Article

UPLC-HRMS Polyphenolic Characterization, Contents and Antioxidant Activity of *Zingiber officinale* Roscoe rhizomes from Costa Rica

Mirtha Navarro-Hoyos [1]*, Mónica Acuña-Quirós [1], María Isabel Quirós-Fallas [1], Felipe Vargas-Huertas [1], Krissia Wilhelm-Romero [1], Felipe Vásquez-Castro [1], Diego Alvarado-Corella [1] and Andrés Sánchez-Kopper [2]

[1] Bioactivity & Sustainable Development (BIODESS) Group, Department of Chemistry, University of Costa Rica (UCR), San Jose 2060, Costa Rica; mnavarro@codeti.org (M.N.-H.), monica.acunaquiros@ucr.ac.cr (M.A.-Q.), maria.quirosfallas@ucr.ac.cr (M.I.Q.-F.), luis.vargashuertas@ucr.ac.cr (F.V.-H.), krissia.wilhelm@ucr.ac.cr (K.W.-R.); felipe.vasquezcastro@ucr.ac.cr (F.V.-C), luis.alvaradocorella@ucr.ac.cr (D.A.-C.)

[2] CEQIATEC, Department of Chemistry, Costa Rica Institute of Technology (TEC), Cartago 7050, Costa Rica; ansanchez@itcr.ac.cr (A.S.-K.)

* Correspondence: mnavarro@codeti.org; Tel.: +506-8873-5539

Received: 15 March 2022; Accepted: xx March 2022; Published: xx March 2022

Abstract

Zingiber offcinale Roscoe rhizomes have been associated with multiple health benefits, such as blocking blood clotting, digestive and antinausea effects and aid in respiratory conditions. These effects have been linked with their polyphenolic main metabolites, gingerols and shogaols. Herewith, we report a detailed study on the polyphenolic profile and in the contents of main gingerols and shogaol as well as the antioxidant activity of extracts from *Z. offcinale* rhizomes (n = 17) produced in Costa Rica. Using UPLC-QTOF-ESI MS, a total of 34 polyphenols were identified, grouped in twelve types of structures. In addition, our findings on the main metabolites using UPLC-DAD show all rhizomes complying with total gingerols (TG) content established by the United States Pharmacopeia (USP). At individual level, samples SR-1 and NR-4 show the higher contents and also exhibit the highest Folin-Ciocalteu (FC) reducing capacity results as well as the best DPPH antioxidant values. In addition, Pearson correlation analysis results showed positive correlation ($p < 0.05$) between TG and 6-gingerol with FC results and negative correlation ($p < 0.05$) between 6-gingerol, TG and FC with DPPH results. In turn, Principal Component Analysis (PCA) indicated variability in the composition associated with their region of origin and confirmed that NR-3, NR-4, and especially SR-1 stand out significatively, showing the highest PC1 because of its particularly high TG, 6-gingerol and antioxidant activities. Finally, results from controlled experimental plots of *Z. officinale* rhizomes (n = 6) indicated influence on metabolites content with higher values for shorter harvest time and high tunnel cultivation. Therefore, our findings indicate the value of *Z. officinale* in the elaboration of products with potential benefits for health, delivering extracts with higher levels of gingerols than previous reports and exhibiting high antioxidant activity.

Keywords: *Zingiber officinale***;** gingerols, shogaols, polyphenols**;** medicinal herbs**;** UPLC; QTOF-ESI MS

1. Introduction

The use of herbs and shrubs to manage health problems goes back to pre-history and within this ancient knowledge, plants extracts have gained much attention in scientists from all disciplines due to their traditional uses in different health conditions. Among them, *Zingiber officinale* Roscoe rhizomes have been extensively used as food ingredient because of its strong and unique flavor. This species originated in Southeast Asia mainly from India but at present is well distributed in East Asia, Australia and Africa as well as in America and its use in traditional medicine is well documented in Sanskrit, Chinese, Greek, Arabic, and Roman ethnomedicine [1].

Ginger has been used to block excessive clotting of blood in arteries and veins, to reduce cholesterol, and to fight against arthritis, high blood pressure, infections, lung diseases, cold, cough and as pain killer [2-5]. It was also used as a digestive aid and antinausea remedy, to treat bleeding disorders, rheumatism, baldness, toothache, snakebite, and respiratory conditions [6].

Most of the above-mentioned health complications have some of their foundation on the molecular oxidant status. This so-called oxidative stress due to free radicals in the organism has demonstrated to play a relevant role in human diseases, linked to oxidant stress and inflammatory processes contribute to aging and age-related diseases [7] independently from various risk factors (i.e. age, race, diet, environment and behavior). This is why the use of antioxidants and among them, polyphenols in pharmacology are intensively studied [8]. The proper intake of antioxidant will help eliminating free radicals in the body, improving the health by lowering the risk of various degenerative and infectious diseases.

Research indicates that numerous compounds are present in ginger as shown by chemical analyses, for instance terpenes and polyphenols including phenolic acids [9] [10]. From this important diversity of compounds, gingerols and shogaols are the most prominent [11][12], being these last compounds the result of gingerols dehydration [13]. Both polyphenol types are responsible for the broad bioactivity described [14-16]

The worldwide spread use of this ancient herb as spice and within the natural medicine market has made it one of the most relevant in terms of economic trade, being cultivated in more than 35 countries around the world. In 2019, the global production of ginger was over four thousand tons with India as the top producing country accounting for 43.81% of total global production and Nigeria second, accounting for 16.94% [17]. This trade potential drove producers to establish ginger cultivation in Costa Rica to supply local market and to export to Europe, however no sufficient scientific evidence exists on the contents and therefore on the quality and bioactive potential of their local products. The application of HPLC spectroscopy has shown to be a reliable method to utilize the metabolic profiling of ginger samples and to further discriminate their quality and determine the influence of different factors as geographical origin, harvest time, drying and processing techniques [18], which in turn change the bioactive potential as antioxidant [19].

Hence, our work objective consisted in obtaining extracts of *Z. officinale* Roscoe rhizomes cultivated in different parts of Costa Rica and under different growth and harvesting conditions (n=17) to characterize their polyphenols through UPLC-QTOF-ESI MS and to assess the main gingerols and shogaols contents using UPLC-DAD. In addition, our work aimed to determine their antioxidant activity through FC and DPPH methods and to apply correlation studies and Principal Component Analysis (PCA) to the data obtained. Finally, to the best of our knowledge, this is the first detailed study on *Z. officinale* Roscoe from Central America.

2. Materials and Methods

2.1. Zingiber officinale Roscoe rhizomes, Chemicals, and Reagents

Rhizomes from *Zingiber officinale* Roscoe (var. Grand Cayman) were acquired in ripe state from producers from different regions in Costa Rica, namely five in the Northern region (NR-1, NR-2, NR-3, NR-4, NR-5), three from the Northwestern region (NW-1, NW-2, NW-3), three from the Western region (WR-1, WR-2, WR-3) and one from the Southern region (SR-1). Five additional samples grown under controlled conditions in an experimental plot were acquired from NR-5 producer, namely NR-6, NR-7, NR-8 (var. Grand Cayman) and NR-9, NR-10 (var. Hawaiian). Solvents of ACS or HPLC grade such as acetonitrile, methanol and ethanol were purchased from Baker (Center Valley, PA, USA). Reagents such as capsaicin, sodium molibdate, gallic acid, 2,2-diphenyl-1-picrylhidrazyl (DPPH), and sodium tungstate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Extraction of Phenolic Compounds from Z. officinale Roscoe rhizomes.

Z. officinale Roscoe rhizomes were rinsed with water, cut in thin slices and dried in a stove at 40°C until constant weight. The dried material was grinded and preserved in closed containers protected from light. To determine the best conditions for extraction of *Z. officinale* samples, a Factorial Design (FD) was performed on 1 g of dried material using two solvents (methanol and ethanol), two different solvent volumes (25 and 50 mL) and 24 hours extraction time with two different stirring periods, namely the first 8 hours or continuous during the 24 hours. The sequence of eight experiments is summarized in Table 1 (section Results and Discussion). After the extraction, the samples were filtrated and adjusted to a volume of 50 mL using the respective extraction solvent. The efficiency of the extractions was determined using UPLC-DAD (UltiMate U3000, Thermo Scientific, USA) for the quantification of main gingerols according to the United States Pharmacopeia (USP) chromatographic method [20]. Briefly, quantification for these compounds was carried out against the calibration curve of capsaicin standard, using a Synergi 4u Polar-RP80A (150 x 4.6 mm x 4 µm (Phenomenex Inc., CA, USA) at 30°C. Elution was performed with solvent A, water, solvent B, methanol, and solvent C acetonitrile, and was applied as isocratic elution program with 44% A, 1% B and 55% C. The DAD was operating at 250–420 nm. The limit of detection (LOD) and the limit of quantification (LOQ) were 3 and 8 ppm respectively. Once the optimal conditions were established, 1 g of *Z. offcinale* dried samples (n = 17) were extracted using 50 mL of methanol, for 24 hours, under stirring for the first 8 hours. Afterwards, the samples were filtrated and adjusted to a volume of 50 mL. The extracts were analyzed under the UPLC-DAD conditions just described and quantification was achieved for five gingerols (6-gingerol, 8-gingerol, 10-gingerol, 6-gingerdiol and 6-dihydrogingerdione) and 6-shogaol.

2.3. Analysis of Z. officinale extracts by UPLC-DAD and UPLC-ESI-MS

A Thermo PDA eλ photodiode array detector (DAD) coupled with a Thermo UltiMate U3000 UPLC system was used for quantification of five gingerols contents, namely 6-gingerol, 8-gingerol, 10-gingerol, 6-gingerdiol and 6-dihydrogingerdione, and 6-shogaol in the different extracts (n = 17) under the conditions and the calibration curve described in section 2.2. On the other hand, to characterize *Z. officinale* polyphenols, measurements were performed with a method developed in our laboratory, using a Xevo G2-XS QTOF (Waters, UK) coupled with an AQUITY H Class UPLC system with a quaternary pump. ESI source parameters were set to a capillary voltage of 2 kV, sampling cone of 20 eV, source temperature 150 °C, source offset 10 °C, desolvation temperature of 450 °C, cone gas flow 0 L/h, and desolvation gas flow 900 L/h. The measure was performed in MSe high-resolution negative mode using an acquisition mass range from 100 m/z to 1000 m/z and a scan rate of 0.5 s, where fragmentation was carried out using Independent Data Acquisition for all eluting compounds with collision energy ramp from 20 V to 30 V storing at the high energy function. Instrument calibration was performed in the mass range of the measurement with sodium formate. Lock mass correction was applied directly to the

measurement using leucine enkephalin infusion measured each 30 s during the run. The data was analyzed using MassLynx V4.2 software from Waters. 1 µL of sample was injected with a flow of 0.5 mL/min using a using a Synergi 4u Polar-RP80A (150 x 4.6 mm x 4 µm (Phenomenex Inc., CA, USA) at 30°C using a chromatographic gradient starting at 40% B increasing to 100% B at 30 minutes, the gradient was held for 7 minutes and then the column was equilibrated for 5 minutes to initial conditions. Solvents used in the mobile phase were A water with 0.1% formic acid and B acetonitrile with 0.1% formic acid.

2.4. Folin-Ciocalteu Determination

Folin-Ciocalteu assay was performed through a modified Singleton and Rossi method, using the Folin–Ciocalteu (FC) reagent, composed of phosphomolybdic and phosphotungstic acids. As previously described [21], the method consists of mixing 0.5 mL of FC reagent and 10 mL of Na_2CO_3 (7.5%) with 0.5 mL of the respective *Z. officinale* extract, previously dissolved in acidified MeOH (0.1% HCl) to assure extract dissolution regardless of the solvent used for plant material extraction. Volume was completed to 25 mL with water. A blank was prepared following the same procedure but using 0.5 mL of MeOH (0.1% HCl) instead of the extract. Both blank and extract mixtures were kept in the dark for 1 h at room temperature, and subsequently absorbance was measured at 750 nm. Absorbance values were extrapolated in a gallic acid calibration curve to obtain FC reducing capacity results, expressed as mg gallic acid equivalents (GAE)/g of extract. Analyses for each sample were performed in triplicate.

2.5. DPPH Radical-Scavenging Activity

DPPH evaluation was performed following a previously reported method [22]. Briefly, a 0.25 mM solution of 2,2-diphenyl-1-picrylhidrazyl (DPPH) reagent was prepared using methanol as solvent. Next, 0.5 mL of the DPPH solution were mixed with 1 mL of *Z. officinale* extract at different concentrations, and resultant solutions were incubated for 30 min at 25° C in the dark. Blanks were prepared for each concentration and DPPH absorbance was measured at 517 nm. The inhibition percentage was determined as shown in the following equation:

$$Inhibition\ percentage\ (\%) = \frac{\left(Abs_{blank} - Abs_{sample}\right)}{Abs_{blank}} * 100$$

The percentage of the radical-scavenging activity of the sample was plotted against its concentration to calculate IC_{50}, which corresponds to the amount of sample necessary to reach the 50% radical-scavenging activity. Each sample was analyzed in three independent assays.

2.6. Statistical Analysis

In order to determine if the total gingerols (TG) content and individual 6-gingerol, 8-gingerol, 10-gingerol, 6-gingerdiol, 6-dihydrogingerdione, and 6-shogaol contents measured by UPLC-DAD have an influence in the FC reducing capacity and DPPH antioxidant activity, Pearson correlation analyses were carried out between the above-mentioned variables and FC and DPPH values. One-way analysis of variance (ANOVA) with Tukey post hoc as statistical tests were applied to TG, 6-gingerol, 8-gingerol, 10-gingerol, 6-gingerdiol and 6-dihydrogingerdione, 6-shogaol, FC and DPPH evaluations to determine significant difference ($p < 0.05$) between samples. Statistical analysis assigned superscript letter "a" to the highest value and subsequent letters or combination of letters were assigned to decreasing values. Two-way ANOVA were applied to the individual polyphenols quantification, FC and DPPH results for the experiments (n=6) comparing varieties, cultivation mode and harvesting maturity. Principal

Component Analysis (PCA) was applied to summarize the data from *Z. officinale* extracts taking into consideration all nine variables. R (version 1.2.1335) was used as the statistical program.

3. Results and Discussion

3.1. Extraction from Z. officinale Rosc. Rhizomes

Extraction results of the main compounds from *Z. officinale* rhizomes (Grand Cayman var.) grown open field and harvested in ripe state at 40 weeks in different parts of Costa Rica (n = 12), were obtained through the process described in the respective Materials and Methods section. The efficiency of the extraction was evaluated through UPLC-DAD quantification in sample NR-1 of the main gingerols, namely 6-gingerol, 8-gingerol and 10-gingerol in order to obtain the Total Gingerols (TG) contents. The factorial design was performed using solvent, solvent:material ratio and stirring time as factors, considering previous reports [23] and the United States Pharmacopeia (USP) monograph on *Z. officinale* [20]. Factors levels included solvents ethanol and methanol, two different solvent volumes accounting for 25:1 and 50:1 solvent:substrate ratios, and 24 hours extraction time with two different stirring periods, namely during the first 8 hours or continuous during 24 hours. The results are summarized in Table 1.

Table 1. UPLC-DAD quantification of 6-gingerol, 8-gingerol and 10-gingerol in *Z. officinale* sample NR-1 under various solvents, extraction volumes and stirring time conditions.

Experiment	Solvent	Volume (mL)	Stirring time (hours)	TG [1, 2, 3]
1	Ethanol	25	8	19.38 [b] ± 1.1
2	Methanol	50	24	27.79 [ab] ± 2.0
3	Methanol	50	8	30.40 [a] ± 2.4
4	Ethanol	25	24	19.50 [b] ± 0.9
5	Ethanol	50	24	26.01 [ab] ± 2.3
6	Ethanol	50	8	20.16 [b] ± 1.5
7	Methanol	25	24	21.20 [ab] ± 1.3
8	Methanol	25	8	21.93 [ab] ± 1.4

[1] TG: Total Gingerol contents are expressed as mg/g dry material [2] TG values are expressed as mean ± standard deviation. [3] Different superscript letters indicate differences are significant at $p < 0.05$ using one-way analysis of variance (ANOVA) with a Tukey post hoc as statistical test.

Statistical analysis of the results did not show a significant effect ($p < 0.05$) for any variable, 2-way or 3-way interactions. However, by excluding the stirring factor showing the least effect, the FD findings indicated a significant difference ($p < 0.05$) for the solvent and the volume factors, with methanol and 50:1 solvent: substrate ratio yielding better results. In addition, the analysis indicated the highest fitted mean associated with the combination of these two conditions and 8 hours stirring time. These results indicating methanol as an adequate solvent align with previous reports [23] while the solvent:sample ratio of 50:1 and the 8 hours stirring time during the 24 hours total extraction time represent USP settings [20]. Therefore, considering the results, these conditions were chosen to perform the extraction of *Z. officinale* Roscoe samples ($n = 17$).

3.2 Polyphenolic Profile by UPLC-ESI-MS Analysis

The UPLC-ESI-MS analysis described in the Materials and Methods section, allowed identifying 34 ploy phenolic compounds, including four paradols, five gingerols, two methyl-gingerols, three shogaols, three gingerdiols, three dihydrogingerdiones, three acetoxygingerols, one methylacetoxygingerol, one acetoxygingerdiol, one methylacetoxygingerdiol, four diacetoxygingerdiols, and four methyldiacetoxygingerdiols. Table S-1 summarizes the analysis results for these compounds and Figure 1 shows the chromatograms for the 34 secondary metabolites.

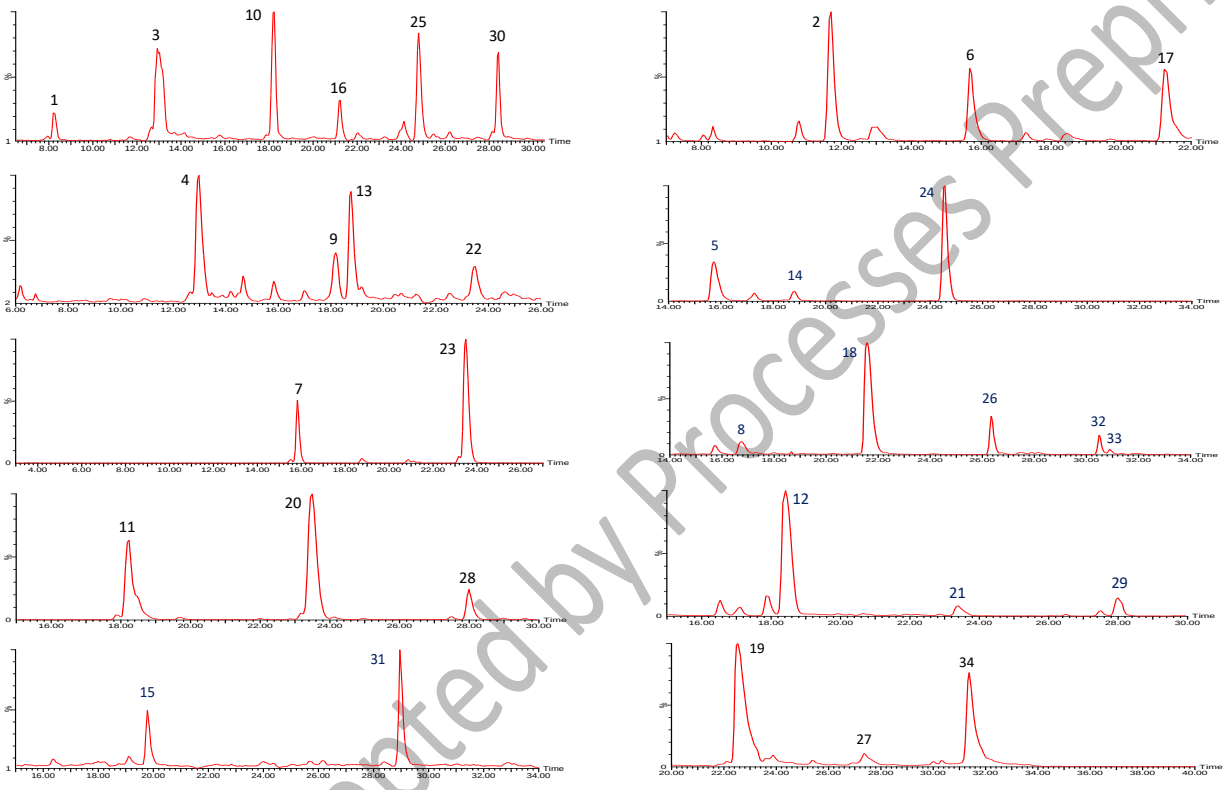

Figure 1. UHPLC QTOF-ESI MS Extracted ion chromatograms of phenolic compounds from *Z. offcinale* rhizomes

The compounds identified had in common a 1, 3, 4-trisubstituted benzene ring consisting of a 3-methoxy group, a 4-hydroxyl or 4-methoxy group, and an unbranched alkyl chain. Most compounds in this study present a homologous series that leads to an increase in hydrophobicity due to greater alkyl chain length, which further supports the observed higher retention times [11].

The first group is constituted by paradols, a series of compounds with a ketone at C-3 of the alkyl chain, as shown in Figure 2. In addition, all four compounds demonstrated consistent fragmentation behavior according to benzylic cleavage with a product ion of m/z 137 (Figure 2) [24]. Peak 4 (Rt = 12.98 min) correspond to 6-Paradol, providing a sodium adduct $[M+Na]^+$ at m/z 301.1882 with a molecular formula $C_{17}H_{26}O_3Na$, and a pseudomolecular ion $[M+H]^+$ at m/z 279.1882 ($C_{17}H_{27}O_3$). Peaks 9 (Rt = 18.19 min), 13 (Rt = 18.77 min) and 22 (Rt = 23.48 min) are tentatively assigned to 8-Paradol, 7-Paradol and 10-Paradol with their respective pseudomolecular ion $[M+H]^+$ at 307.2195 ($C_{19}H_{31}O_3$), 293.2095 ($C_{18}H_{29}O_3$) and 335.2509 ($C_{21}H_{35}O_3$), and presenting $[M+Na]^+$ adducts at m/z 329.2185, 315.2032 and 357.2505, respectively.

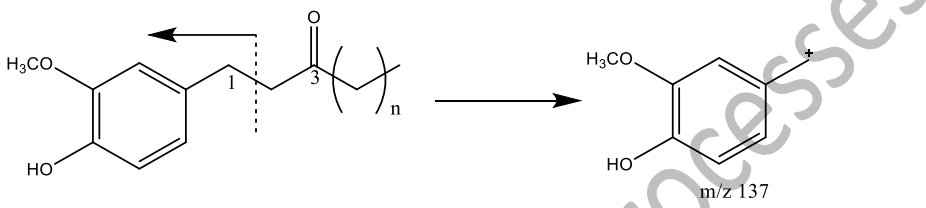

Figure 2. Fragmentation pathway for paradol compounds 4 (n = 6), 9 (n = 7), 13 (n = 8), 22 (n = 10)

The second group correspond to gingerols, which are ginger most known pungent compounds [25], and includes peaks 1 (Rt = 8.20 min), 3 (Rt = 12.93 min), 10 (Rt = 18.23 min), 23 (Rt = 23.52 min) and 30 (Rt = 28.43 min). Peak 1, with a $[M+Na]^+$ ion at m/z 289.1512 ($C_{15}H_{22}O_4Na$), was tentatively assigned to 4-gingerol. In turn, peak 3 had $[M+Na]^+$ at m/z 317.1831 ($C_{17}H_{26}O_4Na$) and was assigned to 6-gingerol, while peak 10 with $[M+Na]^+$ at m/z 345.2137 ($C_{19}H_{30}O_4Na$) was identified as 8-gingerol. Meanwhile, peak 23 had $[M+Na]^+$ at m/z 373.2438 ($C_{21}H_{34}O_4Na$) and was assigned to 10-gingerol and finally, peak 30 with $[M+Na]^+$ at m/z 401.2653 ($C_{23}H_{38}O_4Na$) was identified as 12-gingerol. All these peaks had an adduct corresponding to $[M+H-H_2O]^+$ at m/z 249.1518, 277.1831, 305.2136, 333.2457 and 361.2740, respectively.

The fragment at m/z 179 observed in gingerols (Figure 3) is produced following the path of fragmentation from dehydrated pseudomolecular ions $[M+H-H_2O]^+$ [12]. This ion is decomposed into fragments at m/z 177, 145 and 117 by successive losses of 2 H^+ (2 Da), CH_3OH (32 Da) and CO (28 Da), respectively. In turn, the formation of the fragment at m/z 177 could derive directly from the dehydrated pseudomolecular ions $[M+H-H_2O]^+$ by the loss of CH_3-$(CH_2)_n$-CHO (n = 2, 4, 6, 8 and 10 for 4-gingerol 6-gingerol, 8-gingerol, 10-gingerol and 12-gingerol, respectively) [11]. This ion, in turn, gives rise to the fragment at m/z 137 due to the loss of lateral chain $CHCHCH_2$ (40 Da).

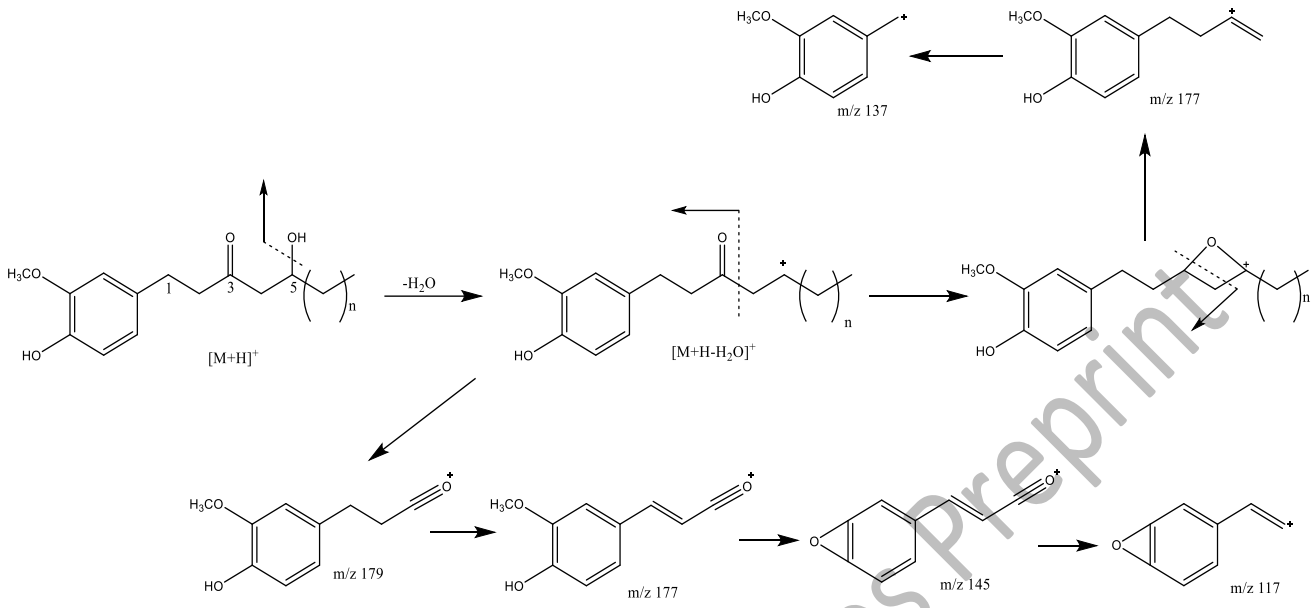

Figure 3. Fragmentation pathway for gingerol compounds 1 (n = 4), 3 (n = 6), 10 (n = 8), 23 (n = 10), 30 (n = 12)

Peaks 7 (Rt = 15.82 min) and 25 (Rt = 24.76 min) correspond to methyl-6-gingerol and methyl-8-gingerol, respectively, based on the adduct $[M+Na]^+$ at m/z 331.1985 ($C_{18}H_{28}O_4Na$) and 359.2364 ($C_{20}H_{32}O_4Na$), and the presence of the dehydrated form $[M+H-H_2O]^+$ at m/z 291.1988 and 319.2360. The MS2 spectrum of methyl gingerols reveals a fragmentation pattern similar to gingerols with fragments at m/z 191 and 151 (Figure 4) [11].

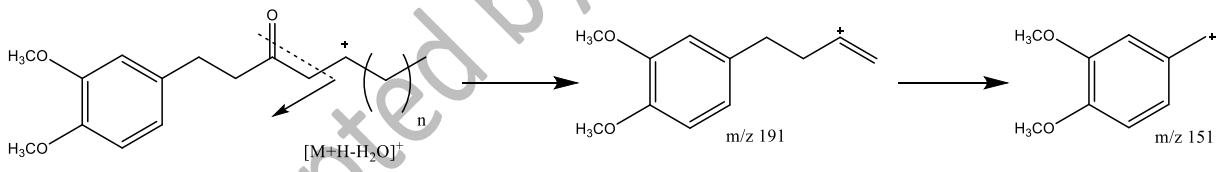

Figure 4. Fragmentation pathway for methylgingerol compounds 7 (n = 6), 25 (n = 8)

The third group is composed by shogaols, compounds that correspond to the dehydrated form of gingerols, conversion that is mainly produced at higher temperature [25]. Among shogaols, peaks 11 (Rt = 18.27 min), 20 (Rt = 23.34 min) and 28 (Rt = 27.94 min) present pseudomolecular ions $[M+H]^+$ at m/z 277.1795 ($C_{17}H_{25}O_3$), 305.2117 ($C_{19}H_{29}O_3$) and 333.2430 ($C_{21}H_{33}O_3$) and were tentatively assigned to 6-shogaol, 8-shogaol and 10 shogaol, respectively. The breaking of the benzyl bond produces the fragment seen at m/z 137 (Figure 5) [24].

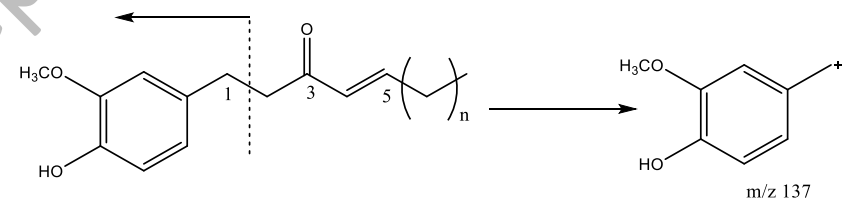

Figure 5. Fragmentation pathway for shogaol compounds 11 (n = 6), 20 (n = 8), 28 (n = 10).

Another group of compounds are gingerdiols, the ketone reduction products of gingerols [11]. Gingerdiols present two hydroxyl groups (−OH groups) which dehydrate and provide adducts $[M+H-2(H_2O)]^+$ and $[M+H-H_2O]^+$, the first one being the predominant adduct for peaks 6 (Rt = 15.72 min) and 17 (Rt = 21.23 min) at m/z 289.2173 ($C_{19}H_{29}O_2$) and 317.2378 ($C_{21}H_{33}O_2$), respectively, corresponding to 8-gingerdiol ($C_{19}H_{32}O_4$) and 10-gingerdiol ($C_{21}H_{36}O_4$). These peaks present likewise the adducts $[M+H-H_2O]^+$ at m/z 307.2273 and 335.2504, and $[M+Na]^+$ at m/z 347.2245 and 375.2632, respectively. Peak 2 (Rt = 11.71 min) presents an adduct $[M+H-H_2O]^+$ at 279.1975 ($C_{17}H_{27}O_3$) and was tentatively identified as 6-gingerdiol ($C_{17}H_{28}O_4$), showing also adducts $[M+H-2(H_2O)]^+$ and $[M+Na]^+$ at m/z 261.1873 and 319.1875, respectively. Gingerdiols did not fragment via C4-C5 cleavage, which may indicate the ketone group on C-3 is critical for the fragmentation pathway [24]. The main fragments found were due to dehydration and the fragment at m/z 137 corresponds to the benzyl moiety (Figure 6).

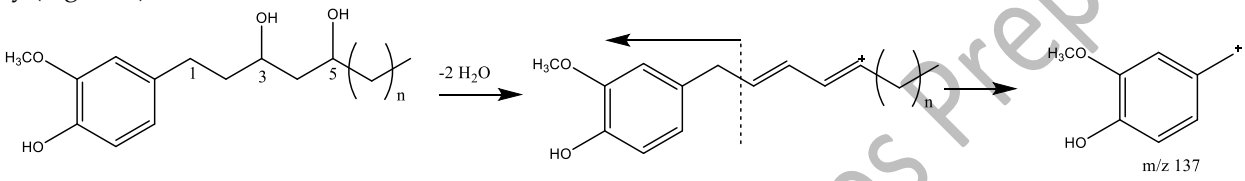

Figure 6. Fragmentation pathway for gingerdiol compounds 2 (n = 6), 6 (n = 8), 17 (n = 10)

.

A related group correspond to dehydrogingerdiones. Peaks 19 (Rt = 22.53 min), 27 (Rt = 27.37 min) and 34 (Rt = 31.37 min) were tentatively identified as 1-dehydro-6-gingerdione, 1-dehydro-8-gingerdione and 1-dehydro-10-gingerdione with a pseudomolecular ion $[M+H]^+$ at m/z 291.1494 ($C_{17}H_{23}O_4$), 319.1909 ($C_{19}H_{27}O_4$) and 347.2220 ($C_{21}H_{31}O_4$), respectively. These dehydrogingerdiones present a double bond between C1 and C2 yielding a fragment ion at m/z 177 due the keto group on the alkyl chain that causes fragmentation on the protonated molecules (Figure 7) [24].

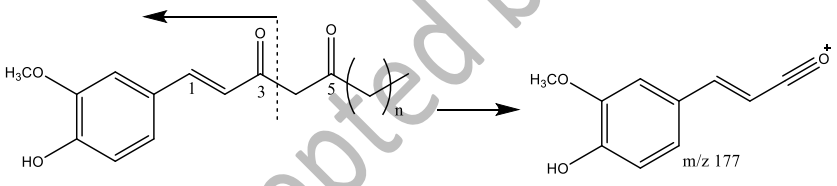

Figure 7. Fragmentation pathway for dehydrogingerdione compounds 19 (n = 6), 27 (n = 8), 34 (n = 10)

In turn, the group of acetoxy gingerols includes peaks 12 (Rt = 18.42 min), 21 (Rt = 23.40 min) and 29 (Rt = 27.99 min) with sodium adduct ions $[M+Na]^+$ observed at m/z 359.1815 ($C_{19}H_{28}O_5Na$), 387.2239 ($C_{21}H_{32}O_5Na$) and 415.2560 ($C_{23}H_{36}O_5Na$) respectively, corresponding to acetoxy-6-gingerol, acetoxy-8-gingerol and acetoxy-10-gingerol. Loss of the neutral acetyl group (60 Da), as illustrated in Figure 8, generates the fragments $[M+Na-C_2H_4O_2]^+$ at m/z 299 for acetoxy-6-gingerol, m/z 327 for acetoxy-8-gingerol, and 355 for acetoxy-10-gingerol [12].

In addition, peak 16 (Rt = 21.18 min) with a pseudomolecular ion $[M+H]^+$ at m/z 351.2173 ($C_{20}H_{31}O_5$), was tentatively assigned to methyl-acetoxy-6-gingerol, with an adduct $[M+Na]^+$ at m/z 373.2072. This peak holds an additional CH_2 group, thus an increase of 14 Da generating a product ion at m/z 151 (Figure 8) corresponding to the methylated aromatic moiety [11].

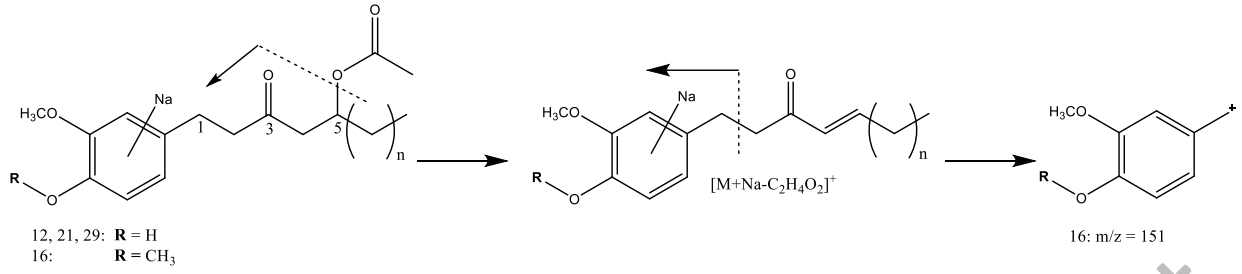

Figure 8. Fragmentation pathway for acetoxygingerol compounds 12 (n = 6), 21 (n = 8), 29 (n = 10) and methylacetoxygingerol 16 (n = 6).

The group of acetoxy gingerdiols holds an acetoxy group in C-5 (Figure 9). Peak 5 (Rt = 15.68 min) was tentatively assigned to 5-acetoxy-6-gingerol and peak 14 (Rt = 18.82 min) was tentatively identified as methyl-5-acetoxy-6-gingerdiol. Both peaks present a predominant adduct $[M+Na]^+$ at m/z 361.1989 ($C_{19}H_{30}O_5Na$) and 375.2150 ($C_{20}H_{32}O_5Na$) respectively, but also show adducts $[M+H]^+$ (m/z 339.2177 and 353.2322) and $[M+H-H_2O]^+$ (m/z 321.2059 and 335.2222). Their fragmentation is mainly due to the loss of the acetoxy group $[M+H-C_2H_4O_2]^+$ yielding ions at m/z 279 for peak 5 and m/z 293 for peak 14 [11]. In addition, fragment at m/z 151 is also observed for peak 14 producing the methylated aromatic moiety (Figure 9).

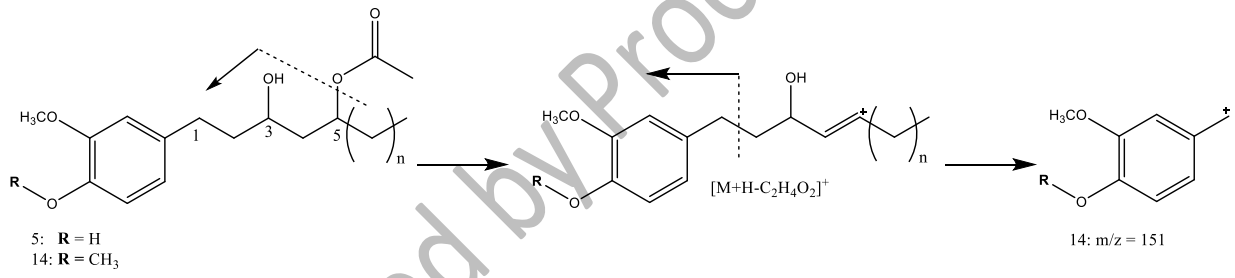

Figure 9. Fragmentation pathway of acetoxygingerdiol 5 (n = 6) and methyl-acetoxygingerdiol 14 (n = 6).

Diacetoxy gingerdiols correspond to gingerdiols acetylated in both hydroxyl groups at C-3 and C-5. Peaks 8 (Rt = 16.74 min), 18 (Rt = 21.57 min), 26 (Rt = 26.34 min) and 32 (Rt = 30.49 min) present predominant $[M+Na]^+$ adducts at m/z 375.1880 ($C_{19}H_{28}O_6Na$), 403.2200 ($C_{21}H_{32}O_6Na$), 431.2443 ($C_{23}H_{36}O_6Na$), and 459.2726 ($C_{25}H_{40}O_6Na$), respectively. These peaks were tentatively assigned to diacetoxy 4-gingerdiol (peak 8), diacetoxy 6-gingerdiol (peak 18), diacetoxy 8-gingerdiol (peak 26) and diacetoxy 10-gingerdiol (peak 32). Their fragmentation pattern corresponds to the loss of an acetyl group $[M+H-C_2H_4O_2]^+$ yielding ions at m/z at 293, 321, 349, and 377. In addition the loss of as a second acetyl group $[M+H-2(C_2H_4O_2)]^+$ produces ions at m/z 233, 261, 289, 317, respectively [25]. The fragment at m/z 137 was present for all peaks corresponding to the benzylic moiety (Figure 10).

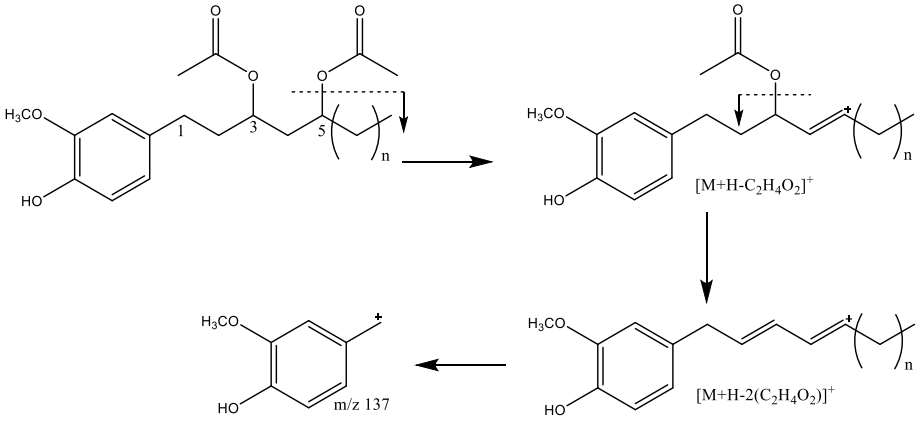

Figure 10. Fragmentation pathway for diacetoxy-gingerdiol compounds 8 (n = 4), 18 (n = 6), 26 (n = 8), 32 (n = 10)

Finally, four methylated diacetoxygingerdiols were identified. For instance, methyldiacetoxy-4-gingerdiol, methyldiacetoxy-6-gingerdiol, methyldiacetoxy-8-gingerdiol and methyldiacetoxy-10-gingerdiol were tentatively assigned to peaks 15 (Rt = 19.79 min), 24 (Rt = 24.51 min), 31 (Rt = 28.96 min) and 33 (Rt = 30.87 min). Their predominant adducts correspond to $[M+Na]^+$ at m/z 389.1938 ($C_{20}H_{30}O_6Na$), 417.2334 ($C_{22}H_{34}O_6Na$), 445.2689 ($C_{24}H_{38}O_6Na$) and 473.2980 ($C_{26}H_{42}O_6Na$) respectively. The fragment $[M+H-C_2H_4O_2]^+$ is due to the loss of one acetyl group corresponding to fragments at m/z 307, 335, 363 and 391. In addition, fragments from the loss of both acetyl moieties $[M+H-2(C_2H_4O_2)]^+$ yield ions at m/z at 247, 275, 303 and 331, respectively (Figure 11). The fragments at m/z 297 (peak 24), 325 (peak 31) and 353 (peak 33) were assigned to the loss of two acetyls from de sodium adduct $[M+Na-2(C_2H_4O_2)]^+$. On the other hand, the fragments at m/z 151 and 191 in all peaks correspond to the methylated fragments that occur in diacetoxygingerols [12].

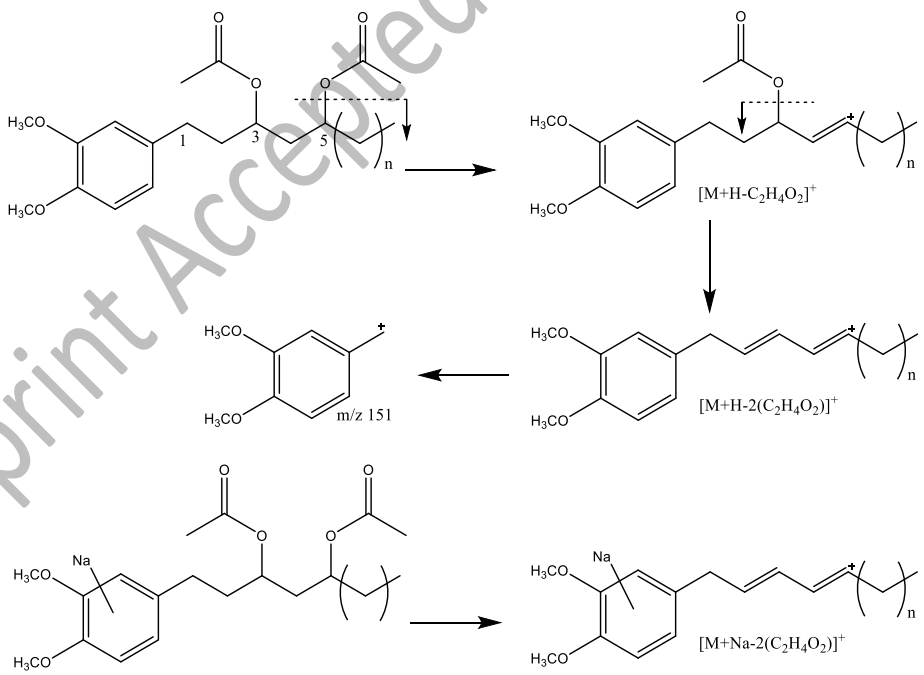

Figure 11. Fragmentation pathway for methyl-diacetoxygingerdiol compounds 15 (n = 4), 24 (n = 6), 31 (n = 8), 33 (n = 10)

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Comparing with the literature, the findings in these twelve ginger samples from Costa Rica are in agreement with previous reports on structures diversity accounting for the different compounds found in this study, including gingerols and gingerdiols and their methyl and acetoxy derivatives, as well as dihydrogingerdiones, paradols and shogaols [11-12, 24-25].

3.3. Total Gingerol and Shogaol Contents in Z. officinale extracts

UPLC-DAD analysis allowed the quantification of 6-gingerol, 8-gingerol, 10-gingerol, 6-gingerdiol and 6-dehydrodingerdione, to determine the total gingerols (TG) in each sample, as well as 6-shogaol contents while 8 and 10-shogaols were not quantifiable. The results are summarized in Table 2.

Table 2. Total gingerol (TG) and 6-shogaol content for extracts of *Z. officinale* rhizomes.

Product	TG (mg/g) [1,2,3]	6-gingerol (mg/g) [1,2,3]	8-gingerol (mg/g) [1,2,3]	10-gingerol (mg/g) [1,2,3]	6-gingerdiol (mg/g) [1,2,3]	Dehydro-6-gingerdione (mg/g) [1,2,3]	6-shogaol (mg/g) [1,2,3,4]
NR-1	31.99 ^{e,f} ± 1.26	22.04 ^{e,f} ± 0.88	4.76 ^{c,d} ± 0.12	3.29 ^{f} ± 0.20	1.43 ^{e,f} ± 0.06	0.47 ^{h} ± 0.03	1.11 ^{d} ± 0.04
NR-2	37.75 ^{c,d} ± 0.18	25.51 ^{b,c} ± 0.88	5.53 ^{b,c} ± 0.59	4.25 ^{c,d} ± 0.15	1.61 ^{d,e} ± 0.01	0.85 ^{e,f} ± 0.04	0.78 ^{f} ± 0.03
NR-3	39.59 ^{b,c} ± 1.66	27.40 ^{b} ± 0.50	6.57 ^{b} ± 0.65	3.59 ^{e,f} ± 0.37	1.00 ^{g} ± 0.09	1.02 ^{d} ± 0.07	0.44 ^{g} ± 0.04
NR-4	42.05 ^{b} ± 0.96	30.18 ^{a} ± 0.42	2.72 ^{e} ± 0.06	4.41 ^{c} ± 0.11	1.78 ^{d,e} ± 0.32	2.95 ^{a} ± 0.10	0.41 ^{g} ± 0.01
NR-5	30.44 ^{f} ± 0.08	20.36 ^{f,g} ± 0.04	3.37 ^{d,e} ± 0.05	4.13 ^{c,d} ± 0.04	1.06 ^{f,g} ± 0.02	1.54 ^{b} ± 0.01	N.Q.
NW-1	33.68 ^{e,f} ± 1.00	18.71 ^{g} ± 0.43	5.48 ^{b,c} ± 0.36	5.43 ^{b} ± 0.14	3.34 ^{b} ± 0.14	0.72 ^{f,g} ± 0.01	1.53 ^{c} ± 0.00
NW-2	38.64 ^{b,c} ± 0.47	24.66 ^{c,d} ± 0.27	5.33 ^{b,c} ± 0.10	6.13 ^{a} ± 0.06	1.85 ^{d} ± 0.03	0.67 ^{g} ± 0.05	1.80 ^{b} ± 0.01
NW-3	34.65 ^{d,e} ± 2.40	24.47 ^{c,d} ± 0.79	2.92 ^{e} ± 1.36	4.26 ^{c,d} ± 0.24	1.51 ^{d,e} ± 0.06	1.49 ^{b} ± 0.03	N.Q.
SR-1	48.17 ^{a} ± 1.08	30.54 ^{a} ± 0.60	8.45 ^{a} ± 0.35	5.26 ^{b} ± 0.05	3.13 ^{b} ± 0.07	0.79 ^{f,g} ± 0.04	1.49 ^{c} ± 0.04
WR-1	23.52 ^{g} ± 0.59	11.38 ^{i} ± 0.28	3.71 ^{d,e} ± 0.10	3.92 ^{d,e} ± 0.06	4.00 ^{a} ± 0.13	0.51 ^{h} ± 0.05	1.06 ^{d} ± 0.01
WR-2	23.26 ^{g} ± 0.53	15.76 ^{h} ± 0.32	0.67 ^{f} ± 0.01	3.32 ^{f} ± 0.13	2.53 ^{c} ± 0.03	0.99 ^{d,e} ± 0.05	1.90 ^{a} ± 0.07
WR-3	32.98 ^{e,f} ± 1.99	22.71 ^{d,e} ± 1.48	3.22 ^{e} ± 0.09	3.60 ^{e,f} ± 0.14	2.25 ^{c} ± 0.20	1.21 ^{c} ± 0.09	0.96 ^{e} ± 0.02

[1] mg/g of dry sample. [2] Values are expressed as mean ± standard deviation (S.D.). [3] Different superscript letters in the same column indicate differences are significant at $p < 0.05$ using one-way analysis of variance (ANOVA) with a Tukey post hoc as statistical test. [4] N.Q. = Not quantifiable.

Results for TG content range from 23.26 to 48.17 mg/g dry sample. One-way ANOVA followed by Tukey post hoc test indicates a significant difference ($p < 0.05$) between samples from the Northern region and Western region, with samples from the first region presenting high TG content, while samples from the second region show low TG content. At individual level, SR-1 rhizomes (48.17 mg/g dry sample) from the Southern region constitute the sample with higher TG content, followed by samples NR-4 (42.05 mg/g of dry sample) and NR-3 (39.59 mg/g of dry sample) from Northern region. The lowest TG content was presented by samples WR-2 (23.26 mg/g dry sample) and WR-1 (23.52 mg/g dry sample).

At individual gingerols level, samples from Northern region presented the highest percentage of 6-gingerol, with an average of 72% of 6-gingerol in respect to TG content, compared to the Northwestern region with 68%, Southern region with 63% and Western region with 61%. Regarding 8-gingerol, sample SR-1 from the Southern region showed the highest percentage of 8-gingerol, which represented 18% of the TG content, while samples from the

Western region showed the lowest 8-gingerol content, with an average of 10% in respect to TG content. Samples from the Northwestern region showed the highest 10-gingerol contents, with an average 16% of TG contents.

All samples analyzed presented a Total Gingerol (TG) content higher than 0.8% and a Total Shogaol, represented by 6-shogaol, equal or lower than 0.18%, as established by the United States Pharmacopeia (USP) [20]. Previous results for rhizomes from India reported samples with 6-gingerol contents between 1030-3049 μg/g of fresh sample, 8-gingerol contents from 105-312 μg/g of fresh sample and 10-gingerol contents between 105-425 μg/g of fresh sample [26]. Samples from Costa Rica analyzed in the present work showed higher gingerol contents, with 6-gingerol content between 2401-4211 μg/g of fresh sample, 8-gingerol content from 141-1165 μg/g of fresh sample and 10-gingerol content between 453-1507 μg/g of fresh sample. Samples analyzed also showed higher 6-gingerol content than a sample from China, which presented a content of 4.31 mg/g of dry material, while samples from Costa Rica presented contents ranging from 11.38-30.54 mg/g of dry material [27].

Costa Rican ginger also presented higher gingerol contents than other samples from China and Malaysia [28][29]. On the other hand, shogaol content was found to be lower than samples from Malaysia, where 6-shogaol content was between 2.20 and 7.49 mg/g of dry sample [28], while the highest 6-shogaol content found in all 12 samples analyzed in the present work was found to be 1.90 mg/g of dry sample.

3.4. Folin-Ciocalteu determination of Z. officinale Extracts

Recent studies [30][31] in polyphenolic compounds of diverse structure have showed that Folin-Ciocalteu assay, broadly used to measure total polyphenolic contents, is an adequate method to evaluate the polyphenolic reducing capacity, which occurs through a single electron transfer mechanism [32][33]. Table 3 summarizes the results for Folin-Ciocalteu (FC) reducing capacity of extracts from *Z. officinale* rhizomes ($n = 12$), performed as described in Section 2.3. from Materials and Methods.

Table 3. Folin-Ciocalteau (FC) reducing capacity for extracts of *Z. officinale* rhizomes.

Product	FC (mg/g) [1,2,3]	Product	FC (mg/g) [1,2,3]
NR-1	99.5 ^{c,d} ± 0.9	NW-2	103.7 ^{c} ± 1.4
NR-2	97.1 ^{d,e} ± 0.9	NW-3	95.2 ^{d,e} ± 1.5
NR-3	121.1 ^{b} ± 1.2	SR-1	150.5 ^{a} ± 1.9
NR-4	146.7 ^{a} ± 4.8	WR-1	49.8 ^{h} ± 0.9
NR-5	64.2 ^{g} ± 1.2	WR-2	49.5 ^{h} ± 0.8
NW-1	93.7 ^{e} ± 1.0	WR-3	74.9 ^{f} ± 1.1

[1] mg of gallic acid equivalent/g extract. [2] Values are expressed as mean ± standard deviation (S.D.). [3] Different superscript letters indicate differences are significant at $p < 0.05$ using one-way analysis of variance (ANOVA) with a Tukey post hoc as statistical test.

Results for FC reducing capacity in *Z. officinale* extracts show variability, with values ranging from 49.5 mg of gallic acid equivalents (GAE)/g extract up to 150.5 mg GAE/g extract. One-way ANOVA followed by Tukey post hoc test indicated significant difference ($p < 0.05$) between samples from Northern and Western regions. Samples from Northern region show some of the highest values with an average FC content of 105.7 mg GAE/g compared to results for samples from Western region, which hold an average 45% lower (58.0 mg GAE/g). Individually, WR-2 shows the lowest value (49.5 mg GAE/g) among all 12 samples, while SR-1 shows the highest FC reducing capacity (150.5 mg GAE/g), followed by NR-4 (146.7 mg GAE/g) and NR-3 (121.1 mg GAE/g).

Comparing with the literature, results obtained for FC assay were shown to be higher than values reported in the literature for *Z. officinale* samples from Malaysia (18.44 mg GAE/g dry material), Sudan (60.34 mg GAE/g dry material) and China (9.69 mg/g dry material) [28] [34] [35]. On the other hand, the UPLC analysis results proved to agree with FC determinations, where samples with the highest TG content, SR-1 (48.17 mg/g), NR-3 (39.59 mg/g) and NR-4 (42.05 mg/g) also presented the highest FC values. A similar trend was observed for the samples with the poorest contents of TG, namely WR-1 (23.52 mg/g) and WR-2 (23.26 mg/g), which was also in agreement with FC lowest findings.

Finally, as shown in Figure 12a, correlation analysis between TG and FC reducing capacity in samples determined by UPLC demonstrated to have significant positive correlation (r = 0.941, $p < 0.05$), thus suggesting a role of gingerols contents in the reducing capacity FC, in agreement with previous reports on polyphenols [36] [37].

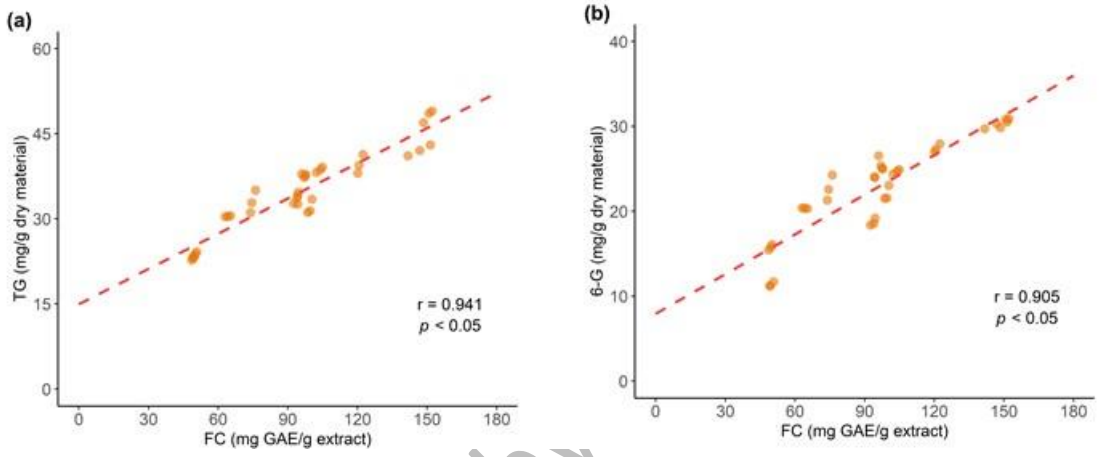

Figure 12. Correlation of total polyphenolic (TP) contents and UPLC-DAD results on **(a)** Total Gingerol (TG) **(b)** 6-gingerol contents.

In turn, correlation analysis between FC and the individual phenolic compounds showed correlation only with 6-gingerol and 10-gingerol, where the highest correlation is presented by 6-gingerol (r = 0.905, $p < 0.05$), as showed in Figure 12b, therefore aligning on the antioxidant role of this compound as previously reported [38][39].

3.5. DPPH Antioxidant Activity of Z. officinale Extracts

The capacity for free radicals scavenging can be suitably evaluated by a reaction with a stable free radical such as 2,2-diphenyl-1-picrylhidrazyl (DPPH) [40]. Recent kinetic studies for this method have indicated that the rate-determining step implies a fast electron transfer from phenoxide anions to DPPH, thus protic organic solvents enabling this mechanism [41]. In order to perform the evaluation of DPPH assay in *Z. officinale* rhizomes ($n = 12$), the method was applied as described in the Materials and Methods section 2.4, and results are summarized in Table 4.

Table 4. DPPH antioxidant activity of extracts from *Z. officinale* rhizomes.

Product	IC_{50} (μg/mL) [1,2,3]	Product	IC_{50} (μg/mL) [1,2,3]
NR-1	41.80 ^{c,d} ± 0.50	NW-2	35.05 ^{e} ± 0.07
NR-2	28.89 ^{f} ± 1.83	NW-3	40.20 ^{c,d} ± 0.14
NR-3	24.66 ^{f,g} ± 1.22	SR-1	22.53 ^{g} ± 0.85
NR-4	23.98 ^{g} ± 0.19	WR-1	64.64 ^{a} ± 4.31
NR-5	46.84 ^{b} ± 1.36	WR-2	64.98 ^{a} ± 0.33
NW-1	37.47 ^{d,e} ± 0.82	WR-3	44.06 ^{b,c} ± 1.22

[1] IC_{50} µg/mL of extract. [2] Values are expressed as mean ± standard deviation (S.D.). [3] Different superscript letters indicate differences are significant at $p < 0.05$ using one-way analysis of variance (ANOVA) with a Tukey post hoc as statistical test.

The results for the DPPH antioxidant activity evaluation show the same trend observed for the determination of FC reducing capacity and UPLC total gingerol (TG) contents. One-way ANOVA followed by a Tukey post-hoc test evidenced significant difference ($p < 0.05$) for samples depending on their region of origin. Samples from the Northern region presented higher antioxidant activity, with an average IC_{50} of 33.23 µg/mL. In contrast, samples from the Western region showed an average IC_{50} of 57.89 µg/mL, corresponding to the lowest antioxidant activity.

At individual level, sample SR-1, from Southern region presented the highest antioxidant activity, with an IC_{50} of 22.53 µg/mL, while sample WR-2 presented the lowest antioxidant activity, with an IC_{50} 65% lower corresponding to 64.98 µg/mL. These findings are consistent with the results obtained for FC and TG. Results obtained for DPPH evaluation of the twelve analyzed samples showed better or similar antioxidant activity than values previously reported for samples from Malaysia (27.2 µg/mL) and Sudan (29.87 µg/mL) [28] [34].

Correlation evaluations between DPPH antioxidant activity and FC and TG results were performed. As shown in Figure 13, results present a high negative correlation between DPPH values and TG results from UPLC-DAD quantification (r =-0.947, $p < 0.05$), suggesting a role for gingerols in the antioxidant activity as reported earlier for these metabolites [38]. This antioxidant activity has been attributed to the hydroxyl groups present in the main compounds of ginger extracts [42]. In addition, a high negative correlation was found between DPPH results and FC reducing capacity (r = -0.920, $p < 0.05$), aligning with previous reports showing correlation between different antioxidant related assays for these metabolites [28].

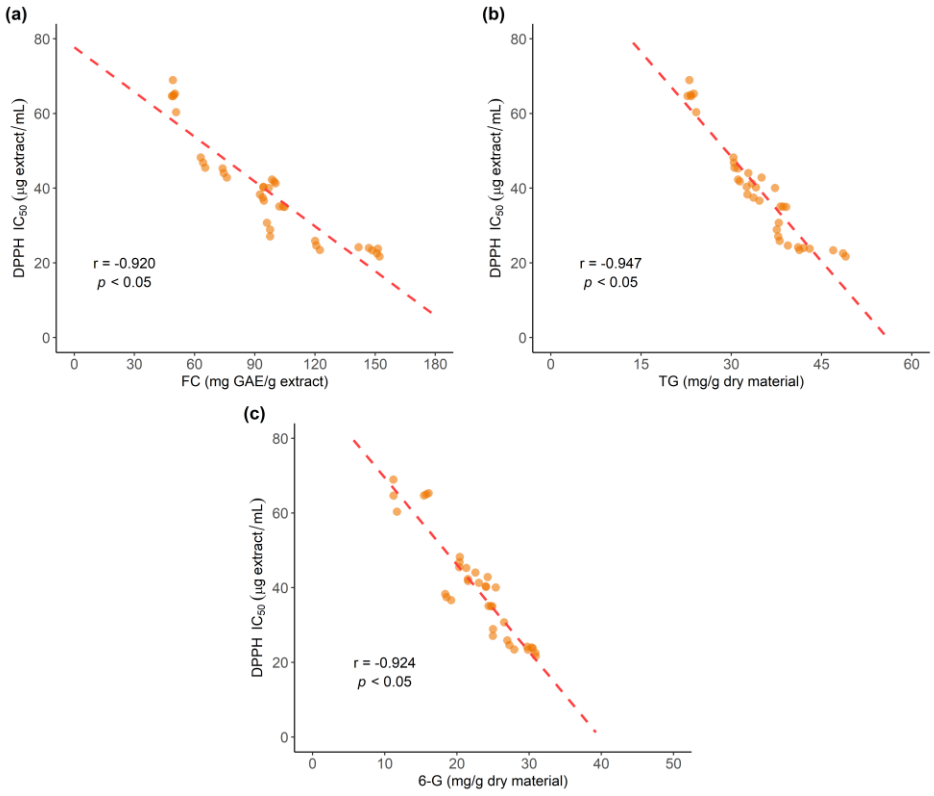

Figure 13. Correlation of antioxidant activity assessed by the DPPH method with (**a**) Reducing capacity by Folin-Ciocalteau; (**b**) TG by UPLC; and (**c**) 6-gingerol by UPLC.

Further, correlations between DPPH with individual gingerols (6-gingerol, 8-gingerol, 10-gingerol, 6-gingerdiol, 6-dehydrodingerdione) and 6-shogaol were also evaluated. A negative correlation was found only between DPPH values and 6-gingerol contents determined by UPLC-DAD (r = -0.924, $p < 0.05$), as shown in Figure 13. This would be consistent with 6-gingerol acting as a major contributor to antioxidant potential as formerly reported [38] [39].

Previous reports on correlation studies between polyphenols contents and antioxidant activities show variability of results with some studies indicating low or no correlation [30][40], other studies indicating structure dependence [43] while correlation is reported in several other studies [44] [45] [46]. The present findings for total gingerols (TG) content, FC reducing capacity and DPPH antioxidant activity are in agreement with the last group of studies reporting correlation between antioxidant activity and polyphenols content and aligning thus with findings suggesting these metabolites play an important role contributing to higher antioxidant activity.

3.6. Evaluation of Z. officnale Roscoe rhizomes from experimental plots

As mentioned earlier, five additional *Z. offcinale* rhizomes were acquired from NR-5 producer, which were grown in experimental pilots for Grand Cayman and Hawaiian cultivars. Table 5 summarizes the characteristics for these samples.

Table 5. Characteristics of *Z. officinale* Roscoe rhizomes from experimental plots

Product	Cultivar	Harvest (weeks)	Growth environment
NR-5	GC	40	Open-field
NR-6	GC	20	Open-field
NR-7	GC	40	High tunnel
NR-8	GC	20	High tunnel
NR-9	HW	40	High tunnel
NR-10	HW	20	High tunnel

[1] Cultivars: Grand Cayman (GC), Hawaiian (HW).

Sample NR-6, similarly to NR-5 corresponds to Grand Cayman variety, which is the main cultivar in Costa Rica, however it was harvested at 20 weeks instead of the ripe commercial maturity of 40 weeks. In turn, NR-7 and NR-8 correspond to Grand Cayman cultivars harvested at 40- and 20-weeks maturity respectively but both cultivated in a high tunnel environment instead of the open-field environment for NR-5 and NR-6, thus allowing to evaluate the influence of this factor. Finally, NR-9 and NR-10 rhizomes correspond to a Hawaiian cultivar grown in a high tunnel environment and harvested at 40 and 20 weeks respectively. Table 6 summarizes the results for the quantification of the main gingerols and 6-shogaol, following the same extraction and UPLC-DAD methods previously described.

Table 6. Phenolic content of *Z. officinale* rhizomes from experimental plots

Sample	TG (mg/g) [1,2,3]	6-gingerol (mg/g) [1,2,3]	8-gingerol (mg/g) [1,2,3]	10-gingerol (mg/g) [1,2,3]	6-gingerdiol (mg/g) [1,2,3]	6-dehydrogin-gerdione (mg/g) [1,2,3]	6-shogaol (mg/g) [1,2,3]
NR-5	30.44 ^{e} ± 0.08	20.36 ^{d} ± 0.04	3.37 ^{d} ± 0.05	4.13 ^{d} ± 0.04	1.06 ^{c} ± 0.02	1.54 ^{e} ± 0.01	N.Q.[4]
NR-6	38.91 ^{d} ± 0.82	26.12 ^{c} ± 0.08	2.50 ^{e} ± 0.34	6.13 ^{c} ± 0.17	1.85 ^{a,b} ± 0.14	2.31 ^{d} ± 0.09	1.76 ^{c} ± 0.07
NR-7	51.84 ^{c} ± 1.37	35.60 ^{b} ± 0.62	4.32 ^{c} ± 0.10	5.54 ^{c} ± 0.45	2.13 ^{a} ± 0.17	4.26 ^{b} ± 0.04	1.83 ^{c} ± 0.12
NR-8	54.67 ^{b,c} ± 0.98	36.94 ^{b} ± 0.54	3.59 ^{d} ± 0.28	10.24 ^{b} ± 0.01	1.65 ^{b} ± 0.11	2.27 ^{d} ± 0.04	0.67 ^{d} ± 0.05
NR-9	56.91 ^{b} ± 0.62	36.32 ^{b} ± 0.13	5.61 ^{b} ± 0.10	9.99 ^{b} ± 0.09	1.56 ^{b} ± 0.16	3.45 ^{c} ± 0.15	2.86 ^{b} ± 0.06
NR-10	73.89 ^{a} ± 1.71	45.94 ^{a} ± 1.01	7.49 ^{a} ± 0.21	13.16 ^{a} ± 0.34	1.76 ^{b} ± 0.05	5.55 ^{a} ± 0.12	3.20 ^{a} ± 0.05

[1] mg/g of dry sample. [2] Values are expressed as mean ± standard deviation (S.D.). [3] Different superscript letters in the same column indicate differences are significant at $p < 0.05$ using one-way analysis of variance (ANOVA) with a Tukey post hoc as statistical test. [4] Not Quantifiable (N.Q.).

Results for the TG content range from 30.44 to 73.89 mg/g of dry sample. One-way ANOVA followed by Tukey post hoc test shows a significant difference ($p < 0.05$) between samples grown open-field (OF) and samples grown under high tunnel (HT) conditions, with HT samples presenting higher TG content. At individual level, gingerol profile analysis showed product NR-10 (73.89 mg/g dry sample) to be the sample with higher TG content, followed by samples NR-9 (56.91 mg/g dry sample) and NR-8 (54.67 mg/g dry sample). The lowest TG content was presented by sample NR-5 (30.44 mg/g dry sample).

Two-way ANOVA was performed to evaluate the effect of the cultivar, harvesting maturity and growth condition on the TG and 6-shogaol contents. For TG content, results showed a significant difference ($p < 0.05$) in all three parameters, with higher gingerol content obtained with HW cultivar, grown under HT environment and harvested at 20 weeks. On the other hand, results for 6-shogaol content showed a significant difference ($p < 0.05$) only for the cultivar, where HW cultivar presented the higher shogaol content, with levels not aligning with USP requirements (6-shogaol content ≤ 0.18%) [20]. In turn, obtaining better results by harvesting at 20 weeks is in agreement with previous reports attributing a higher percentage of these metabolites content in ginger oleoresin at 6 months in respect 9-months harvesting, due to the fast increase of rhizome weight and fiber development approaching maturity [47].

For these samples, Foln-Ciocalteau reducing capacity and DPPH antioxidant activity assays were also applied and results are summarized in Table 7.

Table 7. Folin-Ciocalteau and DPPH results for *Z. officinale* rhizomes from experimental plots

Product	FC (mgGAE/g) [1,2,3]	DPPH IC_{50} (μg/mL) [2,3,4]
NR-5	64.20 [f] ± 1.15	46.84 [a] ± 1.36
NR-6	109.70 [e] ± 1.70	34.19 [b] ± 0.21
NR-7	156.98 [c] ± 3.25	22.38 [d] ± 0.48
NR-8	141.89 [d] ± 2.83	25.02 [c] ± 0.35
NR-9	178.02 [b] ± 0.20	18.15 [e] ± 1.00
NR-10	202.65 [a] ± 2.06	15.56 [f] ± 1.22

[1] mg of gallic acid equivalent/g extract [2] Values are expressed as mean ± standard deviation (S.D.). [3] Different superscript letters indicate differences are significant at $p < 0.05$ using one-way analysis of variance (ANOVA) with a Tukey post hoc as statistical test. [4] IC_{50} μg/mL of extract.

Results for FC reducing capacity range from 64.20 up to 202 mg GAE/g extract. One-way ANOVA followed by Tukey post hoc test indicated significant difference ($p < 0.05$) between all samples. Samples NR-5 and NR-6, both grown in OF, showed the lowest reducing capacity, consistent with results obtained in the TG content analysis. Samples NR-9 and NR-10 corresponding to Hawaiian cultivar showed the highest values with an average FC of 190.33 mg GAE/g compared to results for NR-7 and NR-8 corresponding to GC cultivar grown under the same conditions, which hold an average of 149.43 mg GAE/g, hence 21% lower. Results for the DPPH antioxidant activity evaluation show the same trend observed for the determination of FC reducing capacity and UPLC TG contents with samples NR-9 and NR-10 showing the lowest values, 18.15 and 15.56 μg/mL respectively, therefore better antioxidant activity. On the other hand, sample NR-5 presented the lowest antioxidant activity, with an IC50 of 46.84 μg/mL.

Two-way ANOVA tests were performed to evaluate the effect of the cultivar, growth condition and harvesting weeks on the FC reducing capacity and DPPH antioxidant activity. Results showed a significant difference ($p < 0.05$) for all three parameters, with higher reducing capacity and better antioxidant activity obtained for HW cultivar, grown under HT and harvested at 20 weeks. Comparison of these results with the prevalent conditions for *Z. officinale* cultivation in Costa Rica show the impact on variability that these factors entail. For instance, high-tunnel (HT) practices are known to protect crops, reducing pest related affectation and extending growing season, having influence in nutritional contents [48], which could be associated with present results observed for ginger rhizomes under HT conditions. In turn, Hawaiian variety, originally from Philippines [49], introduced more recently in the country, could seem more promising due to higher TG content. However, depending on the product final use, for instance as source for dietary supplement, these rhizomes no compliance with USP in respect to the maximum amount of shogaols [20] would imply an advantage for the more widespread Grand Cayman variety in Costa Rica.

Finally, correlation evaluations between TG contents with DPPH antioxidant activity and FC results were performed and findings indicated a high positive correlation between TG values and reducing capacity determined by the Folin–Ciocalteau method ($r = 0.961$, $p < 0.05$) and high negative correlation between TG results and DPPH values ($r = -0.930$, $p < 0.05$). These results align with the findings on the twelve rhizomes from different regions in Costa Rica and also with previous reports for gingerols [28] and other polyphenolic structures [44-46].

In sum, for these experimental plots, the use of a high-tunnel environment is a good alternative for cultivation towards fresh produce commercialization and for obtaining high contents of gingerols. Regarding harvesting maturity, 40 weeks complies with commercial preferences for fresh produce in respect to larger fresh rhizomes and prices paid by kilogram, however, producers are looking for commercial alternatives due to the increasing competition on fresh products, therefore, the growing demand for nutritional supplements and better prices paid for value-added products such as dry ginger powder and extracts represent an opportunity for harvesting the ginger at earlier maturity aligning with previous recommendations on this being beneficial for farmers and the oleoresin industry [47]. Hence, these results constitute an important first step to feedback local producers on the potential for shorter harvest time, offering products with higher contents of gingerols.

3.7. *Principal Component Analysis for Polyphenolic Extracts of Z. officinale rhizomes*

A statistical Principal Component Analysis (PCA) was performed for the *Z. officinale* rhizomes ($n = 12$) harvested at term, considering all nine variables, specifically TG, all five individual gingerols and shogaol contents, FC and DPPH values. Two components, namely PC1 and PC2, were obtained (loadings > 0.44). The first component (PC1) represented 52.11% of total variance and showed a negative correlation with TG, 6-gingerol and FC and was positively correlated with DPPH. The second component (PC2) described 23.13% of the total variance and was positively correlated to 6-gingerdiol and 6-shogaol.

As illustrated in the graphical representation of the two components (Figure 14), *Z. officinale* samples are distributed along PC1, showing variability for the above-mentioned gingerol contents, reducing capacity and antioxidant activity. Some samples have particularly high values in PC1, for instance WR-1 and WR-2, which means poorer TG and 6-gingerol contents, as well as low FC and DPPH antioxidant activities. The graph shows samples from the Northern and Northwestern regions with similar PC1 values, except for samples NR-3 and NR-4, due to their higher TG, 6-gingerol, FC and DPPH values in respect to the other samples from those regions. The lowest PC1 value pertains to rhizomes SR-1, which agrees with this sample exhibiting the highest TG, 6-gingerol contents, FC, and DPPH antioxidant activity.

Samples are also distributed along PC2, showing variability in 6-gingerdiol and 6-shogaol contents for the 12 rhizomes. All samples from the Northern region present low PC2, therefore low 6-shogaol and 6-gingerdiol contents. On the other hand, samples from the Northwestern region,

present the highest variability in PC2. The highest PC2 values are presented by samples NW-1, NW-2 and SR-1, indicating higher content of the above-mentioned compounds.

Finally, our findings show that although differences were observed in the composition ranges between products from different regions, sample SR-1 stands out significatively, showing the highest PC1 and PC2 values, due to its rich content of the gingerols and shogaol previously mentioned and highest FC reducing capacity and DPPH antioxidant activity.

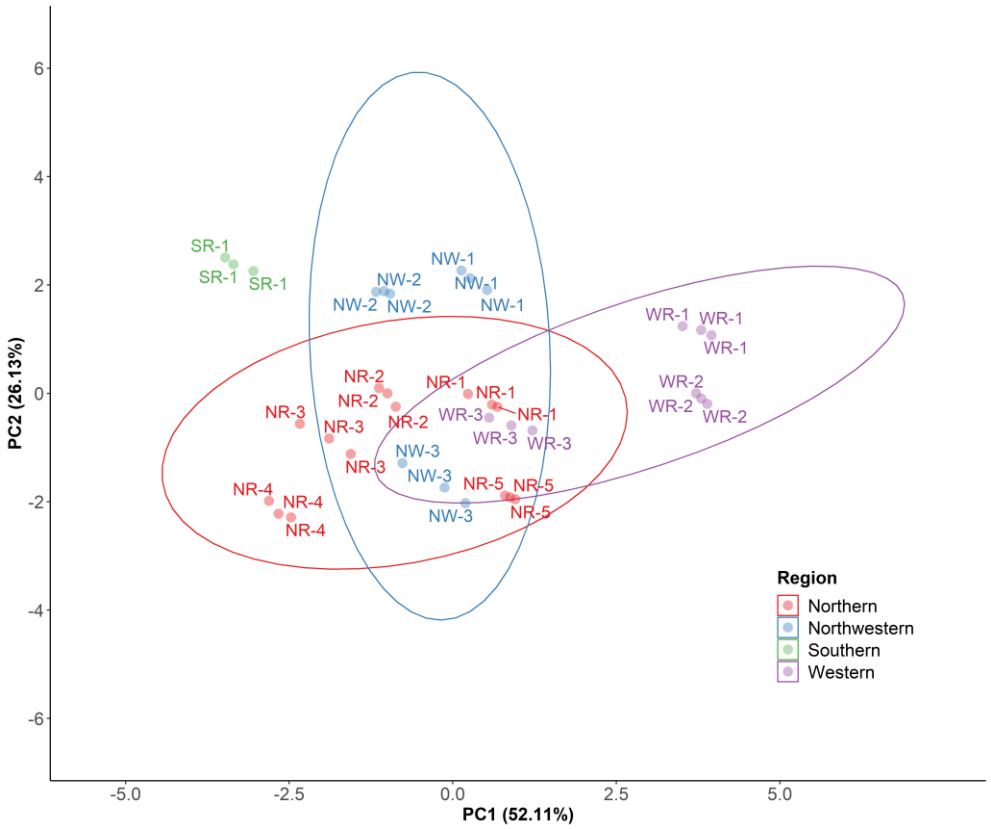

Figure 14. Plane defined by two first principal components (PC1 and PC2) resulting from the PCA analysis of *Z. officinale* rhizomes (n = 12) contents. Regions: Northern (NR), Northwestern (NW), Southern (SR), Western (WR)

Overall, findings for *Z. officinale* rhizomes clearly indicate polyphenolic composition analogous to previously reported results [12][25] and higher gingerols content in all Costa Rican samples in respect to literature [26][28-29]. These results and the antioxidant activity values obtained for these extracts suggest their potential benefits in respect to other biological activities [50][51] and towards their application in the dietary supplements industry. Further, PCA indicating location influence as well as results from the controlled experimental studies at variable harvest times and growth conditions, suggest the importance of future studies regarding environment, soil, and other cultivation conditions to assess their influence in gingerols and shogaols contents for these Costa Rican rhizomes as well as further *in-vitro* and *in-vivo* research on other biological activities.

4. Conclusions

Findings for *Z. offcinale Roscoe* rhizomes from Costa Rica through HRMS-QTOF analysis, clearly indicate analogous polyphenolic structures to previous reports, however UPLC-DAD quantification of the main gingerols show much higher contents than literature for all Costa Rican samples. In addition, all samples from Grand Cayman cultivar align with USP requirements in high total gingerols and low shogaol content while PCA demonstrates the potential coming from homogeneous results for samples from the Northern region as well as suggest the importance to study further

growth conditions, such as soil type, fertility, humidity, since for instance SR-1, rhizomes constituting the only sample from the Southern region, where production is more recent, stand out by showing the highest gingerols content and antioxidant activity.

In sum, the *Z. offcinale* Roscoe extracts evaluated in this paper clearly exhibit a potential benefit concerning their gingerols content and their capacity to protect against oxidative stress, due to their antioxidant activity values. Therefore, the promotion of these products as functional food and their consumption as dietary supplements could be beneficial. Overall results set the foundation for future ginger-based products developed with rhizomes from Costa Rica and future work towards the elaboration of standardized products linked to important antioxidant activity and detailed chemical profile, nonetheless, further studies are needed to assess the physicochemical stability and other bioactive properties such as anti-inflammatory and immunostimulant effects, directly linked to the antioxidant properties described in this work.

Author Contributions: Conceptualization, M.N.-H.; formal analysis, M.N.-H, M.A.-Q., M.Q.-F, F.V.-H., K.W.-R., and A.S.-K.; funding acquisition, M.N.-H.; investigation, M.N.-H, M.A.-Q, M.A.-Q., F.V.-H.; methodology, M.N.-H. and A.S.-K.; writing—original draft, M.N.-H., M.Q.-F., F.V.-H., D.A.-C., K.W.-R., F.V-C.; writing—review and editing, M.N.-H., M.A.-Q, M.Q.-F., F.V.-H., K.W.-R., D.A.-C., F.V.-C. and A.S.-K.

Funding: This work was partially funded by grants from the University of Costa Rica (115-B8-150, 115-C0-001, 115-C1-515 and ED-2033).

Acknowledgments: Authors also thank the support from the Costa Rican Institute of Technology.

Conflicts of Interest: The authors declare no conflicts of interest.

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