

Article

Organic Amendment for Disinfecting Soil Alters the Metabolites in *Spinacia oleracea*

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Abstract: Solar radiation and the incorporation of compost into soil is a practice for disinfecting soil that could have some other effects on spinach cultivation. The quality of spinach leaves after two types of soil disinfection, non-amended soil (NAS) and compost-amended soil (CAS), was compared in order to find biomarkers of both types of disinfection. These practices did not show significant differences in nutrient composition (N-P-K) compared to the control soil (CS). However, the amount of harmful nutrients such as NO_2^- was significantly lower in CAS ($65.74 \text{ mg kg}^{-1} \text{ FW}$) and NAS ($49.99 \text{ mg kg}^{-1} \text{ FW}$) than in CS ($114.39 \text{ mg kg}^{-1} \text{ FW}$). In addition, NO_3^- levels did not exceed the EU-recommended limit ($<3500 \text{ mg kg}^{-1} \text{ FW}$). Both types of disinfected soils produced higher concentrations of total phenols and more individual flavonoids in spinach leaves than the control. Combined chemometric analysis of the HRAMS data showed different clustering depending on the type of disinfection (NAS and CAS). Fifteen metabolite compounds could be identified, seven of which are beneficial for health and were more abundant in spinach grown in CAS compared to that grown in NAS or CS. Such beneficial metabolites measured with non-target analysis as markers of CAS-grown spinach. However, more studies are necessary in order to determine the differences between the metabolites of spinach grown in NAS or CAS.

Keywords: HRAMS; mineral nutrients; bioactive compounds; soil disinfection; compost; *Spinacia oleracea* L.



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1. Introduction

Solar radiation elevates soil temperatures through passive heat that can be used for controlling the pests and pathogens found in soil [1,2]. Previous studies have shown increased biological control against soil-borne pathogens in soil exposed to solar radiation (*Ralstonia solanacearum*, *Phytophthora* spp., *Pythium* spp., *Fusarium* spp., *Rhizoctonia solani*, *Sclerotinia* spp., *Sclerotium* spp., and *Verticillium dahlia*) [3,4]. Furthermore, soil exposed to solar radiation releases more soluble nutrients (inorganic N forms, extractable P and K, available cations, and dissolved organic matter), improving plant growth and yield [3] and increasing beneficial metabolites in crop leaves [3].

The efficiency of soil disinfection through solar radiation can sometimes be significantly improved by combining it with organic amendments, such as green and animal manures, crop residues, or composts. Adding compost as an organic amendment increases

soil microbial communities and microbial activity, improves soil exchange capacity, produces lower bulk density, and promotes populations of beneficial microorganisms; in addition, it increases plant growth and helps to achieve sustainable plant production [2,3].

Leafy green vegetables are part of a healthy diet and are rich in nutrients and phytochemicals that affect their taste and nutritional characteristics [5]. Baby leaf spinach is one of the healthiest vegetables due to the nutrients (vitamins and minerals) and health-promoting bioactive compounds (vitamins A, β -carotene, lutein, and zeaxanthin) it contains [6]. Spinach is abundant in potassium, calcium, magnesium, sodium, phosphorus, and iron. These nutrients and minerals are essential for ordinary biological functions. The most important phytochemicals in spinach are carotenoids: β -carotene [5,6]. However, spinach leaves can also accumulate high levels of non- or anti-nutrients, such as oxalates and nitrates, which can be considered harmful to animal and human health [7,8].

Bioactive compounds (poly(phenols)) are natural nutritional constituents in plants and food products that promote human health [9]. The total flavonoid content in spinach is influenced by its genotype, growth stage, and postharvest handling. Advanced breeding lines of spinach with enhanced disease resistance tend to have higher levels of individual and total flavonoids. Ref. [10] showed that spinach has a high concentration of natural antioxidants (carotenoids and (poly)phenols) and high antioxidant capacity. In addition, an increase in phenolic compounds in fennel leaves has also been shown after a disinfection process using vermicompost [11,12]. Antioxidants protect living cells against the harmful effects of free radicals and other reactive oxygen species. Some phytochemicals have an antioxidative action, for example, carotenoids, flavonoids, and other phenolics [13]. Previous studies have shown that spinach grown in soil treated with organic amendments showed higher leaf quality due to an increase in nutrients, antioxidant enzymes, photosynthetic pigments, and bioactive compounds (polyphenols, flavonoids, and carotenoids) [14–17]; this is due to the changes that organic amendments cause in the properties and structure of the soil [17].

Advanced technologies based on high-resolution accurate mass spectrometry (HRAMS) combined with analytical separation techniques (either gas (GC) and/or liquid chromatography (LC)) are particularly useful for screening thousands of unknown metabolites in a wide range of concentrations. It is no longer necessary to limit these compounds to a targeted series [7]. For this reason, this paper innovates using a non-target analysis on spinach leaves to elucidate biomarkers that distinguish between spinach leaves grown in soil disinfected by solar radiation or compost radiation that has not been previously conducted, accompanied by an analysis of the other most used representative parameters of plant quality.

Our hypothesis was that soil disinfection through solar radiation and compost (NAS and CAS) could improve free radical scavenging, nutrients, and bioactive compounds (polyphenols, carotenoids, and flavonoids) in spinach. We also hypothesized that new compounds that act as novel biomarkers of NAS and CAS could be identified through non-target analysis using HRAMS on baby leaf spinach. Therefore, our objectives were to show how the uptake of free radicals, nutrients, and bioactive compounds in spinach leaves improves after exposure to soil solar radiation and/or the addition of compost (NAS and CAS) and to identify the biomarkers in spinach leaves of both treatments (NAS and CAS) with respect to the non-disinfected soil using a very current and novel technique such as HRAMS.

2. Materials and Methods

2.1. Field Experiments and Sampling

The experiments were carried out from July 2020 to February 2021 in an agricultural field in Cartagena (37°46'27.2" N 1°01'22.7" W, SE Spain). The treatments were as follows: (a) non-amended soil (NAS), where the soil plot was covered with transparent, non-perforated polyethylene film (PE, 30 μ m thick, Serplasa® (Murcia, Spain) 120 g/m²); (b) compost-amended soil (CAS), where the soil plot was mixed with agro-food compost (described below) and covered with the above-mentioned transparent polyethylene cover;

and (c) control soil (CS), a soil plot with no treatment and no plastic cover. The three treatments covered a surface of 100 m², divided into random triplicate plots, with 2 m separation between each of them and the edges of the field to avoid border effects.

The agro-food compost, made with 72% vineyard pruning residues and 28% leek residues, had a pH of 8.93; EC 4.86 mS cm⁻¹; TOC 324 g kg⁻¹; TN 25.8 g kg⁻¹; P 3.8 g kg⁻¹; and K 20.9 g kg⁻¹. This compost was added to the soil at a proportion of 1.5 kg per m² using a rotovator.

In July 2020, the transparent polyethylene cover was arranged over the experimental plots and sealed with soil bags to avoid gas leaks. Once the treatments were incorporated, the soil was moistened to 60 ± 2% water-holding capacity with a drip irrigation system. In November 2020, the polyethylene covers were removed, and the plots were in the open air until December 2020, when the spinach seeds (*Spinacia oleracea* L., variety Nembus, Rijk zwaan Iberica S.A. (Almería, Spain)), were sown. The experiment finished when the plants were harvested (after 40 days of growth), in February 2021. During cultivation, the same amount of nutrients was added to all the plots using commercial fertilization (Inorganic NPK fertilizer 15-15-15 compound NPK fertilizer-Fertiberia). The crop was grown under commercial conditions depending on the requirements of the crop and the farmer. From each triplicate soil treatment, thirty mixed and homogenized spinach leaves of forty days' growth (8–12 cm) were cut, washed, and stored at −80 °C until they were analyzed.

2.2. Determining of Nutrients/Analytical Techniques Used for Spinach Characterisation

The spinach leaves were dried at 100 °C for mineral analysis. The total nitrogen (TN) was determined via dry combustion at 950 °C using an elemental analyzer (Truspec CN, Leco, St. Joseph, MI, USA). Total organic carbon (TOC), nitrates (NO₃⁻), nitrites (NO₂⁻), ammonium (NH₄⁺), oxalates (C₂O₄²⁻), sulfates (SO₄²⁻), phosphates (PO₄²⁻), sodium (Na⁺), potassium (K⁺), calcium (Ca²⁺), and magnesium (Mg²⁺) were extracted from nine samples per treatment. A total of 0.2 g of dry samples were extracted (the dry matter content was determined by drying 70 g of fresh matter in an oven at 60 °C to constant weight) with 50 mL distilled water and was subjected to continuous agitation in an orbital shaker (Stuart SSL1, Stone, Preston, UK) for 45 min at 110 rpm at 50 °C. Ion concentrations were determined via ion chromatography using a Metrosep A SUPP 5 column (Metrohm AG, Zofingen, Switzerland) at a flow rate of 0.7 mL min⁻¹ for anions and a Metrosep C 2-250 column at a flow rate of 1.0 mL min⁻¹ for cations, following the manufacturer's instructions [18].

2.3. Determination of Free Radical Scavenging and Natural Antioxidants

Antioxidant capacity and total contents of flavonoids, phenolics, carotenoids, and chlorophylls were determined using a methanolic extraction of 50 mg of freeze-dried leaves (Telstar Lyoquest 85 plus, Eco, Barcelona, Spain) in 1.5 mL of pure methanol. This was then vortexed and incubated overnight at 4 °C. After incubation, it was centrifuged at 10,000 rpm for 5 min at 4 °C. Antioxidant capacity was evaluated in terms of free radical-scavenging capacity [19], with the modifications described by [20]. The total flavonoids were determined as described by [21]. The total phenolic content was determined using the Folin–Ciocalteu colorimetric method [22]. The total contents of chlorophyll (Chla, Chlb) and carotenoids (Car) were measured at the 652, 665, and 470 nm wavelengths, respectively, in a UV–visible spectrophotometer (8453, Agilent, Santa Clara, CA, USA). The contents were calculated using equations developed by [23] and available elsewhere: Chl a = 16.72 × A₆₆₅ − 9.16 × A₆₅₂, chlorophyll b (Chl b = 34.09 × A₆₅₂ − 15.28 × A₆₆₅), Total Chl = Chl a + Chl b; and Car = (1000 × A₄₇₀ − 1.63 × Chl a − 104.96 × Chl b)/221. Both Chl and Car were expressed as mg kg⁻¹ of fresh weight (FW). Three replicates were obtained from each treatment, which were analyzed in triplicate (n = 9).

2.4. Analysis of Flavonoids

Freeze-dried leaf samples were extracted using MeOH:H₂O:CH₂O₂ (25:24:1 V:V:V) via 1 min vortex shaking, agitation in an orbital shaker for 30 min, and a US bath for 1 h. The samples were then kept at 4 °C overnight and again sonicated for 1 h. Finally, they were centrifuged at 10,000 rpm for 15 min. The supernatant was collected and filtered through 0.22 µm PVDF, 13 mm Ø membrane syringe filters into HPLC vials.

The high-performance liquid chromatography coupled with photodiode array detection and electrospray ionization/ion trap mass spectrometry (HPLC-DAD-ESI/MSⁿ) analyses were carried out in an Agilent HPLC 1200 (Agilent Technologies, Waldbronn, Germany) coupled with a mass detector in series. The characteristics of the HPLC system and the protocol to separate the compounds followed those established by [24]. The compounds were quantified by HPLC-DAD using an external standard curve of quercetin-3-rutinoside (Sigma Aldrich, Barcelona, Spain).

2.5. Non-Target Analysis Using High-Resolution Accurate Mass Spectrometry (HRAMS) Analysis and Data Processing

To evaluate the variations in the natural bioactive compound content of the spinach leaves grown in different soils (CS, NAS, and CAS), a non-targeted analysis was conducted. UHPLC Dionex™ Ultimate 3000 (Thermo Scientific™, San Jose, CA, USA) was used for LC separation. The protocol followed for this evaluation was that of [25].

Compound Discoverer™ software version 3.2 (Thermo Scientific) with the mzCloud and ChemSpider library search as the workflow to identify potential marker compounds was used. This tool detects these compounds based on an exact mass of precursor ions, with a mass tolerance of 5 ppm. To reduce chemical interferences from the matrix, a mass alignment tolerance of 5 ppm, an intensity tolerance of 30%, a total intensity threshold of 1×10^5 , a maximum shift of 0.5 min, and a signal-to-noise (S/N) threshold of 10 were set as filters.

In the second step, the data was manually processed to identify the chemical markers. They were separated by their retention time (t_R), accurate mass values for the precursor and fragment ions (m/z), elemental composition, and mass deviation (ppm) values. From the HRAMS, different peaks were detected for three diagnostic ions (one precursor ion and at least two fragment ions). A mass accuracy of $\leq \pm 5$ ppm was applied to tentatively determine their structure. Xcalibur 4.0, Mass Frontier 7.0, and TraceFinder 4.1 software (Thermo Scientific) were used to review the data and check the diagnostic ions obtained in the MS and tandem mass spectrometry (MS²) spectra. The data obtained on fragment ions contributed to a more robust identification. Mass Frontier 7.0 software was used to match each fragment obtained in the MS² mode by LC-Q-Orbitrap-MS in combination with the database.

Finally, principal component analysis (PCA) was used to differentiate the statistical significance of the three treatments after processing the HRAMS data for each set of samples.

2.6. Statistical Analysis

The data were analyzed using IBM Statistics SPSS® 23.0 software, and a one-way analysis of variance (ANOVA) test was performed for the parameters. When the F-statistic was significant, differences between the treatments were determined using Tukey's b test at a 95% confidence level. Before the ANOVA test, the normality and homogeneity of the variances were checked using the Shapiro–Wilk and Levene tests, respectively. The standard error of the mean values of each parameter was also determined. Principal component analysis (PCA) was performed using the Compound Discover™ software version 3.2.

3. Results

3.1. Mineral Elements in Spinach Leaves

The NO₃[−] and NO₂[−] contents of the spinach leaves showed the lowest value for NAS, followed by CAS, and, finally, for CS treatment (Table 1). The SO₄^{2−}, Na⁺, Ca²⁺, and Mg²⁺ contents also showed the lowest values for NAS, followed by CAS and, lastly, the CS

treatment (Table 1). For total N, NH_4^+ , PO_4^{3-} , and K^+ contents, no significant difference among the treatments were observed (Table 1). Likewise, no significant difference was found for oxalate content ($\text{C}_2\text{O}_4^{2-}$) (Table 1).

Table 1. Spinach leaves quality.

	CS	NAS	CAS	<i>p</i> -Value
Total N (g kg^{-1})	48.81 $\pm$ 0.08	48.21 $\pm$ 0.46	51.61 $\pm$ 3.09	NS
TOC (g kg^{-1})	331.47 $\pm$ 3.04	329.43 $\pm$ 0.32	329.90 $\pm$ 10.66	NS
NO_3^- (mg kg^{-1} FW)	228.35 $\pm$ 30.64 a	101.77 $\pm$ 8.90 c	161.22 $\pm$ 18.88 b	***
NO_2^- (mg kg^{-1} FW)	114.39 $\pm$ 15.61 a	49.99 $\pm$ 13.33 c	65.74 $\pm$ 3.29 b	***
NH_4^+ (mg kg^{-1} FW)	4.27 $\pm$ 0.1	3.99 $\pm$ 0.22	4.11 $\pm$ 0.42	NS
$\text{C}_2\text{O}_4^{2-}$ (mg kg^{-1} FW)	3328 $\pm$ 207	3331 $\pm$ 139	3494 $\pm$ 379	NS
SO_4^{2-} (mg kg^{-1} FW)	219 $\pm$ 25 a	134 $\pm$ 8 c	179 $\pm$ 20 b	***
PO_4^{2-} (mg kg^{-1} FW)	623 $\pm$ 34	567 $\pm$ 60	602 $\pm$ 82	NS
Na^+ (mg kg^{-1} FW)	332 $\pm$ 26 a	248 $\pm$ 49 b	220 $\pm$ 35 b	***
K^+ (mg kg^{-1} FW)	5114 $\pm$ 667	4524 $\pm$ 188	5102 $\pm$ 740	NS
Ca^{2+} (mg kg^{-1} FW)	121 $\pm$ 6 a	111 $\pm$ 13 ab	107 $\pm$ 13 b	*
Mg^{2+} (mg kg^{-1} FW)	494 $\pm$ 21 a	422 $\pm$ 24 b	441 $\pm$ 63 b	**

Values followed by different letters indicate significant differences at the corresponding *p*-value. *p*-value indicate the differences between treatments at * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001 and NS: non-significant. Values are the mean $\pm$ standard deviation (*n* = 9). CS: control soil; NAS: non-amended soil; CAS: compost-amended soil; TOC: total organic carbon; NO_3^- : nitrates; NO_2^- : nitrites; NH_4^+ : ammonium, $\text{C}_2\text{O}_4^{2-}$: oxalates, SO_4^{2-} : sulfates, PO_4^{2-} : phosphates, Na^+ : sodium, K^+ : potassium, Ca^{2+} : calcium, Mg^{2+} : magnesium. FW: fresh weight.

3.2. Antioxidant Capacity, Total Flavonoids, and Phenol Contents in Spinach Leaves

Table 2 shows the quality of the spinach leaves measured for their antioxidant capacity, in terms of total flavonoids, and total phenols. The antioxidant capacity and the content of total flavonoids were not significantly different in the spinach leaves of the different treatments. However, the total phenols in leaves from NAS and CAS treatments showed significantly higher contents (974.31 mg GA kg^{-1} FW and 932.47 mg GA kg^{-1} FW, respectively) than CS treatment (856.32 mg GA kg^{-1} FW) (Table 2).

Table 2. Free radical scavenging capacity and natural antioxidant tests in spinach leaves.

	CS	NAS	CAS	<i>p</i> -Value
Antioxidant capacity (mg DPPH Kg^{-1} FW)	51.21 $\pm$ 6.32	61.31 $\pm$ 4.57	58.12 $\pm$ 3.39	NS
Total Flavonoids (mg Rutin kg^{-1} FW)	4114 $\pm$ 97	4095 $\pm$ 195	3865 $\pm$ 128	NS
Total Phenols (mg GA kg^{-1} FW)	856 $\pm$ 53 b	974 $\pm$ 48 a	932 $\pm$ 30 ab	*
Carotenoids Total (mg kg^{-1} FW)	185 $\pm$ 9	185 $\pm$ 12	177 $\pm$ 3	NS
Chlorophyll a (mg kg^{-1} FW)	548 $\pm$ 18	557 $\pm$ 27	509 $\pm$ 7	NS
Chlorophyll b (mg kg^{-1} FW)	77.13 $\pm$ 2.65 b	89.83 $\pm$ 5.12 a	91.45 $\pm$ 7.74 a	*

Values followed by different letters indicate significant differences at the corresponding *p*-value. *p*-value indicate the differences between treatments at * *p* < 0.05 and NS: non-significant. Values are the mean $\pm$ standard deviation (*n* = 9). CS: control soil; NAS: non-amended soil; CAS: compost-amended soil. FW: fresh weight.

3.3. Total Contents of Carotenoids, Chlorophyll A, and Chlorophyll B in Spinach Leaves

Regarding photosynthetic pigments, the total contents of carotenoids, chlorophyll a, and chlorophyll b were analyzed (Table 2). The total contents of carotenoids and chlorophyll a were not significantly different among the treatments. However, chlorophyll b showed significantly higher values for NAS (89.83 mg kg^{-1} FW) and CAS (91.45 mg kg^{-1} FW) treatments than for the CS treatment (77.13 mg kg^{-1} FW) (Table 2).

3.4. Glycosylated Flavonoid Identification and Quantitation in Spinach Leaves

Eight target glycosylated flavonoids were identified using HPLC-DAD-ESI-MSn on the spinach leaves, including the glucuronides and acylated di- and tri-glycosides of 6-oxygenated flavonoid methylated and methylenedioxyderivatives (Table 3). The leaves had significantly higher flavonoid contents in NAS and CAS treatments compared to CS treatment (Table 3). The most abundant flavonoid found in the leaves was spinacetin glucuronide (0.887–1.065 mg g^{-1} DW). The second-most plentiful was spinacetin 3-O-

gentiobioside (0.530–0.646 mg g^{−1} DW), followed by 5,3',4'-trihydroxy-3-methoxy-6:7-methylenedioxyflavone-4'-glucuronide (0.476–0.580 mg g^{−1} DW), and patuletin 3-glucosyl-(1-6) [apiosyl (1-2)] glucoside (0.445–0.530 mg g^{−1} DW).

Table 3. The eight abundant/predominant flavonoid compounds mg g^{−1} (D.W.) on spinach leaves.

	CS	NAS	CAS	<i>p</i> -Value
Patuletin 3-glucosyl-(1-6)[apiosyl(1-2)]glucoside	0.445 ± 0.006 b	0.530 ± 0.016 a	0.489 ± 0.018 a	***
Patuletin 3-O-gentiobioside	0.214 ± 0.003 c	0.243 ± 0.008 a	0.223 ± 0.009 b	***
Spinacetin-3-glucosyl-(1-6)[apiosyl(1-2)-glucoside]	0.325 ± 0.006 b	0.394 ± 0.014 a	0.382 ± 0.015 a	***
Spinacetin 3-O-gentiobioside	0.530 ± 0.012 b	0.646 ± 0.022 a	0.612 ± 0.024 a	***
Spinacetin glucuronide (Spinatoside)	0.887 ± 0.064 b	1.065 ± 0.029 a	1.006 ± 0.043 a	**
Jaceidin glucuronide	0.281 ± 0.007 c	0.350 ± 0.012 a	0.324 ± 0.013 b	***
5,3',4'-trihydroxy-3-methoxy-6:7-methylenedioxyflavone-4'-glucuronide	0.476 ± 0.010 c	0.580 ± 0.017 a	0.526 ± 0.024 b	***
5,4'-dihydroxy-3,3'-dimethoxy-6:7-methylenedioxyflavone-4'-glucuronide	0.127 ± 0.011 c	0.172 ± 0.006 a	0.152 ± 0.007 b	***

Values followed by different letters indicate significant differences at the corresponding *p*-value. *p*-value indicate the differences between treatments at ** *p* < 0.01; *** *p* < 0.001 and NS: non-significant. Values are the mean ± standard deviation (n = 4). CS: control soil; NAS: non-amended soil; CAS: compost-amended soil. D.W.: dry weight.

3.5. Tentative Spinach Leaf Marker Identification Using HRAMS

A total of fifteen bioactive compounds were identified in the spinach leaves, using an extraction of 98% water and 2% methanol, on which an HRAMS analysis was performed. These bioactive compounds were of different types, including phenolic acids, amino acids and amine derivatives, vitamins, and others. They showed accurate mass measurements of the precursor ions at [M + H]⁺ *m/z* 165.0551, 318.2625, 166.0867, 204.1832, 220.1183, 205.0976, 288.1447, 154.0868, 270.0763, 289.1884, 285.0764, 201.2451, 243.2920, 243.2920, correlating to the exact theoretical mass of *m/z*, 164.0473, 317.2547, 165.0789, 203.1754, 219.1105, 204.0898, 287.1369, 153.0790, 269.0685, 288.1806, 284.0686, 200.2373, 242.2842, 228.2686, and 228.2686, respectively, with *m/z* deviations or mass errors below ±1 ppm. The possible empirical formulas proposed for the protonated molecules [M + H]⁺ are described in Table 4. Other molecules were also obtained at [M + H]⁺, but they were not identified.

Table 4. Natural food components tentatively identified as biomarkers in spinach leaves by HRAMS analysis.

<i>t_R</i>	Precursor Ion			Fragment Ions			Tentatively Identified Compounds ^b
	Accurate Mass Value (<i>m/z</i>)	Proposed Formula	Mass Deviation ^a (ppm)	Accurate Mass Value (<i>m/z</i>)	Proposed Formula	Mass Deviation ^a (ppm)	
1.7	164.0473	C ₉ H ₈ O ₃	−0.2	51.0229	C ₄ H ₃	−1.4	(E)-p-coumaric acid (Phenolic acid)
				63.0230	C ₅ H ₃	0.9	
				65.0386	C ₅ H ₅	0.4	
				77.0386	C ₆ H ₅	−0.2	
				91.0541	C ₇ H ₇	−1.4	
				95.0490	C ₆ H ₇ O	−1.5	
				103.0541	C ₈ H ₇	−1.4	
				107.0491	C ₇ H ₇ O	−0.7	
				109.0648	C ₇ H ₉ O	0.5	
				123.0440	C ₇ H ₇ O ₂	−0.4	
3.2	317.2547	C ₁₅ H ₃₃ N ₄ O ₃	0.2	147.0441	C ₉ H ₇ O ₂	0.1	2-Methyl-2-propanyl (2-aminoethyl) [[3-(diethylamino) propyl] carbamoyl] carbamate (Other compounds)
				58.0651	C ₃ H ₈ N	−0.2	
				74.0236	C ₂ H ₄ O ₂ N	−0.4	
				86.0963	C ₅ H ₁₂ N	−1.3	
				102.0549	C ₄ H ₈ O ₂ N	−0.6	
				113.0711	C ₅ H ₆ ON ₂	1.3	
				129.1022	C ₆ H ₁₃ ON ₂	0.4	
				144.1022	C ₇ H ₁₄ O ₂ N	1.8	
				189.1709	C ₉ H ₂₁ O ₂ N ₂	0.9	

Table 4. Cont.

t _R	Precursor Ion			Fragment Ions			Tentatively Identified Compounds ^b
	Accurate Mass Value (m/z)	Proposed Formula	Mass Deviation ^a (ppm)	Accurate Mass Value (m/z)	Proposed Formula	Mass Deviation ^a (ppm)	
3.3	165.0789	C ₉ H ₁₁ N O ₂	−0.3	51.0229	C ₄ H ₃	−0.7	L-Phenylalanine (Amino acid)
				65.0386	C ₅ H ₅	0.4	
				77.0385	C ₆ H ₅	−0.8	
				79.0543	C ₆ H ₇	0.5	
				91.0541	C ₇ H ₇	−1.1	
				95.0490	C ₆ H ₇ O	−1.9	
				102.0463	C ₈ H ₆	−1.1	
				103.0541	C ₈ H ₇	−1.2	
				120.0807	C ₈ H ₁₀ N	−0.7	
				131.0491	C ₉ H ₇ O	−0.3	
3.5	203.1754	C ₁₀ H ₂₃ N ₂ O ₂	−0.1	58.0651	C ₃ H ₈ N	−0.8	1,1-Dimethylethyl N-(4-aminobutyl)-N-methylcarbamate (Other compounds)
				74.0236	C ₂ H ₄ O ₂ N	−0.4	
				102.0548	C ₄ H ₈ O ₂ N	−1.5	
				144.1019	C ₇ H ₁₄ O ₂ N	−0.2	
				203.1753	C ₁₀ H ₂₃ O ₂ N ₂	−0.5	
3.7	219.1105	C ₉ H ₁₇ N O ₅	−0.4	56.0130	C ₂ H ₂ ON	−1.7	D-Pantothenic acid (Vitamin)
				59.0490	C ₃ H ₇ O	−2.3	
				70.0287	C ₃ H ₄ ON	−0.9	
				85.0647	C ₅ H ₉ O	−1.1	
				90.0547	C ₃ H ₈ O ₂ N	−2.3	
				95.0490	C ₆ H ₇ O	−1.3	
				98.0235	C ₄ H ₄ O ₂ N	−1.9	
				116.0338	C ₄ H ₆ O ₃ N	−3.3	
				142.0861	C ₇ H ₁₂ O ₂ N	−0.9	
				166.0862	C ₉ H ₁₂ O ₂ N	−0.6	
				184.0967	C ₉ H ₁₄ O ₃ N	−0.4	
3.9	204.0898	C ₁₁ H ₁₂ N ₂ O ₂	−0.2	74.0237	C ₆ H ₄ O ₂ N	0.5	DL-Tryptophan (Amino acid)
				91.0542	C ₇ H ₇	−0.4	
				102.0549	C ₄ H ₈ O ₂ N	−0.9	
				118.0651	C ₈ H ₈ N	−0.1	
				130.0652	C ₉ H ₈ N	0.8	
				159.0919	C ₁₀ H ₁₁ N ₂	1.2	
				170.0606	C ₁₁ H ₈ ON	3.1	
				188.0707	C ₁₁ H ₁₀ O ₂ N	0.3	
4.8	287.1369	C ₁₃ H ₂₁ N O ₆	0.3	69.0335	C ₄ H ₅ O	−0.2	BOC-D-GLU(OALL)-OH (Amino acid)
				85.0282	C ₄ H ₅ O ₂	−2.9	
				97.0283	C ₇ H ₇	−0.6	
				145.0499	C ₆ H ₉ O ₄	2.6	
4.9	153.0790	C ₈ H ₁₁ N O ₂	0.3	53.0386	C ₄ H ₅	−0.2	Dopamine (Other compounds)
				65.0384	C ₅ H ₅	−2.5	
				81.0699	C ₆ H ₉	0.9	
				91.0541	C ₇ H ₇	−0.9	
				108.0806	C ₇ H ₁₀ N	−1.2	
5.0	269.0685	C ₁₁ H ₁₆ N ₂ O	−0.2	79.0543	C ₆ H ₇	1.3	Tocainide (Other compounds)
				91.0540	C ₇ H ₇	−2.1	
				105.0698	C ₈ H ₉	−0.5	
				150.0909	C ₉ H ₁₂ ON	−3.2	
				176.1067	C ₁₁ H ₁₄ ON	−1.7	
8.1	288.1806	C ₁₄ H ₂₆ N O ₅	−0.9	55.0542	C ₄ H ₇	−0.8	(2S)-2-[(<i>Tert</i> -butoxycarbonyl)amino]-3-(4-hydroxycyclohexyl)propanoic acid (Other compounds)
				69.0700	C ₅ H ₉	2.1	
				72.0569	C ₄ H ₁₀ N	−0.1	
				86.0726	C ₅ H ₁₂ N	−3.8	
				118.0858	C ₅ H ₁₂ O ₂ N	−3.7	
				224.1646	C ₁₃ H ₂₂ O ₂ N	0.6	
				242.1751	C ₁₃ H ₂₄ O ₃ N	0.3	

Table 4. Cont.

t_R	Precursor Ion			Fragment Ions			Tentatively Identified Compounds ^b
	Accurate Mass Value (m/z)	Proposed Formula	Mass Deviation ^a (ppm)	Accurate Mass Value (m/z)	Proposed Formula	Mass Deviation ^a (ppm)	
10.4	284.0686	$C_{16}H_{12}O_5$	0.7	68.9970	C_3HO_2	−1.0	Olmelin (Phenolic acid)
				84.0206	$C_4H_4O_2$	0.2	
				112.0155	$C_5H_4O_3$	0.3	
				270.0527	$C_{15}H_{10}O_5$	1.7	
11.1	200.2373	$C_{13}H_{30}N$	−0.1	57.0698	C_4H_9	1.7	N-Methyldodecylamine (Other compounds)
				71.0856	C_5H_{11}	4.2	
				85.1013	C_6H_{13}	4.6	
12.0	242.2842	$C_{16}H_{36}N$	0.5	57.0698	C_4H_9	1.7	Bis(2-ethylhexyl) amine (Other compounds)
				71.0852	C_5H_{11}	−1.4	
12.1	228.2686	$C_{15}H_{34}N$	−0.1	57.0697	C_4H_9	0.1	Dodecyltrimethylammonium (Other compounds)
				71.0854	C_5H_{11}	1.4	
				95.0853	C_7H_{11}	0.1	
12.2	228.2686	$C_{15}H_{34}N$	−0.1	57.0697	C_4H_9	0.1	Pentadecan-8-amine (Other compounds)
				71.0854	C_5H_{11}	1.4	
				95.0853	C_7H_{11}	0.1	

t_R : retention time; ^a Values reported in the worst case; ^b Marker tentatively identified.

Figure 1 shows the areas of the peaks identified using HRAMS on spinach leaves for the three treatments, corresponding to the different metabolites identified. The metabolites were as follows: 2-Methyl-2-propenyl (2-aminoethyl) {[3-(diethylamino) propyl] carbamoyl} carbamate; 1,1-Dimethylethyl N-(4-aminobutyl)-N-methylcarbamate; (2S)-2-[(Tert-butoxycarbonyl) amino]-3-(4-hydroxycyclohexyl) propanoic acid; Olmelin; N-Methyldodecylamine; Dodecyltrimethylammonium; and Pentadecan-8-amine. The latter two showed larger areas in CAS treatment than in CS and NAS treatments. Only the area of Bis (2-ethylhexyl) amine was more extensive in CS treatment than in the disinfected ones (Figure 1). The rest of the metabolites L-Phenylalanine, DL-Tryptophan, D-Pantothenic acid, Dopamine, and Tocainide only showed significant differences with respect to CS.

A principal component analysis (PCA) model was created for spinach leaves (Figure 2) using HRAMS data. The first principal component (PC) accounting 76.1% of the observed variation, whereas PC2 allowed to explain an additional 20.8% of the total variation, accounting for a total 96.9%. In summary, the model showed efficient separation between CS, NAS, and CAS treatments.

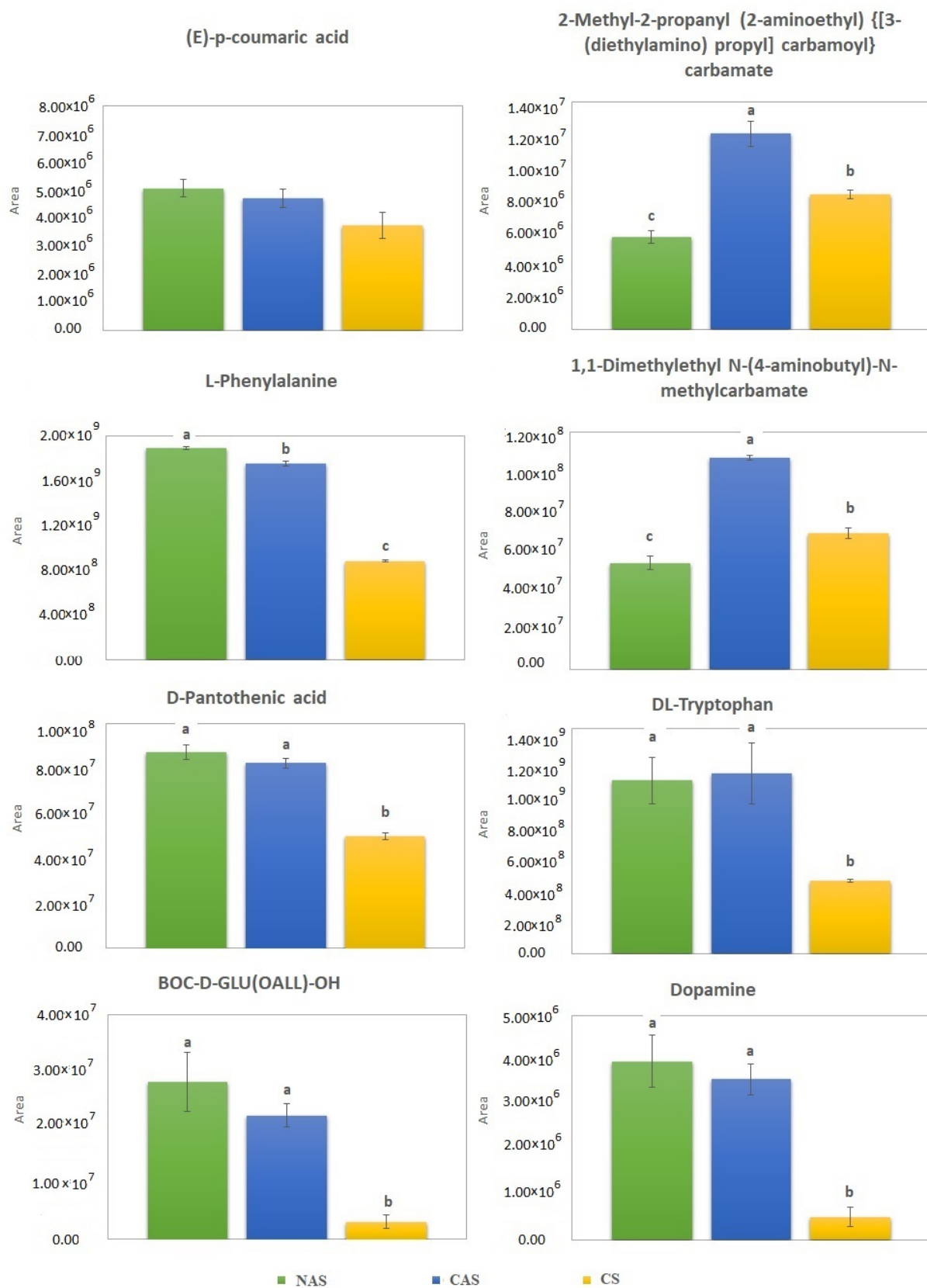


Figure 1. Cont.

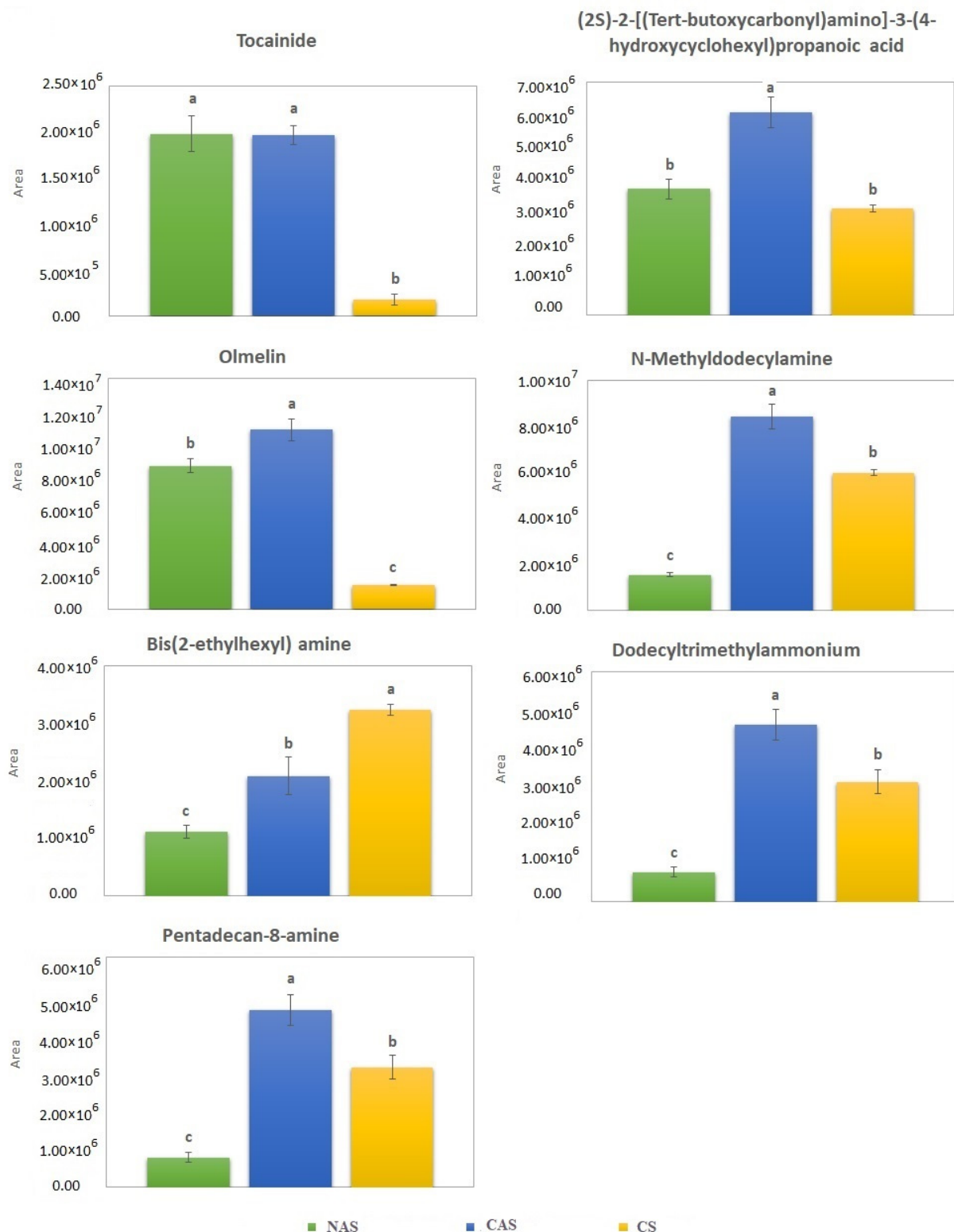


Figure 1. Area of the peaks identified by HRAMS on spinach leaves. Area measured as intensity in the Orbitrap mass analyzer. The bars represent the mean $\pm$ standard error, $n = 3$. A one-way ANOVA was performed, when there were significant differences ($p < 0.05$), a Tukey test was performed to show the significant differences. The different letters (a, b and c) show the significant differences between each treatment. The significantly highest value has a letter 'a', the second a 'b' and the third a 'c'. When there are no significant differences there aren't letters CS: control soil; NAS: non-amended soil; CAS: compost-amended soil.

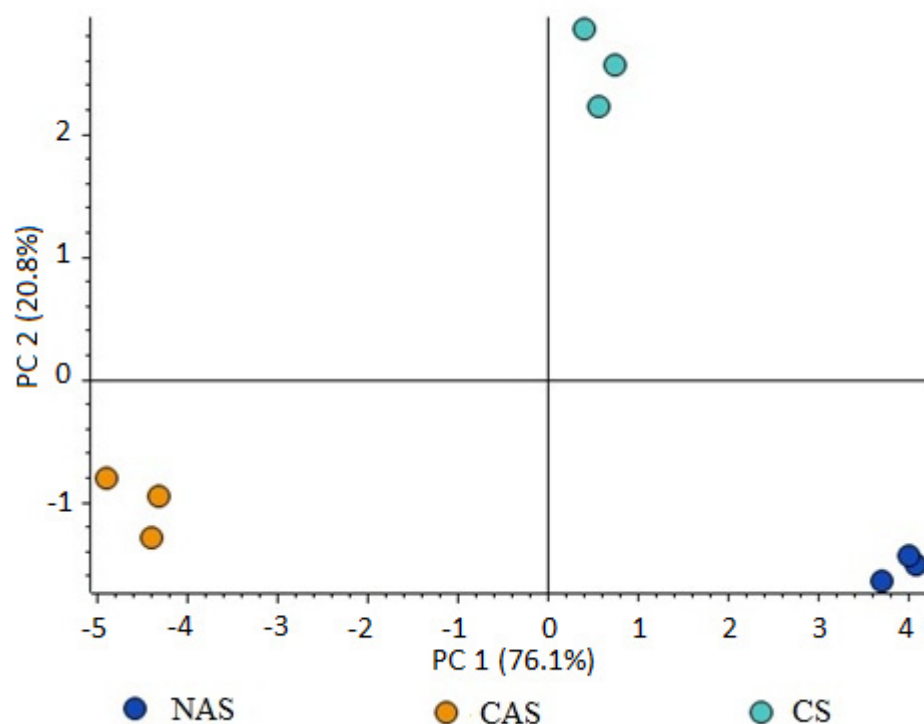


Figure 2. Principal coordinated analysis (PCA) of biomarkers identified by HRAMS data in spinach leaves. CS: control soil; NAS: non-amended soil; CAS: compost-amended soil. In parenthesis, the total explained variation for each principal component.

4. Discussion

In this study, targeted and untargeted analyses of spinach leaves have been used to explore the use of the soil disinfection techniques, through solar radiation and the application of agri-food compost (NAS and CAS) before spinach seeds were sown to boost the quality and metabolite profile of spinach leaves (*Spinacia oleracea* L., variety Nembus) and to find biomarkers of disinfection protocol.

NAS and CAS have been shown to induce qualitative and quantitative changes in the soil ecosystem, primarily by increasing the availability of certain soil nutrients [3]. This increases nutrient solubility, promotes faster plant growth, and results in higher yields due to weed and disease control. These disinfection techniques have also led to changes in the microbial composition of the soil [3]. Ref. [26] demonstrated that the combination of compost and radiation disinfection increased soil microbial activity, diversity, and functionality.

4.1. Effects of Radiation and Compost Disinfection on Spinach Quality

Minerals play a crucial role in versatile biological processes during the stages of plant growth and development. The values obtained were within the range of those found by other authors who has experimented on lettuce leaves [27]. The nitrate content in spinach leaves showed values under the legal EU limits ($<3500 \text{ mg kg}^{-1} \text{ FW}$). Spinach is prone to accumulate NO_3^- in degrees that vary depending on the cultivation system used [28]; the spinach leaves accumulated more nitrates when we added N to the soil in inorganic form than when N was incorporated in the form of an organic amendment [28]. Our values were very similar to those found by [29], who reported values below $400 \text{ mg kg}^{-1} \text{ FW}$ for organically cultivated Swiss chard. Solar radiation is a key factor in spinach cultivation, where high-intensity light favors the plant's metabolism by trapping nitrogen in nitrogenous organic compounds, such as amino acids, proteins, chlorophyll, etc., which reduces nitrate content. Although the nitrate content in all our samples was below EU limits, the lowest values were found in NAS and CAS treatments. Similarly, this is in line with previous studies [24,29], which demonstrated that a strawberry crop

accumulated a greater amount of nitrates in a conventional crop compared to an organic crop. This could be due to the potential organic mineralization caused by changes in the microbial community and the increased microbial activity due to higher temperatures during the process [2,26]. This produces nitrates and the nitrification processes are thus provoked by the inhibition of nitrifying bacteria. Spinach leaves grown in CAS showed higher nitrate content than those grown in NAS, probably due to the nitrogen content of the stable organic matter and the maturity of the compost added [29]. Similar behavior was observed in nitrite contents considered harmful to human health [30]. The range of nitrites in spinach leaves was low (49.99–114 mg kg⁻¹ FW). Nitrite exists as an intermediate metabolite in nitrate assimilation, and it showed similar behavior to nitrates in the different disinfection treatments. Nitrite can be found in leaves when plants are exposed to stressful conditions [30]; however, they do not accumulate it under favorable conditions. In terms of the other nutrients, no differences in N-P-K contents were observed among the different disinfection processes, but lower values of sodium, calcium, and magnesium were found in the disinfected treatments, probably caused by less nitrate in the root medium, since these parameters are positively related [31].

Despite its many health benefits, spinach is one of the foods highest in oxalate content [32]. Oxalates are considered harmful to health due to their potential to limit the bioavailability of essential nutrients such as calcium, magnesium, and zinc [33]. The values observed in the spinach leaves in our study ranged between 3328 and 3494 mg kg⁻¹ FW, similar to those found by [34] in spinach grown in soil amended with compost. These values can be considered low, according to [35]. The soil disinfection treatments did not affect the oxalate content in spinach leaves. It could be possible that exposure to NH₄⁺ in soil significantly reduces the accumulation of oxalates by inhibiting nitrate uptake [36].

4.2. Free Radical Scavenging Capacity and Natural Antioxidant Tests

Total carotenoids and chlorophyll a and b are pigments necessary for photosynthesis [29]. Furthermore, total carotenoids and chlorophyll a were not affected by the disinfection treatments, but there was higher chlorophyll b content in the NAS and CAS than in CS. This could be due to the bioactive compounds (amino acids, nucleic acids, phosphatides, alkaloids, enzymes, hormones, and vitamins) present in the disinfection treatments known to be integral parts of chlorophyll molecules [29].

The antioxidant capacity of spinach is caused by the large amounts of ascorbic acid and phenolic compounds it contains, especially its flavonoid content [36]. These flavonoids activate systemic resistance mechanisms [37]. The spinach leaves did not show high antioxidant capacity nor flavonoid content in the disinfection treatments, but higher total phenol content was observed; this was probably due to greater microbial activity from increased organic matter as temperatures rose [38]. Ref. [39] found a correlation between antioxidant capacity and total phenols in lettuce plants grown in compost and compost tea treatments, suggesting that phenolic compounds could be the major contributor to antioxidant capacity, thus activating or priming induced systemic resistance mechanisms. They concluded that the compost feedstock used were rich in compounds that activated an oxidative process in plants [40].

4.3. Glycosylated Flavonoid Identification in Spinach Leaves

Spinach is classified as a low-flavonoid vegetable [41]. No significant differences in total flavonoids were observed between the disinfection treatments (NAS and CAS) and CS treatment. The analysis of different flavonoid compounds showed compound profiles similar to those previously observed in spinach crops by [36,41,42]. As bioavailability and antioxidant activity differ among individual flavonoid compounds [43], a change in flavonoid composition may affect the flavonoid bioactivity and potential health benefits of the vegetable, even though the total flavonoid concentration is the same [41].

According to [42], Patuletin 3-glucosyl-(1-6) [apiosyl(1-2)]glucoside, Patuletin 3-O-gentiobioside, Spinacetin-3-glucosyl-(1-6)[apiosyl(1-2)]glucoside, Spinacetin glucuronide

(Spinatoside), Jaceidin glucuronide, 5,3',4'-trihydroxy-3-methoxy-6:7-methylenedioxyflavone-4'-glucuronide, and 5,4'-dihydroxy-3,3'-dimethoxy-6:7-methylenedioxyflavone-4'-glucuronide provided the leaves with resistance to diseases in the breeding program. In terms of the structure—activity relationship, the 5,3',4'-trihydroxy-3-methoxy-6:7-methylenedioxyflavone-4'-glucuronide, and patuletin glycosides all exhibited high antioxidant activity and contributed to the flavonoid compounds in 34% of the total flavonoid content among treatments. The spinacetin glycosides and spinatoside-4'-glucuronide showed comparably lower, but still relatively high, antioxidant activity, contributing to 53% [44].

4.4. Non-Target Metabolite Profiles in Spinach Leaves

Different studies on the metabolite profile of spinach leaves have found different metabolites depending on the application of N to the soil [45], processing after harvest [46], the application of glycine and nitrates to the soil [47], and increased concentrations of salt in the soil [48]. However, there are no studies on non-target metabolites in spinach leaves to differentiate spinach crops sown and grown in soils disinfected via solar radiation and/or compost amendment. In our study, several differences have been found as consequences of soil disinfection. Metabolite profiles varied among the soil disinfection types.

Compounds identified as Pentadecan-8 amine and Olmelin, overall, were significantly more abundant in CAS than in NAS and CS (Figure 1). Pentadecan-8 amine could be a marker of compost disinfection and is associated with antibiofilm activity and potentiation against pathogens like mycobacterium [49], while Olmelin, observed previously in spinach leaves, is considered beneficial to human health [50,51]. In general, the main differences were found between spinach grown in disinfected and non-disinfected soils. Urethanes [[[2-Methyl-2-propenyl (2-aminoethyl)] [3-(diethylamino) propyl]- carbamoyl carbamate] and [1,1-Dimethylethyl N-(4-aminobutyl)-N-methylcarbamate]] are biodegradable metabolites and promote antimicrobial and antifungal activity against pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus*, *Trichophyton mentagrophytes*, and *Candida albicans* [52–54]. Ref. [53] showed a higher abundance of urethanes in radiation and compost-amended soils than in non-disinfected soil. No differences were observed among treatments for the (E)-p-coumaric acid metabolite. However, the L-phenylalanine-tryptophan and dopamine metabolites synthesized by spinach leaves [55], which are considered beneficial for humans [56–59], showed a clear increase with NAS and CAS treatments.

5. Conclusions

The effects of NAS and CAS produced a reduction in the content of anti-nutritional compounds (NO_3^- , NO_2^-). Both disinfection treatments showed higher total phenol compounds and higher contents of some individual flavonoid compounds. However, these analyses did not find differences between NAS and CAS. Non-target analysis using HRAMS had sufficient resolution to identify compost priming in spinach leaves, such as pentadecan-8 amine and olmelin. The biocontrol activity of these metabolites is reported to be beneficial to human health, adding value to spinach [56–59]. Also, in spinach grown on both NAS and CAS, essential compounds for human nutrition, such as L-phenylalanine, tryptophan, and dopamine were synthesized by the leaves. However, more studies are needed to verify whether these metabolites are markers of CAS and to improve spinach quality.

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