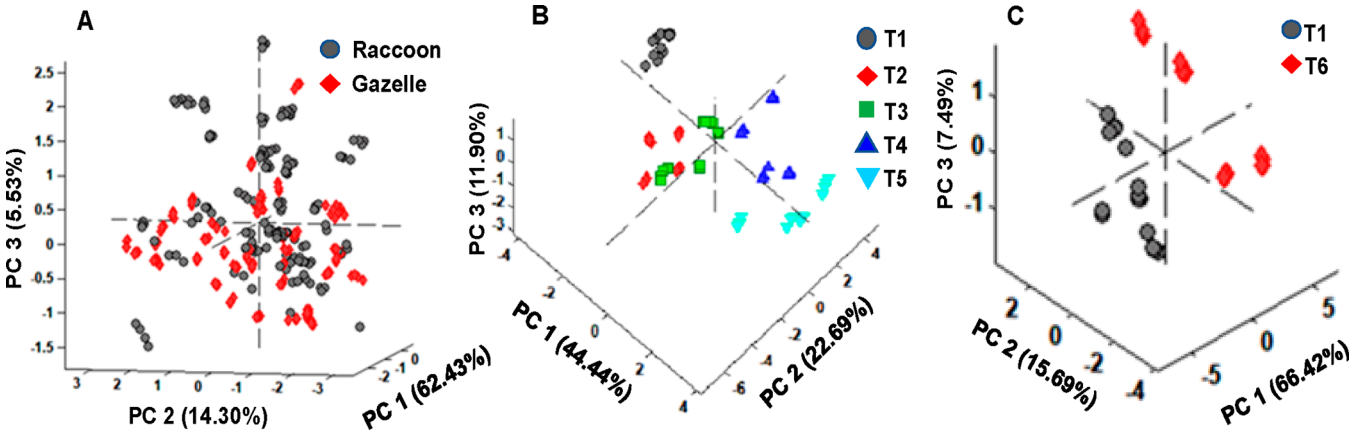

Figure 1. Typical example of principal component analysis (PCA) of the near-infrared spectral (NIR) data of spinach. (A) PCA of the spinach cultivars Raccoon and Gazelle. (B) PCA of the NIR data of Raccoon samples grown under five different salt treatments (T1−T5). (C) PCA of the NIR data of Raccoon samples grown under T1 and T6.

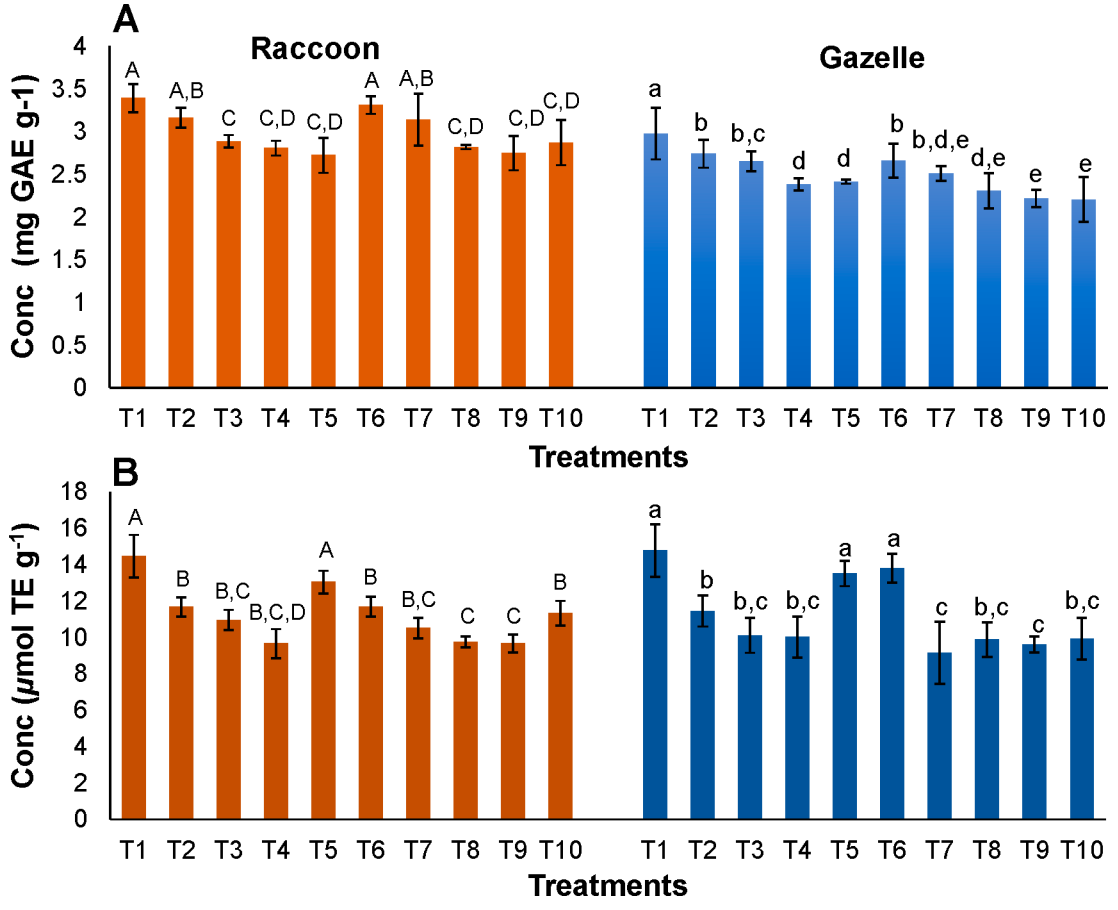

Figure 2. (A) Total phenolic content (TPC) in gallic acid equivalents in freeze-dried spinach extracts from 10 salinity treatments measured by a Folin−Ciocalteu assay. (B) Antioxidant capacity of the same extracts using the FRAP assay. The results are summarized as averages of at least three replicates ± the standard deviation. Different letters at the top of bars represent the statistical difference ($P \leq 0.05$) between all 10 salinity treatments mean (T1−T10) for each genotype, according to pairwise comparisons by the Tukey−Kramer HSD.

selective ion monitoring (SIM) mode. An XBridge BEH Amide XP column (2.5 μm, 2.1 mm × 100 mm) (Waters Corp., Milford, MA) equipped with a Waters VanGuard HS S T3, 1.8 μm precolumn was used for the separation of the sugars. A binary solvent system consisting of solvent A (water with 0.1% ammonium hydroxide) and solvent B (acetonitrile with 0.1% ammonium hydroxide) was used as the mobile phase. The solvent gradient was set as follows: 3% A from 0 to 0.2 min, 3% to 60% A from 0.2 to 15 min, 60% A from 15.0 to 17.5 min, 60% A to 3% A from 17.5 to 17.8 min, and 3% A from 17.8 to 20.0 min. Mass spectra were obtained using heated electrospray ionization (HESI) in negative ion mode within a mass range of m/z 50−1500. The mobile phase flow rate was 0.2 mL/min, and the column temperature was maintained at 35 °C. A spray voltage of 4000 V, a vaporizer temperature of 200 °C, a capillary temperature of 250 °C, a sheath gas pressure of 60 arbitrary units (au), an auxiliary gas pressure of 10 au, a sweep gas pressure of 0 au, a declustering voltage of −6 V, and a collision pressure of 1.0 m Torr were used for mass spectral analysis.

https://dx.doi.org/10.1021/acsfoodscitech.0c00034
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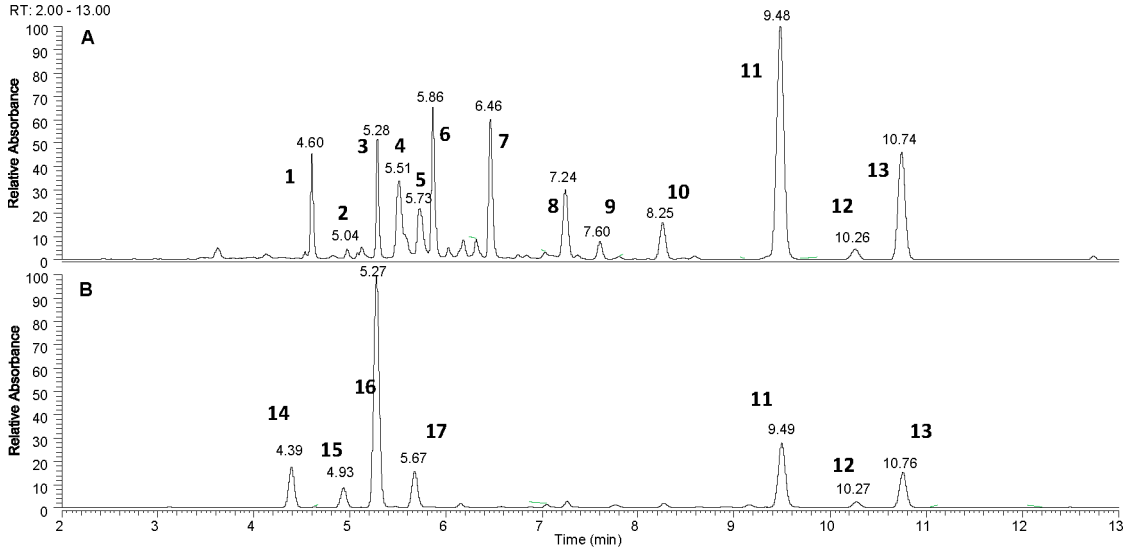

Figure 3. Representative UHPLC-UV-HRESI-MS^n chromatogram (at λ_{max} = 330 nm) of (A) polyphenols in unhydrolyzed spinach samples and (B) phenolic acids in hydrolyzed samples.

Statistical Analysis. All colorimetric and spectroscopic analyses were performed in triplicate from each of the three plant samples (n = 9), and data expressed as the mean ± the standard deviation (SD) using Microsoft Excel. PCA of the NIR spectral data was performed using Solo software (release 7.8.2, Metlab version 7.9.0.529, Eigenvector Research, Inc., Manson, WA). One-way analysis of variance for comparison between samples grown under 10 different salinity treatments was performed using the JMP Pro 15.0.0 statistical software (Cary, NC). The mean comparison for all pairs was made with a Tukey−Kramer HSD test.

RESULTS AND DISCUSSION

Principal Component Analysis. In this study, we analyzed ground freeze-dried spinach shoots, initially by NIR fingerprinting, to determine the overall compositional differences between the two genotypes cultivated at various salt concentrations. PCA of the NIR spectral fingerprinting showed a significant overlap when all samples (two spinach genotypes grown under 10 different treatments) were included for analysis (Figure 1A). However, PCA of Raccoon under one K and five different salinity conditions (treatments T1−T5 or T6−10) showed a distinction between samples irrigated with control and the highest salinity level (Figure 1B). Similarly, PCA showed significant differences between samples of Raccoon genotypes from two different K concentrations (0.25 and 5.0 mmol$_c$ L^{-1}, T1 and T6, respectively) (Figure 1C). Similar clustering patterns were observed with the Gazelle genotype (Figure S1). There are several published reports in which NIR has been used to differentiate crop samples based on genotypes, growing conditions, and phytochemical concentrations.[38−40] Previous results from our laboratory have shown that there were significant differences in the NIR, UV, and MS spectral fingerprints of broccoli samples grown under different selenium concentrations.[41−43] The mass spectral fingerprint results for broccoli extracts showed that the discriminating ions were not associated with phenolic compounds but tentatively associated with polar metabolites, namely, common sugars and organic acids.[41,43] Thus, simple spectral fingerprinting techniques can be potentially used to provide rapid classifications of food samples grown under varying salinity environments. In this study, we adopted a similar approach to investigate the phytochemical (sugars and phenolics) variation in spinach samples grown under five salinity treatments combined with two K levels (Table 1).

Total Phenolic Content and Antioxidant Capacity Analyses. In this study, we investigated the total phenolic content and antioxidant capacity by two colorimetric assays, FC and FRAP, respectively. Similar TPC and antioxidant capacity were exhibited by both genotypes (Gazelle and Raccoon) (Figure 2A,B). An approximately 30% variation in the TPC was observed in plants from different salinity treatments. In general, a gradual decrease in TPC and antioxidant capacity was observed with an increase in salinity from treatment T1 to T4 and from treatment T6 to T9. Insignificant variations in TPC were observed for T4 to T5 or T9 to T10 in the Raccoon genotype. However, in the case of the Gazelle genotype, a marginal increase (<10%) in TPC content was observed with increasing salinity (T4 to T5). These results showed that variation in plant secondary metabolites might be due to stress treatment (in this case, salinity) and/or cultivar. Direct comparison of TPC by the FC assay showed insignificant differences between treatments T1 and T6, T2 and T7, T3 and T8, T4 and T9, and T5 and T10, where the K concentration was increased 20-fold for Raccoon genotype. However, the Gazelle genotype showed a minor decrease in TPC under the same treatments (Figure S3). Similar levels of TPC were reported previously in spinach samples grown under different salinity treatments.[26] The same group also reported that the TPC concentration decreased with an increase in salinity, regardless of K dose.

There was a moderate correlation (r^2 = 0.534) between the colorimetric FRAP and TPC assays. There are a few reports in the literature showing a wide array of correlations between the two assays.[44−46] In some cases, there is a positive correlation, while in other cases, no significant or negative correlations were observed.[44−46] Furthermore, as mentioned above, salinity influences the accumulation of sugar in leaves, and it is also known that the FC assay is impacted by the presence of sugars.[28] Therefore, there is a need to be cautious in interpreting results for phenolic content by colorimetric assays. The colorimetric assay results need to be confirmed from the colorimetric methods with other chromatographic methods,

https://dx.doi.org/10.1021/acsfoodscitech.0c00034
ACS Food Sci. Technol. 2021, 1, 205−214

Table 2. Identification of Unhydrolyzed Polyphenols and Hydrolyzed Phenolic Acids in Freeze-Dried Ground Spinach Shoots

	rt	compound	m/z [M − H]$^-$	fragments
		Polyphenols from Unhydrolyzed Samples		
1	4.60	patuletin-3-*O*-*β*-D-glucopyranosyl-(1→6)-[*β*-D-apiofuranosyl-(1→2)]-*β*-D-glucopyranoside	787.1946	655, 331, 287
2	5.04	patuletin-3-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranoside	655.1611	330, 287
3	5.28	spinacetin-3-*O*-*β*-D-glucopyranosyl-(1→6)-[*β*-D-apiofuranosyl-(1→2)]-*β*-D-glucopyranoside	801.2111	669, 651, 345
4	5.51	patuletin-3-*O*-*β*-D-(2″-*β*-coumaroylglucopyranosyl-(1→6)-[*β*-D-apiofuranosyl-(1→2)]-*β*-D-glucopyranoside	933.2305	787, 769, 655, 331
5	5.73	patuletin-3-*O*-*β*-D-(2″-feruloylglucopyranosyl-(1→6)-[*β*-D-apiofuranosyl-(1→2)]-*β*-D-glucopyranoside	963.2415	787, 655, 331, 175
6	5.86	spinacetin-3-*O*-*β*-D-(2″-*β*-coumaroylglucopyranosyl-(1→6)-[*β*-D-apiofuranosyl-(1→2)]-*β*-D-glucopyranoside	947.2457	669, 651, 345, 175
7	6.46	spinacetin-3-*O*-*β*-D-(2″-feruloylglucopyranosyl-(1→6)-[*β*-D-apiofuranosyl-(1→2)]-*β*-D-glucopyranoside	977.2576	801, 669, 345, 175
8	7.24	spinatoside-4′-glucuronide	521.0934	345, 330, 175
9	7.60	spinacetin-3-*O*-*β*-D-(2″-feruloylglucopyranosyl-(1→6)-*β*-D-glucopyranoside	845.2141	669, 651, 499, 345
10	8.25	jaceidin-4′-glucuronide	535.1107	359, 344, 175
11	9.48	5,3′,4′-trihydroxy-3-methoxy-6:7-methylenedioxyflavone-4-*β*-D-glucuronide	519.0786	343, 283, 175
12	10.26	5,4′-dihydroxy-3-methoxy-6:7-methylenedioxyflavone-4-*β*-D-glucuronide	503.0834	327, 175
13	10.74	5,4′-dihydroxy-3,3-dimethoxy-6:7-methylenedioxyflavone-4-*β*-D-glucuronide	533.0944	357, 327, 175
		Phenolic Acids from Hydrolyzed Samples		
14	4.39	*p*-coumaric acid	163.0400	145, 117
15	4.93	*p*-coumaric acid isomer	163.0401	
16	5.27	ferulic acid	193.0505	175, 149
17	5.67	ferulic acid isomer	193.0506	

such as liquid or gas chromatography coupled with different detectors.

HPLC and LC-MS Analyses of Polyphenols and Phenolic Acids. There are many reports on the phytochemical analysis of spinach samples. The major active phytochemicals detected in spinach samples are flavones, flavanols, methylene-dioxyflavonol, and carotenoids.[21] Spinacetin and patuletin and their derivatives are the most abundant flavonoids detected in spinach by several research groups.[24,47−49] Using UV and LC-HESI-MS/MS followed by a comparison with the literature,[24,47−49] a total of 13 prominent polyphenols were tentatively identified in the spinach shoot aqueous methanol extract (80% MeOH) (Figure 3A). Details of the compounds identified in this study are listed in Table 2.

As purified standards of all identified polyphenols are not readily available, quantification was achieved by measuring the area under each identified peak. Summing of areas under each identified polyphenol was used to quantify total polyphenols. Similar levels of total polyphenols were observed in genotypes Raccoon and Gazelle under greenhouse conditions for all treatments (Figure 4A). Compound **11** (∼35%) was the major polyphenols in both genotypes. On the basis of dry weight, compound **10** (∼15%) was the second major polyphenol in Raccoon, whereas compound **13** (∼14%) was the second prominent polyphenol in Gazelle. The profiles of the other polyphenols in the two genotypes were marginally different. In both genotypes, there was a significant decline in polyphenol content (∼20%) when the EC_{iw} was increased from 1.3 or 1.6 dS m^{-1} (T1 or T6, respectively) to 4.2 or 4.5 dS m^{-1} (T2 or T7, respectively). PCA of the sum of the polyphenol data of the three biological replicates showed a clustering pattern based on five treatments [T1−T5 (Figure S2A)]. This corresponds to an increase in salinity from 6.5 to 30.5 $mmol_c$ L^{-1} Na (5−10-fold increase) with an associate increase in Cl from 12- to 23-fold (Table 1). The trend (decrease with an increase in salinity from T1 to T5 or from T6 to T10) for most identified polyphenols was very similar when an individual polyphenol area was tabulated, except for compound **13**, where no significant difference was observed (Figure S4). In a recent study, water salinity resulted in an increase in shoot Na concentration from 1.4 to 2.95 mol kg^{-1} in Raccoon and from 1.0 to 2.7 mol kg^{-1} in Gazelle at 0.25 $mmol_c$ L^{-1} K, but a larger increase in shoot Cl concentration from 0.25 to 2.0 mol kg^{-1} in Raccoon and from 0.17 to 2.2 mol kg^{-1} in Gazelle at the same K dose.[22] These authors also reported that shoot concentrations of N, P, and K remained constant in all salinity treatments, except that there was 2.0−2.5-fold more when plants were irrigated with K 5.0 $mmol_c$ L^{-1} (from the irrigation water) than in the ones provided only at 0.25 $mmol_c$ L^{-1} K (from the soil solution). Although the salinity of 4.5 dS m^{-1} did not cause any visible stress or biomass decrease for these two spinach genotypes,[22] this small salinity increase caused a significant decrease in the shoot Ca concentration, although not enough for plants to express visual signs of mineral deficiency or toxicities (in the cases of shoot Na and Cl).[22]

To quantify the conjugated phenolic acids, base hydrolysis of a limited number of spinach samples from treatments T1, T5, T6, and T10 was performed. The base-hydrolyzed extracts showed the presence of four prominent phenolic acid peaks in spinach samples (Figure 3B). Two of the phenolic acids had a molecular ion at m/z 163 [M − H]$^-$, and the other two phenolic acids had a molecular ion at m/z 193 [M − H]$^-$. Two of these phenolic acids with retention times (rt's) of 4.3 and 5.2 min were characterized as *p*-coumaric acid and ferulic acid, respectively, based on comparison of rt, UV, and MS spectral data of purified standards. Both of these phenolic acids have been previously detected by other researchers in spinach.[50−52] The other two phenolic acids at rt's of 4.9 and 5.6 min were tentatively characterized as isomers of coumaric acid and ferulic acid, respectively, based on LC-MS analysis of data.[52] As pure standards for all phenolic compounds were not easily available, only the area under the curve was used for comparative analysis to maintain uniformity throughout the manuscript.

The results from the base hydrolysis were compared (T1 vs T5 and T6 vs T10) by computing the total area under the curve for the identified phenolic acids. Both identified phenolic

https://dx.doi.org/10.1021/acsfoodscitech.0c00034
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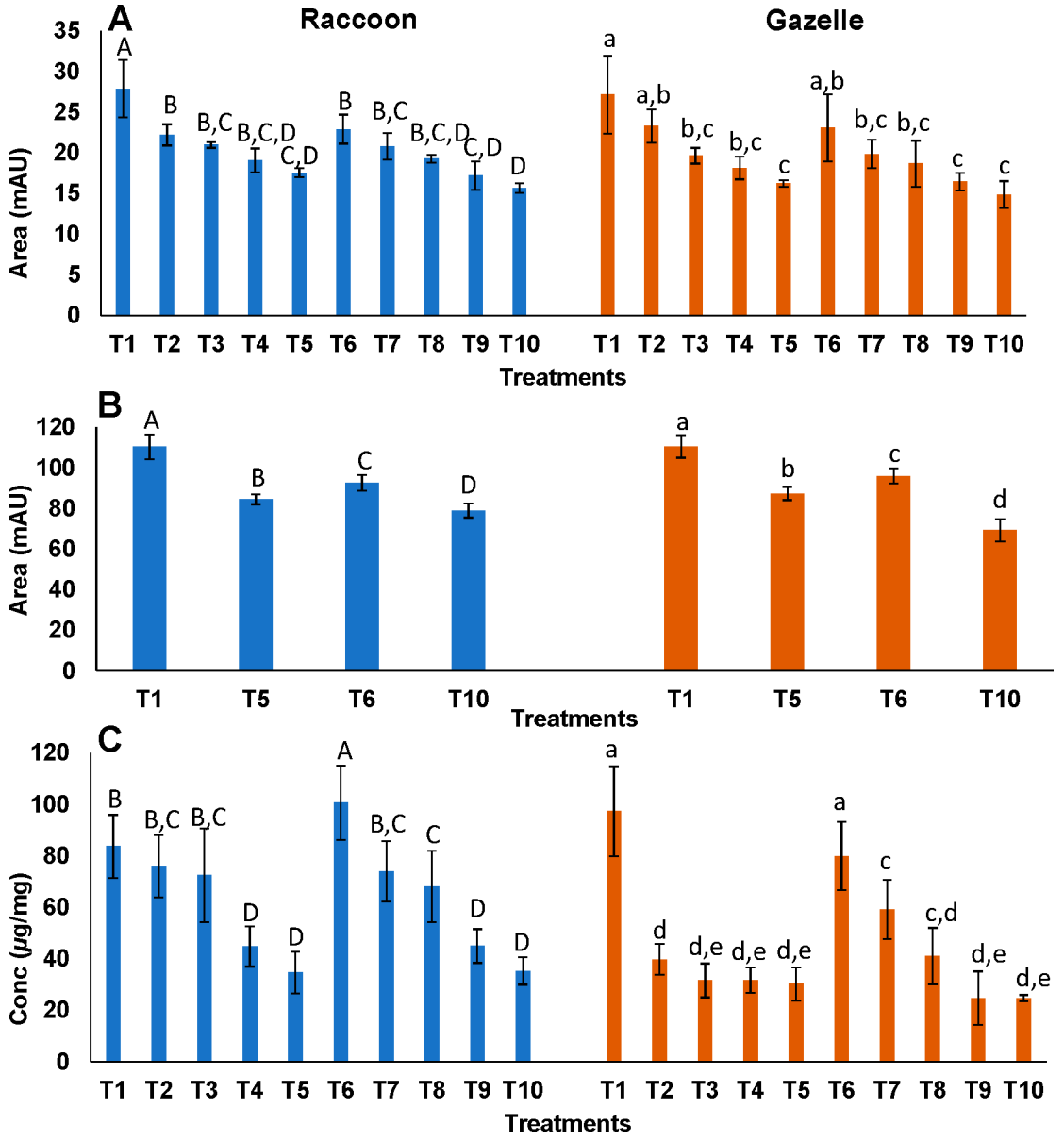

Figure 4. (A) Sum of polyphenols, (B) hydrolyzed phenolic acids, and (C) total soluble sugars (glucose, fructose, and sucrose) in shoots of Raccoon and Gazelle genotypes in response to increasing salinity of irrigation water. The results are summarized as averages of at least three replicates ± the standard deviation. Different letters at the top of bars represent the statistical difference ($P \leq 0.05$) among all 10 salinity treatment means (T1−T10) for each genotype, according to pairwise comparisons by the Tukey−Kramer HSD.

acids *p*-coumaric acid and ferulic acid constituted ∼76% and ∼78%, respectively, of the total identified base-hydrolyzed phenolic acids, and ∼24% and ∼22% were identified as isomeric *p*-coumaric acid and ferulic acid, respectively, in both genotypes. The concentrations of total conjugated phenolic acids in control samples (T1 and T6) were ∼20% higher than those of samples grown under high-salinity conditions (T5 and T10) (Figure 4B). These results showed similar trends, as observed for total free polyphenols.

It has been reported that an increased irrigation water salinity decreases in the rate of growth resulting in a shorter stature and smaller leaves in artichoke and cardoon genotypes.[53] The severity of the salinity response is also influenced by other environmental factors such as temperature, radiation, humidity, and air pollution.[55] Furthermore, exposure to salinity for a prolonged time was previously reported as having a significant impact on phytochemical content.[54] In grapevine, the content of the phenolic compound (rutin) in leaves and roots of four genotypes increased initially after treatment for 24 h with 50 mM NaCl (equivalent to an EC_{iw} of 5.0 dS m^{-1}) but decreased after 7 and 14 days.[55] In the study presented here, we evaluated the effect of salinity in spinach leaves, which were collected after cultivation and irrigation with saline waters for 28 days. The results showed a gradual decrease in both polyphenol and phenolic acid content in plants irrigated with high-salinity water compared to control (Figure 4A,B). Similarly, Hanen et al. reported that the leaf phenolic content was significantly increased at 25−50 mM NaCl and decreased at 150 mM NaCl in *Cynara cardunculus* L.[56] We found that the correlation between the sum of polyphenols identified by HPLC versus the FC assay was moderate (r^2 = 0.648) but weak between HPLC and the FRAP assay (r^2 = 0.357). Although both colorimetric and chromatographic methods produced similar results, i.e., the TPC and

https://dx.doi.org/10.1021/acsfoodscitech.0c00034
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antioxidant capacity were reduced with an increase in salinity, phenolic quantification by the chromatographic method was more precise, indicating that colorimetric determination assays for phenolics may produce erratic quantification due to the interference from sugars and other antioxidants, thus not representing the precise concentration of phenolics in the sample.

Soluble Sugar Analysis. As documented above, sugars are prominent interference agents for the total phenolic Folin–Ciocalteu colorimetric assay.[27,28] Hence, we identified soluble sugars in the aqueous extract of spinach shoots. The three soluble sugars, glucose, fructose, and sucrose, were identified in spinach extract, based on ion chromatography coupled with a PAD detector, with glucose being the most abundant (~50%) in spinach shoots from both genotypes. The structures of the identified sugars were confirmed by mass spectral analysis of a representative number of spinach extracts.

PCA of the sum of the sugar data of three biological and three analytical replicates did not show a distinct clustering pattern based on five treatments [T1–T5 (Figure S2B)]. Similar levels of individual soluble sugars were found in both genotypes (Figure 4C). For instance, under control (low-salinity) irrigation water (T1), soluble sugars were present in the following order (and dry matter concentrations) in the ion chromatogram, glucose (43.9 μg/mg), fructose (14.6 μg/mg), and sucrose (25.1 μg/mg) in Raccoon, whereas Gazelle shoots had glucose (52.4 μg/mg), fructose (10.0 μg/mg), and sucrose (34.9 μg/mg). The sum of soluble sugars ranged from 83.7 ± 13.3 to 97.3 ± 19.2 μg/mg from Raccoon to Gazelle, respectively, under control irrigation (T1). However, the sum of individual soluble sugars in both cultivars irrigated with waters of different salinity decreased as the salt concentration increased (Figure 4C). Higher levels of sugars were found in the low-salinity controls T1 (76.0 μg/mg) and T6 (97.3 μg/mg), suggesting that the increase in K level from 0.25 to 5 $mmol_c\ L^{-1}$ with the same low concentration of 30 $mmol_c\ L^{-1}$ NaCl resulted in a marginal increase in total sugar content in Raccoon but a decrease in Gazelle (~15%). These unexpected results can be investigated in a separate study. Previous work with the same genotypes under the same conditions of salinity and K concluded that shoot biomass was the lowest under the two highest salinity levels (90 and 120 $mmol_c\ L^{-1}$) for both genotypes when K was deficient and for Gazelle even when K was sufficient.[22] Rahneshan et al. investigated the effects of salinity stress on growth, physiological and biochemical parameters, and nutrients in two pistachio (*Pistacia vera* L.) rootstocks.[30] The authors reported that salinity stress significantly increased the level of soluble saccharides at high salinities in leaves and roots of the Badami-Rize-Zarand cultivar, while saccharide levels were significantly decreased at high salinities in leaves and roots.[57] Similarly, tolerant sunflower accessions had more soluble carbohydrates, soluble proteins, total free amino acids, and proline in the leaves than the sensitive accessions.[58]

Sugar profiles were similar in both genotypes, with results showing that there were no effects of potassium doses, but there was a generalized and steady decrease in sugar concentration of spinach shoots with increased salinity. In our study, a gradual increase in NaCl concentration from 30 to 120 $mmol_c\ L^{-1}$ (with 30 $mmol_c\ L^{-1}$ increments) resulted in a gradual decrease in the concentrations of total sugars in both Raccoon and Gazelle (T1–T5 and T6–T10). An approximately 60% reduction in total sugar concentration was observed when NaCl concentrations increased from 30 to 120 $mmol_c\ L^{-1}$. Sugar levels decreased under salt stress, which indicates the increase of glycolysis in shoots as observed before in barley grown under salt stress.[59] A similar consistent decrease in the level of soluble sugars with salinities of 0, 2.5, 5, and 10 g L^{-1} was also reported in two alfalfa varieties.[60] However, in two young olive cultivars, the total soluble sugars in leaves increased with an increase in salinity to 80 mM NaCl (equivalent to an EC_{iw} of 9.5 dS m^{-1}) but decreased with an additional increase in salinity.[31]

In conclusion, there was a moderate correlation between TPC and antioxidant capacity determined by colorimetric assays and the sum of areas of polyphenols identified by chromatographic methods. This may be attributed to varying the soluble sugar content and other antioxidants as mentioned above via interference in shoot samples of different salinity treatments. NIR spectral fingerprinting methods showed significant potential for rapidly classifying samples grown under varying salinity conditions. In general, the concentrations of free polyphenols, phenolic acids, soluble sugars, total phenolic content, and antioxidant capacity gradually decreased with increased salinity. This is the first detailed report on the variations in polyphenols, soluble sugar profiles, total phenolics, and antioxidant capacity determined in two salinity-tolerant spinach cultivars grown under the combined stresses of moderate to high salinity. These results are of significant interest to farmers, researchers, and nutritional professionals working on the use of vegetables produced with alternative irrigation waters as a sustainable strategy for increasing global food production while understanding the impact of growing conditions on crop bioactive nutrients. Spinach cultivars that are salt-tolerant can be used to maintain crop yield and provide adequate nutrition to humans who live in areas afflicted with the salinity of soils and water.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsfoodscitech.0c00034.

Figures S1–S4 (PDF)

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Funding

This work was supported by the Agricultural Research Service, U.S. Department of Agriculture (Project 1235-52000-066-00D).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Samina Shami and Renata Atkinson for their help in experimentation and Drs. Pei Chen and Geng Ping for helping us in acquiring high-resolution mass spectral data.

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ACS Food Sci. Technol. 2021, 1, 205−214