Article

Post Acid Treatment on Pressurized Liquid Extracts of Sorghum (*Sorghum bicolor* L. Moench) Grain and Plant Material Improves Quantification and Identification of 3-Deoxyanthocyanidins

Ádina L. Santana [1,†], Jaymi Peterson [2,†], Ramasamy Perumal [3], Changling Hu [4], Shengmin Sang [4], Kaliramesh Siliveru [1] and Dmitriy Smolensky [2,*]

1   Grain Science and Industry Department, Kansas State University, 1301 N Mid Campus Drive, Manhattan, KS 66503, USA
2   Grain Quality and Structure Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Manhattan, KS 66502, USA
3   Agricultural Research Center, Kansas State University, Hays, KS 67601, USA
4   Nutrition Research Institute Building, North Carolina Agricultural and Technical State University, 150 N Research Campus Dr, Kannapolis, NC 28081, USA
*   Correspondence: dmitriy.smolensky@usda.gov
†   These authors contributed equally to this work.

Abstract: Sorghum is a unique natural food source of 3-deoxyanthocyanidins (3-DA) polyphenols. This work evaluated the effect of acidification on sorghum extracts post pressurized liquid extraction (PLE) and its ability to increase the identification and quantification of 3-DA. The sorghum genotypes included Sumac and PI570366 (bran only) and SC991 (leaf and leaf sheath tissue). The acidification of the PLE extracts was carried out with methanol–HCl solutions at various concentrations (0, 0.5, 1, 2, and 4%, v/v). Changes in color were determined using $L*a*b*$. The overall phenolic composition was estimated with the total phenolic content and the DPPH free radical scavenging assays. Quantitative and qualitative chromatographic methods determined the phenolic profile. Color analysis showed that the redness and color saturation increased after acidification. No statistical difference was found in the total phenolic content of the acidified extracts, except for SC991, which was increased. There were no differences in the antioxidant capacity following acidification in all samples. For chromatographic analysis, luteolinidin was predominant in the extracts and the 3-DA content increased after acid treatment. However, some flavonoid and phenolic acid concentrations decreased following acid treatment, including taxifolin, quercetin, and chlorogenic acid. Interestingly, 0.5% v/v HCl acidification was sufficient to increase the color, allow the detection of 5-methoxyluteolinidin, and to increase luteolinidin and 7-methoxyapigenidin by at least twofold.

Keywords: accelerated solvent extraction equipment; natural colorants; hydrochloric acid treatment; sorghum polyphenols; thin-layer chromatography; HPLC; 3-deoxyanthocyanidins; LC-MS

1. Introduction

In 1998, the Dionex Corp. [1] patented a bench-scale automated piece of apparatus that uses pressurized liquid extraction (PLE) principles. This apparatus and methodology, designated as accelerated solvent extraction (ASE), extracts analytes from a solid or semi-solid matrix with the use of compressed liquids below the subcritical state. Cardenas-Toro et al. [2] noted that the subcritical state of water happens at temperatures ranging between around 150 and 374 °C and at pressures higher than its vapor saturation pressure. When the liquid solvent is hot and pressurized, its dielectric constant decreases and contributes to an increase in the solubility of target compounds in the matrix to the solvent, resulting in rapid extraction in comparison with conventional extraction methods including Soxhlet, maceration, and reflux extraction.

Although PLE can be used for high throughput, there are some limitations to this method. For instance, most phenolic compounds are thermal sensitive and, therefore, extraction temperatures should be studied to evaluate the optimal solubility while minimizing thermal degradation [3]. Additionally, the vessels, valves, and fittings in the ASE are easily corroded by the presence of strong acids and alkali, limiting the use of solvents. The ASE machine is also limited by its inability to pressurize higher than 10 MPa.

Sorghum (*Sorghum bicolor* L. Moench) grain contains a variety of phenolic compounds, including phenolic acids (ferulic and caffeic acids), flavonoids (apigenin, luteolin, and naringenin), and 3-deoxyanthocyanidins (3-DA), namely, luteolinidin, apigenidin, and 7-methoxyapigenidin. The extraction of phenolics from sorghum has been studied worldwide using water [4] and solvent solutions containing acetone [5], ethanol [6,7], and methanol [8–10].

Despite the use of solvents that are generally recognized as safe (GRAS) for sorghum extraction methods, some phenolics are not easily extracted in various solvents. To increase extraction efficiency, acidified methanol at 1% hydrochloric acid (HCl) has been extensively used [8,9]. Hydrolysis can cleave ester bonds to release phenolics and is primarily performed through acid, alkali, or enzyme treatments [11,12]. In acidic medium, there is an increase in hydrogen ions, allowing the protonation of phenolics. This assists the release of phenolics bound to macromolecules (i.e., proteins, lignin, cellulose), subsequently increasing the solubility of hydrophobic compounds [13,14].

Solutions containing HCl are highly corrosive. In academic studies, solutions with low concentrations of HCl were shown to be efficient for their goals. Wizi et al. [14] observed that treating samples with 1% HCl (v/v) increased the colorants luteolinidin and apigenidin by approximately 17% in Chinese sorghum husks extracted by microwave and ultrasonification. Paunović et al. [15] showed that acidification with 5% HCl in an aqueous solution increased the total phenolic content in barley extracts by approximately 200% in comparison with the non-acidified extracts in 30% ethanol (v/v). Ju and Howard observed that the post acidification with HCl at 0.1% (v/v) of PLE extracts of red grape skin enhanced the detection of total anthocyanins similar to acetic acid at 7% (v/v) [16].

For this reason, this work investigates the effect of post acid treatments on phenolic composition in sorghum extracts derived from PLE technology. The goal was to determine whether post-extraction acidification would enhance the phenolic content and increase the amount of detectable phenolics, namely, 3-DA. The advantages of acidification post extraction are the ability to use PLE and obtain high-throughput extractions while limiting the amount of HCl waste generated, leading to greener chemistry when extracting sorghum phenolics.

2. Materials and Methods

2.1. Raw Material and Chemicals

Commercial Sumac bran was purchased from NU Life Markets (Scott City, KS, USA). The sorghum cultivar PI570366 was grown in Puerta Vallarta, Mexico, by Kansas State University (Hays, KS, USA), during the 2017 growing season and stored at −20 °C. The grains were decorticated in-house to produce the bran. Sorghum leaf and leaf sheaths from SC991, a high phenolic genotype, were collected during the 2019 season in Hays (KS, USA), and milled with the aid of a UDY mill (Fort Collins, CO, USA) using a 0.5 mm screen. Glacial acetic acid, acetonitrile, hydrochloric acid, methanol, chloroform, deionized water, formic acid, and ethanol were ordered from Fisher Scientific (Waltham, MA, USA).

The standards used in HPLC analysis include 7-methoxyapigenidin (Cayman, Ann Harbor, MI, USA), taxifolin (HWI, Ruelzheim, Germany), 4-hydroxybenzoic acid, syringic acid, protocatechuic acid, ferulic acid, quercetin, naringenin (Sigma-Aldrich, Darmstadt, Germany), catechin (TCI, Portland, OR, USA), kaempferol, caffeic acid, p-coumaric acid, eriodictyol, luteolin, apigenin, and the chloride salts of luteolinidin, apigenidin, cyanidin, pelargonidin, malvidin, and peonidin (Indofine, Hillsborough, NJ, USA).

Gallic acid hydrate (TCI, Portland, OR, USA), Folin-Ciocalteu (Sigma-Aldrich, Darmstadt, Germany) and sodium carbonate (Fisher Scientific, Waltham, MA, USA) were used for the TPC analysis. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Trolox (Sigma-Aldrich, Darmstadt, Germany) were used for antioxidant potential analysis.

2.2. Pressurized Liquid Extraction (PLE)

The extraction was carried out in Accelerated Solvent Extraction 350 (Dionex, Thermo Fisher, Pittsburgh, PA, USA, Figure 1) apparatus. Then, 1 g of raw material was mixed with approximately 6 g of borosilicate glass beads (3 mm i.d., Chemglass Life Sciences, Vineland, NJ, USA) and inserted into a 10 mL stainless steel extraction vessel (Thermo Fisher, Pittsburgh, PA, USA) packed with cellulose filters at both ends.
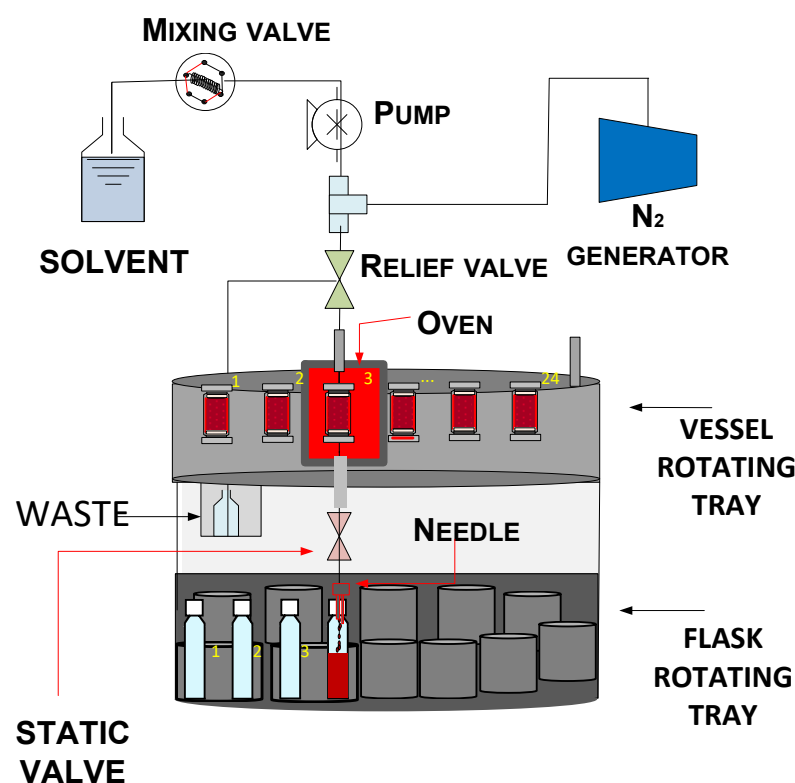

Figure 1. Schematic diagram showing the extraction process using ASE equipment (adapted from Thermo Fisher [17]).

Once the oven reached the set-up temperature, the nitrogen-driven pump pressurized the vessel with fresh solvent at 10.34 MPa, starting the extraction process. Two cycles of 5 min each were selected, i.e., the number of times consisting of vessel heating followed by pressurization with the solvent. The rinse volume was 100% of the vessel volume. Once the rinse cycles finished, a nitrogen stream (99% pure, Calypso DS, F-DGSi Nitrogen Generator, France) flushed the line throughout the vessel for 1 min. Afterwards, the vessel was depressurized, and the PLE finished.

The solvent used was ethanol:water (70:30, v/v) based on prior research suggesting that it is an optimal GRAS solvent for sorghum phenolic extraction [6]. At first, three levels of temperature (80 °C, 100 °C, and 120 °C) were used to select to the optimal temperature to extract phenolics for further acidification. The total extraction time was approximately 20 min. The quantity of solvent spent per experiment varied around 25–30 mL. Such variation is justified by the nature of the raw material and the quantity of solvent remaining in the line before the purge phase. Afterwards, the volume of extracts was adjusted to 30 mL to assure equal volume for all samples followed by the TPC assay (see Figure 2). Two biological replicates were conducted for each temperature tested.
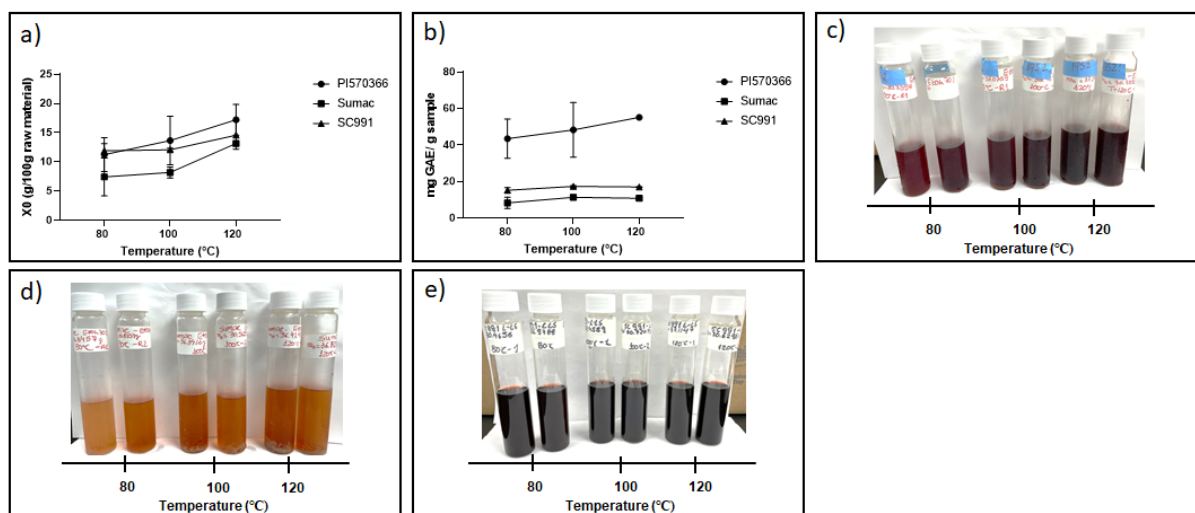
Figure 2. PLE extracts of sorghum with ethanol 70%: the effect of temperature on (**a**) global yield, (**b**) total phenolic content, and the visual representation of extracts (**c**) PI570366, (**d**) Sumac bran, and (**e**) SC991 leaf tissue.

2.2.1. Global Yield of Soluble Extract

The extracts were centrifuged at 4 °C, $3500\times g$, for 10 min (Thermo Fisher, Pittsburgh, PA, USA) to remove any precipitate. The precipitate was discarded, and the supernatant collected and dried with the use of a Rocket Synergy 2 Evaporation System (Thermo Fisher, Pittsburgh, PA, USA) at 60 °C for approximately 2 h. The global yield, or mass of the dried supernatant, was calculated according to Equation (1).

$$X_0 = \left(\frac{m_{EXT}}{m_{RM}} \right) \times 100 \tag{1}$$

where X_0 is the global yield, expressed as g soluble extract per 100 g of raw material, m_{EXT} (g) is the mass of dried extract, and m_{RM} (g) is the mass of raw material used in PLE, i.e., 1 g.

2.2.2. Acidification of PLE Extracts

Since HCl is highly corrosive, it cannot be incorporated into solvents for ASE equipment or any other equipment that is sensitive to strong alkali or acids [17]. In this case, the acidification of extracts with HCl solutions at small volumes was studied to optimize the release of phenolic species to improve the sample preparation of sorghum extracts for analytical measurements.

Dried PLE extracts solubilized with 10 mL of pure methanol served as the control. For acid treatments, extracts were solubilized in 10 mL of methanol–HCl solutions at the following percentages: 0.5%, 1%, 2%, and 4% (*v/v*), and stored at -20 °C for at least 48 h. The acid-treated samples were then dried under nitrogen. All samples, including the untreated dried samples, were resuspended in the solvent used for analysis (90% methanol, 10% ethanol). The samples were homogenized and sonicated with the aid of an ultrasonic bath (ULTRAsonik, Simi Valley, CA, USA) for 20 min. Afterwards, the samples were centrifuged at 4 °C, $3500\times g$ for 10 min (Thermo Fisher, Pittsburgh, PA, USA) to remove any precipitate. The supernatants were used for analysis. Three biological replicates of each acidification treatment were studied.

2.3. Characterization of Extracts

2.3.1. Color Analysis

The effect of acidification in the coloration of the extracts was evaluated using a MiniScan® EZ 4500 portable spectrophotometer (45°/0° geometry, HunterLab, Reston, VA,

USA). Due to poor transmittance, SC991 plant tissue extracts and PI570366 bran extracts were diluted in pure ethanol at 1:10, *v/v* for color analysis (see Figure 3).

Figure 3. CIEBLAB color values for (**a**) PI570366; (**b**) Sumac; (**c**) SC991 leaf and leaf sheaths. Hue values for (**d**) PI570366; (**e**) Sumac; (**f**) SC991 and chroma values for (**g**) PI570366; (**h**) Sumac; (**i**) SC991 and overall changes in physical appearance for (**j**) PI570366; (**k**) Sumac; (**l**) SC991 with increasing acid concentration. The results are represented as mean ± standard deviation of three independent acidified extracts post extraction. Significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, *** $p \leq 0.0001$.

The Commission Internationale de l'Eclairage (CIE) system was used to model *L**, *a**, and *b** color coordinates. The coordinate *a** represents the color trend of red (positive) or green (negative), whereas the *b** coordinate represents the color trend between yellow (positive) and blue (negative). The chroma (*C**) represents the color saturation, and the hue angle (*H**) represents the relative intensity of redness and yellowness, where 0° denotes red, 90° yellow, 180° for green, and 270° for blue. The chroma (*C**) and hue (*H**) values were calculated using *a** and *b** values, as mentioned in Equations (2) and (3), respectively.

$$C^* = \sqrt{(a^*)^2 + (b^*)^2} \tag{2}$$

$$H^* = arctan\left(\frac{b^*}{a^*}\right) \tag{3}$$

2.3.2. Total Phenolic Content Assay

The method previously described by Herald et al. [18] was used to determine the total phenolic content (TPC) using the Folin–Ciocalteu (FC) method. The FC working reagent was prepared by diluting FC with deionized (DI) water at a 1:1 (*v/v*) ratio. The samples were diluted with ethanol (70% *v/v*) at a 1:20 ratio. To a 96-well plate, samples or standards (25 µL) were combined with 75 µL DI water and 25 µL FC working reagent. The plate was allowed to incubate at room temperature for 6 min before 100 µL of 7.5% (*w/v*) Na_2CO_3 was added. The plate was then heat sealed and left to incubate in the dark for 90 min before reading the absorbance at 765 nm. The absorbance was read using a BioTek Synergy 2 multi-detection plate reader (Winooski, VT, USA). The standard curve was prepared by dissolving gallic acid (1 mg/mL) in 70% ethanol and ranged in concentration from 0 to 400 µg/L. Sample absorbances were calculated using the standard curve and expressed as milligram gallic acid equivalents (GAE) per gram of raw material.

2.3.3. DPPH Antioxidant Capacity Assay

Antioxidant capacity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH*) radical method [19]. Briefly, 50 µL of samples were combined with 10 mL of DPPH reagent (0.1 mM in 70% ethanol). The samples and standards were briefly vortexed before incubating in the dark for 30 min. The samples were filtered using a 0.45 µm nylon syringe filter prior to reading absorbance at 517 nm. Absorbance was measured using a UV–Vis spectrophotometer (Shanghai Metash Instruments Co., Ltd., Shanghai, China). The Trolox standard curve in 70% ethanol was plotted (0–100 mg/mL). The results of triplicates were expressed as milligram Trolox equivalents (TE) per gram of raw material.

2.3.4. High-Performance Liquid Chromatography

The 1260 Infinity (Agilent, Santa Clara, CA, USA) HPLC apparatus analyzed the individual phenolics in the samples, based on the protocol of Irakli et al. [20] with adaptations described by Lee and coworkers [21]. The stationary phase consisted of a Kinetex® C18 column (150 mm × 4.6 mm, 100 Å, 2.6 µm, Phenomenex, Torrance, CA, USA) connected to a guard column (SecurityGuard™, Phenomenex, Torrance, CA, USA). The column temperature was set up at 30 °C. The mobile phases consisted of acetonitrile (A), methanol (B), and acidified water at 0.5% glacial acetic acid, *v/v* (C). The elution gradient of A:B:C (*v/v/v*) at 1 mL/min consisted of an initial composition of 5:5:90, followed by 0–5 min of 8:8:84, 5–15 min of 10:10:80, 15–25 min of 25:0:75, 25–35 min of 30:0:70, and 35–45 min of 60:0:40. A post time of 5 min with the initial gradient was used to stabilize the baseline for further runs.

Catechin, eriodictyol, taxifolin, 4-hydroxybenzoic-, protocatechuic- and syringic acids, quercetin, luteolin, and naringenin were investigated at 280 nm. Chlorogenic, caffeic, p-coumaric, and ferulic acids, rutin, apigenin, and kaempferol were investigated at 320 nm.

Luteolinidin, cyanidin, peonidin, malvidin, pelargonidin, apigenidin, and 7-methoxyapigenidin were investigated at 510 nm. The results were expressed as μg/g raw material.

In Sumac and PI570366, apigenidin was the second most prominent 3-DA in the extracts. However, in the acidified SC991 extracts, apigenidin could not be detected by HPLC-DAD using the current protocol, because of peak overlap with 5-methoxyluteolinidin, which was identified by LC-MS. For the calculation of 5-methoxyluteolinidin, we used the luteolinidin calibration curve multiplied by the molecular weight correction factor of 0.93, as described by Speranza and coworkers [22].

Representative chromatograms at 280 nm, 320 nm, and 510 nm, as well as information about the HPLC method validation (precision and accuracy) are available in the Supplementary Materials.

For all studied compounds, the sorghum extracts spiked with phenolics presented linear behavior with a correlation factor (R^2) of 0.998 or better (Table S2.1), which is consistent with the previous findings [23,24]. For one concentration studied, the repeatability and accuracy for the detection of 3-deoxyanthocyanidins, flavonoids, and phenolic acids for the genotypes studied (Table S2.2) were comparable with other validated HPLC methods [23–25].

2.3.5. Thin-Layer Chromatography (TLC)

A qualitative evaluation of the presence of 3-DA and the effect of acidification on the extracts was carried out with silica gel–glass TLC plates (10 × 20 cm, 60G F_{254}, Merck, Darmstadt, Germany) as a stationary phase. Stock solutions of apigenidin, luteolinidin, and 7-methoxyapigenidin at 0.09 mg/mL, in methanol:ethanol (90:10, *v/v*) were prepared. One milliliter of non-acidified and acidified extracts at 1% and 4% HCl were dried under nitrogen (99%, Matteson, Manhattan, KS, USA) and resuspended in 1 mL methanol:ethanol (90:10, *v/v*).

Two microliters of standards and four microliters of extracts were injected into the TLC plates with the use of a chromatographic syringe (Hamilton, Reno, NV, USA). One hundred milliliters of the mobile phase (chloroform:ethanol:glacial acetic acid:methanol, 95:5:1:10, *v/v/v*) were inserted into a 20 × 20 cm closed glass chamber and, subsequently, the TLC plates were eluted with samples and standards injected. After elution, the plates were recorded under visible and under UV regions with a Cole-Parmer viewing cabinet (Vernon Hills, IL, USA) equipped with a UV lamp at 366 nm. The theoretically present 3-DA in the extracts was identified via the quality of the bands eluted, and the retention factor (R_F, cm/cm) calculations of the separated zones, i.e., the R_F values of the compounds in the extracts, were compared to the R_F values of the standards.

2.3.6. LC-MS Identification of 5-Methoxy Luteolinidin

LC-MS analysis was carried out with a Thermo Q Exactive mass spectrometer coupled with a Vanquish LC system (Thermo Scientific, San Jose, CA, USA) incorporated with an electrospray ionization (ESI) for UHPLC-HRMS/MS analysis. Chromatographic separation was performed using an Accucore Gemini NX-C18 column (50 mm × 2.1 mm; i.d. 2.6 μm, Thermo Scientific, San Jose, CA, USA). The binary mobile phase system consisted of water with 0.1% formic acid (FA) (phase A) and acetonitrile (ACN) with 0.1% FA (phase B). The flow rate was 0.2 mL/min, and the injection volume was 3 μL. The column was eluted with a gradient program (2% B from 0 to 1 min, 2% to 45% B from 1 to 2 min, 45% to 80% B from 2 to 6 min, 80% to 100% B from 6 to 6.5 min, and held at 100% for 1 min, then re-equilibrated with 2% B for another 1 min). The mass conditions were optimized using a mixture of apigenidin and luteolinidin. The negative ion polarity mode was set for an ESI ion source with the voltage on the ESI interface maintained at 2.65 kV. Nitrogen gas was used as the sheath gas at a flow rate of 20 AU and the auxiliary gas at 10 AU. The collision-induced dissociation (CID) was conducted with an isolation width of 1.5 Da and a normalized collision energy of 20, 40, and 60 for MS/MS analysis.

2.4. Statistical Analysis

The one-way analysis of variance (ANOVA) with post hoc Tukey's test was used to evaluate the statistical differences. GraphPad Prism version 9.5.1 was used. Data are represented as mean $\pm$ standard deviation of three independent experiments, unless otherwise stated.

3. Results and Discussion

3.1. Selection of Temperature for Obtaining Phenolic Compounds via PLE

Increasing the temperature increased the global yield of Sumac bran, PI570366 bran, and the SC991 leaf and leaf sheaths (Figure 2a). A higher yield of crude extract was not associated with a higher concentration of target compounds, but the association of pressurized solvent with the temperature may allow the selection of other classes of compounds, or the use of temperatures higher than 100 °C may allow the formation of degradation products in the extracts, including hydroxymethylfurfural [26]. Since there was no statistical difference in TPC for the temperatures used (Figure 2b), 100 °C was selected to generate high phenolic extracts for subsequent acid hydrolysis. The TPC detected in PI570366 at 100 °C was 0 mgGAE/g, which is comparable to 45 mgGAE/g found in Chinese whole sorghum grain extracted with subcritical water at 150 °C [4]. For the replicates reproduced at 100 °C, the global yield values were 8.20 $\pm$ 0.94 g/100 g for Sumac, 13.65 $\pm$ 4.16 g/100 g for PI570366, and 12.06 $\pm$ 0.28 g/100 g for SC991 (Figure 2a).

3.2. Acid Hydrolysis of PLE Extracts

3.2.1. Color Analysis

The color, hue, and chroma results of the acidified extracts post PLE extraction are represented in Figure 3. In our extracts, the redness of extracts represented by *a** coordinate increased after acidification, while the lightness (*L**) and yellowness (represented by *b**) decreased (Figure 3a–c).

For all extracts, the hue angle ranged between 0 and 1, which is an interval expected for red color, as highlighted by the *a** values. Interestingly, increasing the HCl concentration had an inverse effect on hue in Sumac and SC991. The hue angles in the extracts were lower than the hue 31–59° range previously reported in high-tannin black sorghum extracts [27]. The color saturation represented by chroma (*C**) increased significantly after acidification in all extracts (Figure 3g–i). Our values differed from those found by Wizi and coworkers [14], who dyed wool fabrics with sorghum leaf sheath extracts acidified with HCl at 1%, and found *L** of 26.4–31.5°, *a** = 15.5–23.7, *b** = 13.1–19.1, H = 38.9–40°, and *C** = 26.4–31.5. Our values also differed from those found in the ethanolic extracts of leaf sheaths harvested from Benin: *L** = 28.1°, *a** = 5.6, *b** = −0.4 [28].

3.2.2. Effect of Acidification on Total Phenolic Content

The highest TPC detected in PI570366 was 27.56 mgGAE/g. In Sumac, the TPC ranged from 17.73 to 26.46 mgGAE/g. This was within the range of 12.03–124.85 mgGAE/g reported by Herald and coworkers [18], who detected polyphenols in non-tannin and high-tannin genotypes [18]. No statistical differences were detected in TPC between non-acidified and acidified extracts, except for SC991 between 0 and 0.5% HCl, which increased from 10.32 mg GAE/g to 15.17 mg GAE/g (Figure 4c). Kayodé and coworkers reported much higher concentrations of 103–411 mg GAE/g TPC in their acidified extracts at 1% HCl (*v*/*v*) obtained from leaf sheaths cultivated from an unknown genotype in Benin [28]. Such differences may be attributed to genotypes, location, and plant growing conditions [29].
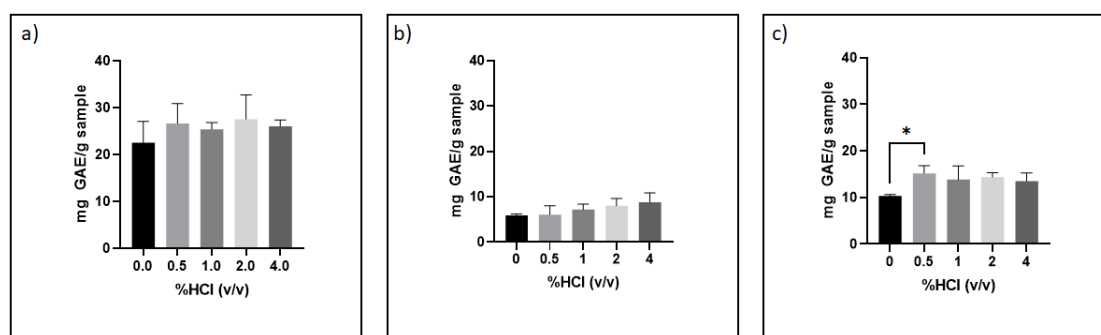
Figure 4. Total phenolic content of (**a**) PI570366, (**b**) Sumac, and (**c**) SC991 at various acid levels. The data represent mean $\pm$ standard deviations of three independent experiments. Significance: * $p \leq 0.05$.

3.2.3. Effect of Acidification on DPPH Antioxidant Capacity

The antioxidant capacity of non-acidified and acidified extracts is represented in Figure 5. Similar to TPC, there were no statistical differences detected after HCl acidification. Interestingly, the phenolic compounds' potential against free radicals is dependent on pH, i.e., at acidic conditions, the phenolics are deprotonated, leading to the H-atom transfer and consequently decreasing the oxidative stress [30]. The variety with the highest DPPH radical scavenging capacity was the PI570366 bran extract, which peaked 1968.24 mg TE/g at 0% HCl. The Sumac bran extracts ranged from 348.93 to 467.59 mg TE/g. This is in agreement with the 35.47–742.63 mg TE/g detected in the bran of tannin and non-tannin lines of sorghum [18], and higher than the 1.65–2.11 mgTE/g detected in the bran of non-tannin white and red sorghum varieties [22]. The SC991 leaves and leaf sheaths ranged from 551.85 mg TE/g to 645.27 mg TE/g. No significant effect of acidification on the antioxidant capacity was found.
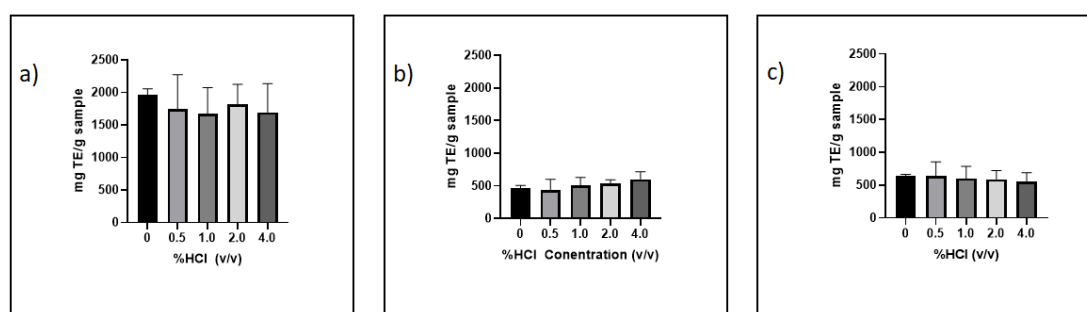

Figure 5. DPPH radical absorbance capacity for of (**a**) PI570366, (**b**) Sumac, and (**c**) SC991 at various acid levels.

3.2.4. Phenolic Profile Detected by HPLC

Kaempferol, syringic acid, rutin, pelargonidin, malvidin, and peonidin were not identified in any of the extracts. In the PLE extracts, the phenolic acids and some flavonoids were found mostly in the control solvent, i.e., methanol (Table 1A–C). Such reduced detection in phenolic species after acidification may be justified by the (A) binding of phenolics to the plant matrix—for instance, p-coumaric acid and ferulic acid are easily released after the alkaline hydrolysis of lignocellulosic materials, since alkaline hydrolysis is suitable to dissolve lignin [31]; (B) degradation of phenolics after interaction with HCl [32]; and (C) precipitation of phenolics because of the reduced solubility. In addition, many phenolics are extracted as glycosides. For instance, flavonoids are generally present in C-glycoside and O-glycoside forms. The linkage between flavonoids and sugar moieties can occur through an OH group to form O-glycosides or through carbon–carbon bonds to form C-glycosides [33]. In sorghum bran genotypes, the glycosides of luteolinidin, apigenidin, kaempferol, luteolin, and apigenin were detected previously by LC-MS [27,34,35].

Table 1. (**A**) Phenolic compounds quantified (µg/g raw material) in acidified PLE extracts obtained from Sumac bran extract. (**B**) Phenolic compounds quantified (µg/g raw material) in acidified PLE extracts obtained from PI570366 bran extract. (**C**) Phenolic compounds quantified (µg/g raw material) in acidified PLE extracts obtained from SC991 leaf and leaf sheath.

(A)							
				% HCl			
Subclass	**Compound**	**λ nm**	*0%*	*0.50%*	*1%*	*2%*	*4%*
3-deoxyanthocyanidin	Luteolinidin	510	60.63 ± 7.88 [b]	101.56 ± 14.09 [ab]	107.89 ± 20.65 [ab]	102.71 ± 30.73 [ab]	132.30 ± 21.94 [a]
	Apigenidin		7.62 ± 0.30 [b]	14.20 ± 1.77 [a]	14.56 ± 0.22 [a]	15.19 ± 2.05 [a]	17.14 ± 1.85 [a]
Benzoic acid	Protocatechuic acid	280	15.96 ± 0.53 [b]	26.36 ± 8.71 [a]	17.27 ± 3.93 [b]	21.19 ± 2.70 [ab]	15.63 ± 1.97 [b]
Flavanol	Catechin	280	67.48 ± 2.32 [a]	7.50 ± 2.53 [b]	4.04 ± 0.91 [b]	12.16 ± 4.07 [b]	3.88 ± 2.79 [b]
Flavanonol	Taxifolin	280	261.32 ± 11.06 [a]	61.22 ± 15.93 [b]	76.39 ± 88.72 [b]	54.61 ± 20.57 [b]	88.14 ± 101.36 [b]
Flavanone	Eriodictyol	280	10.71 ± 0.47 [a]	20.92 ± 13.21 [a]	58.89 ± 49.82 [a]	78.46 ± 35.40 [a]	86.02 ± 99.28 [a]
	Naringenin		0 [#b]	12.20 ± 5.18 [a]	20.12 ± 13.88 [a]	24.12 ± 6.17 [a]	27.67 ± 27.26 [a]
Flavonol	Quercetin	280	26.09 ± 0.56 [a]	13.72 ± 2.28 [a]	12.35 ± 4.63 [a]	11.46 ± 5.19 [a]	11.45 ± 14.01 [a]
Cinnamic acid	Chlorogenic acid	320	14.09 ± 0.24 [a]	0 [#b]	0 [#b]	0 [#b]	0 [#b]
	Caffeic acid		9.25 ± 0.13 [a]	6.16 ± 0.37 [b]	6.09 ± 1.31 [b]	5.38 ± 0.09 [b]	0 [#c]
	p-Coumaric acid		2.53 ± 0.49 [a]	2.06 ± 1.59 [a]	4.23 ± 3.25 [a]	3.53 ± 1.13 [a]	0.78 ± 0.84 [a]
	Ferulic acid		6.28 ± 0.35 [a]	6.59 ± 0.52 [a]	6.94 ± 3.59 [a]	5.87 ± 0.81 [a]	5.10 ± 0.09 [a]
Flavone	Apigenin	320	5.18 ± 0.21 [a]	5.41 ± 0.10 [a]	5.42 ± 0.18 [a]	5.55 ± 0.20 [a]	7.74 ± 4.19 [a]
	Luteolin		27.87 ± 1.26 [b]	43.24 ± 3.00 [ab]	40.12 ± 6.25 [ab]	41.03 ± 4.73 [ab]	46.83 ± 10.19 [a]
Anthocyanidin	Cyanidin	510	0 [#b]	35.95 ± 0.36 [ab]	43.51 ± 13.25 [a]	41.32 ± 4.79 [ab]	60.95 ± 30.81 [a]
(B)							
				% HCl			
Subclass	**Compound**	**λ nm**	*0%*	*0.50%*	*1%*	*2%*	*4%*
3-deoxyanthocyanidin	Luteolinidin	510	613.87 ± 83.52 [b]	1067.40 ± 117.92 [a]	1113.65 ± 96.63 [a]	1197.85 ± 82.93 [a]	1156.78 ± 221.78 [a]
	Apigenidin		85.77 ± 11.17 [b]	161.81 ± 33.35 [ab]	169.41 ± 22.10 [a]	142.17 ± 46.67 [ab]	167.34 ± 20.75 [a]
	7-methoxyapigenidin		129.79 ± 4.70 [b]	672.37 ± 66.07 [a]	744.85 ± 30.73 [a]	835.84 ± 110.99 [a]	726.24 ± 75.45 [a]
Benzoic acid	Protocatechuic acid	280	7.16 ± 0.38 [b]	18.84 ± 1.40 [a]	18.69 ± 1.05 [a]	18.22 ± 4.58 [a]	16.39 ± 5.25 [a]
	4-hydroxybenzoic acid		13.05 ± 3.16 [ab]	21.14 ± 3.69 [a]	18.59 ± 2.36 [a]	17.04 ± 12.36 [a]	0 [#b]

Table 1. *Cont.*

(B)

Subclass	Compound	λ nm	% HCl				
			0%	*0.50%*	*1%*	*2%*	*4%*
Flavanol	Catechin	280	41.62 ± 5.97 b	129.34 ± 21.54 ab	81.94 ± 23.07 b	121.70 ± 51.67 b	308.40 ± 141.11 a
Flavanone	Eriodictyol	280	12.11 ± 1.42 b	75.40 ± 16.50 a	41.43 ± 15.80 ab	58.82 ± 27.78 ab	81.11 ± 10.45 a
	Naringenin		0 #c	21.30 ± 4.64 ab	17.23 ± 3.40 b	24.79 ± 7.47 ab	32.07 ± 2.91 a
Flavone	Luteolin	280	32.22 ± 6.50 b	53.17 ± 6.29 ab	49.19 ± 3.91 ab	56.70 ± 10 a	48.16 ± 4.28 ab
	Apigenin		5.08 ± 0.23 a	5.87 ± 0.67 a	5.70 ± 0.07 a	5.92 ± 0.18 a	5.82 ± 0.64 a
Cinnamic acid	Chlorogenic acid	320	30.05 ± 5.26 a	11.42 ± 1.08 b	11.15 ± 0.95 b	12.13 ± 0.37 b	12.61 ± 1.28 b
	p-Coumaric acid		2.19 ± 1.35 ab	2.93 ± 0.81 a	0 #b	0.94 ± 1.92 ab	0 #b
	Ferulic acid		7.19 ± 0.99 a	12.23 ± 1.93 a	8.48 ± 1.34 a	8.60 ± 8.89 a	7.26 ± 1.09 a
Anthocyanidin	Cyanidin	510	0 #b	67.10 ± 17.20 ab	92.53 ± 11.72 ab	139.79 ± 67.41 ab	165.35 ± 108.58 a

(C)

Subclass	Compound	λ nm	% HCl				
			0%	*0.50%*	*1%*	*2%*	*4%*
3-deoxyanthocyanidin	Luteolinidin	510	295.43 ± 20.10 a	1499.37 ± 508.47 a	1486.01 ± 396.13 a	1514.00 ± 710.02 a	1595.43 ± 692.57 a
	Apigenidin		40.29 ± 7.70 a	NQ	NQ	NQ	NQ
	7-methoxyapigenidin		123.13 ± 17.81 b	1170.42 ± 313.44 a	1515.29 ± 499.65 a	1517.42 ± 358.27 a	1640.87 ± 284.82 a
	5-methoxyluteolinidin		0 #b	1733.41 ± 110.50 a	1861.12 ± 95.90 a	1881.75 ± 355.67 a	1765.45 ± 52.60 a
Benzoic acid	Protocatechuic acid	280	15.75 ± 0.09 a	15.85 ± 0.79 a	16.52 ± 1.18 a	17.37 ± 2.25 a	18.03 ± 2.33 a
	4-hydroxybenzoic acid		56.77 ± 0.93	84.81 ± 57.03	90.72 ± 47.72	35.72 ± 47.18	12.45 ± 6.57
Flavanone	Naringenin	280	9.74 ± 0.40 a	0 #b	0 #b	0 #b	0 #b
Flavone	Luteolin	320	235.44 ± 8.64 a	362.26 ± 22.19 a	301.59 ± 71.29 a	265.14 ± 101.79 a	260.38 ± 96.03 a
	Apigenin		53.49 ± 2.32 a	41.92 ± 3.33 ab	34.18 ± 4.73 b	31.05 ± 7.51 b	28.94 ± 6.60 b
Cinnamic acid	Chlorogenic acid	320	43.71 ± 0.37 a	13.71 ± 2.69 b	0 #c	0 #c	0 #c
	p-Coumaric acid		138.67 ± 4.19 ab	173.01 ± 13.66 a	90.74 ± 48.62 bc	33.79 ± 17.46 cd	21.65 ± 14.56 d

(**A**) Average ± standard deviation of three biological replicates. Different letters indicate statistical differences ($p \leq 0.05$) within each row. # For values below detection threshold, a value of 0 (zero) was assigned for statistical analysis. (**B**) Average ± standard deviation of three biological replicates. Different letters indicate statistical differences ($p \leq 0.05$) within each row. # For values below detection threshold, a value of 0 (zero) was assigned for statistical analysis. (**C**) Average ± standard deviation of three biological replicates. Different letters indicate statistical differences ($p \leq 0.05$) within each row. # For values below detection threshold, a value of 0 (zero) was assigned for statistical analysis. NQ: Apigenidin was not quantifiable when 5-methoxyluteolinidin was present due to peak overlap.

Flavonoids: 3-Deoxyanthocyanidins

In the non-acidified extract, luteolinidin eluted at 18.25 min, apigenidin at 21.42 min, and 7-methoxyapigenidin at 23.8–24 min. Prominent, unknown peaks at 21.95, 22.16, 23.37, and 27.10 min were observed at the 510 nm wavelength (Figure S1.9). The peak at 21.945 min was identified as 5-methoxyluteolinidin by LC-MS. As shown in Figure 6, this unknown compound (RT: 6.75 min) had the molecular ion at m/z 283.0608 ((M − H) − calculated 283.0605) under high-resolution negative ESI mode, which showed one more methyl unit than luteolinidin (m/z 269.0450). The typical fragment ions at m/z 268.0375, 240.0425, and 196.0526 of this unknown compound are similar to those of 5-methoxyluteolinidin reported from sorghum (Peak 75 in Xiong and coworkers [24]). Therefore, this compound was tentatively characterized as 5-methoxyluteolinidin.

Figure 6. Ion chromatogram (**A**) under selected ion mode and MS^2 spectrum (**B**) of 5-methoxyluteolinidin in sorghum samples at negative ESI mode.

In Sumac bran, the luteolinidin increased from 60.63 µg/g to 132.30 µg/g (Table 1A) after acidification at 4% *v/v* HCl, which was significant compared to the control. The luteolinidin in acidified PI570366 bran ranged from 1067.4 to 1197.85 µg/g, which was significantly higher than in the non-acidified extracts (613 µg/g). There were no statistical differences among the HCl treatments (Table 1B). The luteolinidin concentration in non-acidified and acidified PI570366 extracts (Table 1B) was higher than the 319.9 µg/g obtained after the microwave-assisted extraction of non-tannin black sorghum genotypes, as reported by others [9].

In the SC991 leaf extracts, the luteolinidin increased after acidification, but the difference was not significant. According to Mizuno et al. [36], the RedforGreen (RG) mutant genotype contains over 1000-fold more 3-DA than the wild sorghum genotypes. However, the luteolinidin found by Petti et al. [37] in the RG genotype (1768 µg/g) is comparable to the SC991 leaf extracts (1486.01–1595.43 µg/g) reported in Table 1C. This contradicts Mizuno et al.'s [36] findings and support the findings of Petti et al. [37] that some natural genotypes of sorghum may also serve as low-cost sources of 3-DAs, i.e., pigments. 3-DAs provide superior properties such as improved stability after long storage, resistance to thermal processing, acidification, and color bleaching when compared to other naturally derived pigments such as carotenoids [31,34,38].

After acidification, the apigenidin peak at 22.16 min was dominated by 5-methoxyluteolinidin (identified by LC-MS) in SC991 leaf and leaf sheaths. This made apigenidin not quantifiable using the current methods after the appearance of the 5-methoxyluteolinidin peak. Figure S1.10, available in the Supplementary Materials, shows the peak separation after spiking the acidified SC991 extract with apigenidin.

The apigenidin increased twofold after acidification in the Sumac bran extracts after 0.5% *v/v* HCl treatment with no additional significant increase at higher HCl concentrations. In the PI570366 bran extracts, the apigenidin significantly increased at 1% *v/v* HCl with no additional increase at higher HCl concentrations. In the SC991 extracts, apigenidin was not quantified after acid treatment due to the reasons stated previously.

In the Sumac bran extract, 7-methoxyapigenidin was not detected. In the PI570366 bran and SC991 leaf extracts, 7-methoxyapigenidin significantly increased 5-fold and 10-fold with HCl treatments. There was no additional significant increase in 7-methoxyapigenidin at HCl concentrations higher than 0.5% *v/v*. 5-methoxyluteolinidin was only detected in SC991 leaf tissue after the acid treatments; therefore, the amount of 5-methoxyluteolinidin increased from non-detectable to a range of 1733.41–1881.75 μg/g raw material after acid treatment. There were no statistical differences between the acid treatment concentrations.

Overall, the acid treatments greatly improved the quantifiable levels of 3-DA. The increase in the 3-DA concentrations was achievable using 0.5% *v/v* HCl.

Flavonoids: Other Classes

In the Sumac and PI570366 extracts, cyanidin could only be detected after acidification (Figures S1.3 and S1.6), and the highest significance was found at 4% HCl (*v/v*) for both genotypes (Table 1A,B).

A non-identified compound that eluted at 6.55 min (Figure S1.1) and taxifolin (261.32 μg/g) were the most prominent compounds in the non-acidified extracts of Sumac at 280 nm. In Sumac, the acidification significantly decreased taxifolin from 261.32 μg/g to 54.61–88.14 μg/g and catechin from 67.48 μg/g to 3.88–12.16 μg/g.

In the PI570366 extracts, a significant catechin increase was observed at 4% HCl (Table 1B). Condensed tannin monomers such as catechin could not be found in SC991, which was different from the findings of Oboh and coworkers, who detected 16.09 mg/g catechin in Nigerian sorghum stems [39].

In the non-acidified extracts of Sumac and PI570366, a prominent peak at approximately 34.17 min with different spectra (Figure S1.1) was observed, and after acidification, the peak was replaced by naringenin at 33.99 min. In Sumac, no statistical differences in naringenin were found across the HCl treatments. In PI570366, naringenin could only be detected in the acidified extracts (Table 1B). In PI570366, 0.5%, 2%, and 4% HCl significantly increased the naringenin.

Glycosylation, or the hydrolysis of glycosides, converts the theoretical phenolic glycosides present in the extracts into aglycones such as naringenin, which is an aglycone form of naringin. According to Mizuno et al. [36], naringenin is considered the branching point of the metabolic pathway of 3-DA. The acidification favored the release of both naringenin and 3-DA in PI570366. Interestingly, no naringenin was found in the SC991 leaf extracts after acidification (Table 1C), even with the substantial increase in 3-DA.

In the Sumac and PI570366 extracts, no statistical difference in apigenin content was observed within the HCl treatments (Table 1A,B), whereas for the SC991 extracts, apigenin decreased significantly after acidification.

In the grain extracts, luteolin significantly increased in Sumac from 27.87 μg/g in the non-acidified extract to 46.83 μg/g at 4% HCl (Table 1A), and in PI570366 from 32.22 μg/g in the non-acidified extract to 56.70 μg/g at 2% HCl (Table 1B). In the SC991 extracts, no additional significant luteolin increase was observed with increasing HCl percentage, from which the content ranged from 260.38 μg/g to 362.26 μg/g (Table 1C).

After acidification, there were no significant differences in eriodictyol concentrations in Sumac (Table 1A), whereas in PI570366, 0.5%, 2%, and 4% eriodictyol significantly increased from 12.11 μg/g in the non-acidified extract to 75.40 μg/g in 0.5% HCl (Table 1B). No eriodictyol was detected in the SC991 extracts.

Non-Flavonoids

In Sumac, the effect of acidification on the protocatechuic acid was most significant at 0.5% HCl (Table 1A). In PI570366, acidification increased protocatechuic acid from 7.16 μg/g in the non-acidified extract to 16.39–18.84 μg/g in the acidified extracts, representing an increase of over 50% (Table 1B). In SC991, no significant changes in protocatechuic acid were detected throughout the acidification (Table 1C).

In the Sumac extracts, no significant increase was found in p-coumaric after acidification. For PI570366, a 33% increase in the p-coumaric acid content was observed from 2.19 µg/g in the non-acidified extract to 2.93 µg/g detected in the extract acidified with 0.5% HCl, and a significant decrease to zero in the p-coumaric acid concentration was observed with increases toward a higher HCl content (Table 1B). In the SC991 extracts, p-coumaric increased from 138.67 µg/g in the non-acidified extract to 173.01 µg/g in the acidified extract at 0.5% HCl, followed by decrease with the HCl concentration (Table 1C).

Caffeic acid was only detected in the Sumac bran, from which a significant decrease was observed from 9.25 µg/g in the non-acidified extract to 0 µg/g at 4% HCl (Table 1A).

In the Sumac and PI570366 extracts, no significant increase in ferulic acid was observed after HCl treatment (Table 1A,B).

Acidification reduced chlorogenic acid in all products studied. Chlorogenic acid reductions to 0 µg/g were observed in Sumac and SC991 with HCl concentrations. In PI570366, acidification decreased the chlorogenic acid significantly; however, no additional reductions were observed at higher HCl concentrations (Table 1B).

3.2.5. Thin-Layer Chromatography (TLC)

Multiple bands of compounds were detected in non-acidified extracts of SC991 and PI570366 at 366 nm. However, in Sumac, pale purple spots were observed at visible light. The separation of the bands in the stationary phase decreased according to the HCl concentration. The silica gel used as the stationary phase is a polar, and slightly acidic adsorbent. Therefore, the HCl present in some extracts negatively affected the binding ability of the analytes to the stationary phase. There are no analytical standards available for 5-methoxyluteolinidin, so we were not able to establish qualitative TLC comparisons with HPLC results for this compound.

Pure luteolinidin, after elution, resulted in a long purple spot at $R_F = 0.27$ (Figure 7a). Acidification increased the TLC intensity of luteolinidin in SC991 followed by the PI570366 extracts. However, in Sumac, the luteolinidin streaks were almost imperceptible (Figure 7a). Like the HPLC analysis, the TLC showed that luteolinidin is the dominant 3-DA in PI570366 and SC991 extracts. The low concentration of luteolinidin in Sumac detected by HPLC justifies its lack of intensity in the TLC plates.
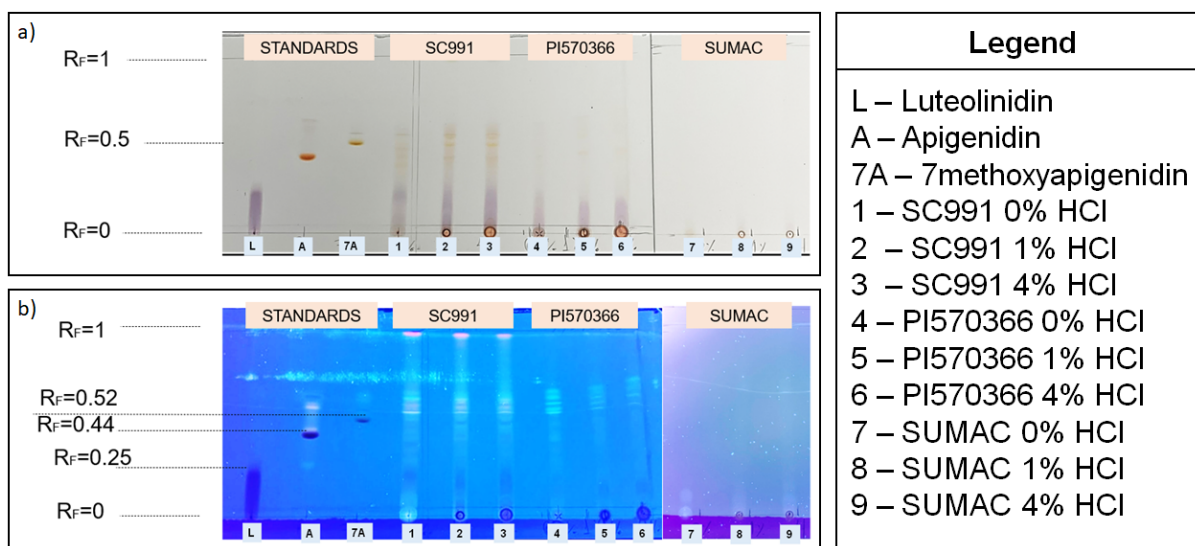

Figure 7. Thin-layer chromatography of 3-deoxyanthocyanidins and the selected extracts at (**a**) visible light and (**b**) 366 nm.

Apigenidin standard elution resulted in a red ($R_F = 0.45$) and pale purple ($R_F = 0.60$ and 0.63) spot under visible light that emitted dark blue and orange fluorescent colors at 366 nm. In the non-acidified SC991 extracts, the orange fluorescent bands matched with

apigenidin at R_F = 0.63. In the acidified extracts, we found that the prominent red spot's intensity decreased (Figure 7b). At R_F = 0.44, red spots of low intensity under visible light and at R_F = 0.52, orange fluorescent spots at 366 nm were observed in the SC991 extracts, which were qualitatively comparable with the apigenidin standard (Figure 7b). In the acidified PI570366 extract at 0.5%, it was possible to observe a pale red spot at R_F = 0.44, comparable to the apigenidin standard. Although detected in Sumac via HPLC, apigenidin could not be qualitatively detected in the TLC plates.

The pure 7-methoxyapigenidin eluted as a dark blue spot at R_F = 0.52, which could be visualized in the acidified SC991 extracts at low intensity, but could not be visualized in the PI570366 and Sumac extracts.

4. Conclusions

In this work, a two-step strategy was developed with aim of (1) extracting sorghum phenolics in a high-throughput process, (2) improving sorghum phenolic detectability with post extraction acid treatments, and (3) evaluating the concentration of acids used for post extraction treatment to improve green chemistry practices.

The color analysis indicated that acidification decreased the luminosity and increased the redness of the extracts. Even with almost no statistical increase found in TPC and antioxidant capacity with acidification, the HPLC analysis showed us diversity in the phenolic species present in each genotype and how the release of the same compounds differed after acidification across the genotypes studied. For instance, in the SC991 extracts, apigenidin was not detected in the acidified extracts by HPLC because 5-methoxyluteolinidin became dominant. Increasing the HCl concentration above 0.5% did not significantly increase the anthocyanidin or the 3-DA. For this reason, our results show that the addition of HCl at concentrations of 0.5% *v/v* would be enough for the detection of the purple colorant cyanidin and 5-methoxyluteolinidin and to increase the concentration of luteolinidin and 7-methoxyapigenidin twofold.

Interestingly, cyanidin was not detected in the Sumac and PI570366 extracts until the acid treatment. Overall, the chlorogenic acid, caffeic acid, taxifolin, and quercetin significantly decreased after the acidification of the extracts. In addition, there were no statistical differences after acidification for protocatechuic and ferulic acids.

The qualitative (TLC) and quantitative (HPLC) analysis of 3-deoxyanthocyanidins showed that luteolinidin is the most abundant compound and its quantified amount increased with HCl addition, highlighting the byproducts of sorghum as a low-cost source of red colorants with desirable properties for industry.

standards consisted of: catechin, chlorogenic acid, p-coumaric acid, taxifolin, cyanidin, apigenidin, quercetin, and apigenin. The concentration of standards was 2.25 µg/g. Figure S2.3 The representative chromatograms of non-spiked extract mixture used for method validation at 280 nm (A), 320 nm (B) and 510 nm (C). Table S2.1. The calibration curve, correlation factor (R^2), limit of detection (LOD) and limit of quantification (LOQ). Table S2.2. Within day and between day precision evaluated at (2.25, 12.5, 22.5 µg/g) in sorghum.

Author Contributions: Conceptualization: Á.L.S., J.P., K.S. and D.S. Methodology: Á.L.S., J.P., K.S., R.P., C.H., S.S. and D.S. Validation: Á.L.S., J.P., R.P., C.H., S.S., K.S. and D.S. Formal analysis: Á.L.S., J.P., R.P., C.H., S.S., K.S. and D.S. Investigation: Á.L.S., J.P., K.S., R.P. and D.S. Resources: K.S. and D.S. Data curation: Á.L.S., J.P., R.P., C.H., S.S., K.S. and D.S. Writing—original draft: Á.L.S., J.P., K.S., R.P., C.H., S.S. and D.S. Writing—review and editing: Á.L.S., J.P., K.S., R.P., C.H., S.S. and D.S. Visualization: Á.L.S., J.P., K.S. and D.S. Supervision: K.S. and D.S. Project administration: K.S. and D.S. Funding acquisition: D.S. All authors have read and agreed to the published version of the manuscript.

Funding: The funding for this work was provided by the USDA-ARS under non-assistance cooperative agreement numbers 3020-43440-002-018S and 3020-43440-002-020S.

Data Availability Statement: Data will be uploaded into https://data.nal.usda.gov/ no later than 12 months after the publication of the manuscript.

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

Disclaimer: Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. USDA is an equal opportunity provider and employer.

Abbreviations

ASE	Accelerated solvent extraction
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
LC-MS	Liquid chromatography–mass spectrometry
PLE	Pressurized liquid extraction
TLC	Thin-layer chromatography
TPC	Total phenolic content
3-DA	3-deoxyanthocyanidins

References

1. Gleave, G.L.; Roethe, N.J.; Kemp, D.W.; Richter, B.E.; Ezzel, J.L. Automated Accelerated Solvent Extraction Apparatus and Method. U.S. Patent 5,785,856, 28 July 1998.
2. Cardenas-Toro, F.P.; Forster-Carneiro, T.; Rostagno, M.A.; Petenate, A.J.; Maugeri Filho, F.; Meireles, M.A.A. Integrated supercritical fluid extraction and subcritical water hydrolysis for the recovery of bioactive compounds from pressed palm fiber. *J. Supercrit. Fluids* **2014**, *93*, 42–48. [CrossRef]
3. Lachoz-Perez, D.; Brown, A.B.; Mudhoo, A.; Martinez, J.; Timko, M.T.; Rostagno, M.A.; Forster-Carneiro, T. Applications of subcritical and supercritical water conditions for extraction, hydrolysis, gasification, and carbonization of biomass: A critical review. *Biofuel Res. J.* **2017**, *14*, 611–626. [CrossRef]
4. Luo, X.; Cui, J.; Zhang, H.; Duan, Y. Subcritical water extraction of polyphenolic compounds from sorghum (*Sorghum bicolor* L.) bran and their biological activities. *Food Chem.* **2018**, *262*, 14–20. [CrossRef]
5. Dykes, L.; Seitz, L.M.; Rooney, W.L.; Rooney, L.W. Flavonoid composition of red sorghum genotypes. *Food Chem.* **2009**, *116*, 313–317. [CrossRef]
6. Cox, S.; Noronha, L.; Herald, T.; Bean, S.; Lee, S.-H.; Perumal, R.; Wang, W.; Smolensky, D. Evaluation of ethanol-based extraction conditions of sorghum bran bioactive compounds with downstream anti-proliferative properties in human cancer cells. *Heliyon* **2019**, *5*, e01589. [CrossRef]
7. Hou, F.; Su, D.; Xu, J.; Gong, Y.; Zhang, R.; Wei, Z.; Chi, J.; Zhang, M. Enhanced Extraction of Phenolics and Antioxidant Capacity from Sorghum (*Sorghum bicolor* L. Moench) Shell Using Ultrasonic-Assisted Ethanol–Water Binary Solvent. *J. Food Process. Preserv.* **2016**, *40*, 1171–1179. [CrossRef]
8. Wu, Y.; Wang, Y.; Liu, Z.; Wang, J. Extraction, Identification and Antioxidant Activity of 3-Deoxyanthocyanidins from Sorghum bicolor L. Moench Cultivated in China. *Antioxidants* **2023**, *12*, 468. [CrossRef] [PubMed]

9. Herrman, D.A.; Brantsen, J.F.; Ravisankar, S.; Lee, K.-M.; Awika, J.M. Stability of 3-deoxyanthocyanin pigment structure relative to anthocyanins from grains under microwave assisted extraction. *Food Chem.* **2020**, *333*, 127494. [CrossRef]

10. Barros, F.; Dykes, L.; Awika, J.M.; Rooney, L.W. Accelerated solvent extraction of phenolic compounds from sorghum brans. *J. Cereal Sci.* **2013**, *58*, 305–312. [CrossRef]

11. Hefni, M.E.; Amann, L.S.; Witthöft, C.M. A HPLC-UV Method for the Quantification of Phenolic Acids in Cereals. *Food Anal. Methods* **2019**, *12*, 2802–2812. [CrossRef]

12. İlbay, Z.; Şahin, S.; Büyükkabasakal, K. A novel approach for olive leaf extraction through ultrasound technology: Response surface methodology versus artificial neural networks. *Korean J. Chem. Eng.* **2014**, *31*, 1661–1667. [CrossRef]

13. Chaves, J.O.; Souza, M.C.; Silva, L.C.; Lachoz-Perez, D.; Torres-Mayanga, P.C.; Machado, A.P.F.; Forster-Carneiro, T.; Vazquez-Espinoza, M.; Gonzalez-de-Peredo, A.V.; Barbero, G.F.; et al. Extraction of Flavonoids From Natural Sources Using Modern Techniques. *Front. Chem.* **2020**, *8*, 507887. [CrossRef] [PubMed]

14. Wizi, J.; Wang, L.; Hou, X.; Tao, Y.; Ma, B.; Yang, Y. Ultrasound-microwave assisted extraction of natural colorants from sorghum husk with different solvents. *Ind. Crops Prod.* **2018**, *120*, 203–213. [CrossRef]

15. Paunović, D.Đ.; Mitić, S.S.; Kostić, D.A.; Mitić, M.N.; Stojanović, B.T.; Pavlović, J.L. Kinetics and Thermodynamics of the Solid-Liquid Extraction Process of Total Polyphenols From Barley. *Adv. Technol.* **2015**, *3*, 58–63. [CrossRef]

16. Ju, Z.Y.; Howard, L.R. Effects of Solvent and Temperature on Pressurized Liquid Extraction of Anthocyanins and Total Phenolics from Dried Red Grape Skin. *J. Agric. Food Chem.* **2003**, *51*, 5207–5213. [CrossRef]

17. Thermo Fisher Scientific. *Dionex ASE 350*; Thermo Fisher Scientific: Waltham, MA, USA, 2011.

18. Herald, T.J.; Gadgil, P.; Tilley, M. High-throughput micro plate assays for screening flavonoid content and DPPH-scavenging activity in sorghum bran and flour. *J. Sci. Food Agric.* **2012**, *92*, 2326–2331. [CrossRef]

19. Plank, D.W.; Szpylka, J.; Sapirstein, H.; Woolard, D.; Zapf, C.M.; Lee, V.; Chen, C.-Y.O.; Liu, R.H.; Tsao, R.; Dusterloch, A.; et al. Determination of Antioxidant Activity in Foods and Beverages by Reaction with 2,2′-Diphenyl-1-Picrylhydrazyl (DPPH): Collaborative Study First Action 2012.04. *J. AOAC Int.* **2012**, *95*, 1562–1569. [CrossRef] [PubMed]

20. Irakli, M.N.; Samanidou, V.F.; Biliaderis, C.G.; Papadoyannis, I.N. Simultaneous determination of phenolic acids and flavonoids in rice using solid-phase extraction and RP-HPLC with photodiode array detection. *J. Sep. Sci.* **2012**, *35*, 1603–1611. [CrossRef]

21. Lee, H.-S.; Santana, Á.L.; Peterson, J.; Yucel, U.; Perumal, R.; De Leon, J.; Lee, S.-H.; Smolensky, D. Anti-Adipogenic Activity of High-Phenolic Sorghum Brans in Pre-Adipocytes. *Nutrients* **2022**, *14*, 1493. [CrossRef]

22. Speranza, S.; Knechtl, R.; Witlaczil, R.; Schönlechner, R. Reversed-Phase HPLC Characterization and Quantification and Antioxidant Capacity of the Phenolic Acids and Flavonoids Extracted From Eight Varieties of Sorghum Grown in Austria. *Front. Plant Sci.* **2021**, *5*, 769151. [CrossRef] [PubMed]

23. Irakli, M.N.; Samanidou, V.F.; Biliaderis, C.G.; Papadoyannis, I.N. Development and validation of an HPLC-method for determination of free and bound phenolic acids in cereals after solid-phase extraction. *Food Chem.* **2012**, *134*, 1624–1632. [CrossRef]

24. Xiong, Y.; Zhang, P.; Warner, R.D.; Shen, S.; Johnson, S. HPLC-DAD-ESI-QTOF-MS/MS qualitative analysis data and HPLC-DAD quantification data of phenolic compounds of grains from five Australian sorghum genotypes. *Data Br.* **2020**, *33*, 106584. [CrossRef] [PubMed]

25. Bae, H.; Jayaprakasha, G.K.; Jifon, J.; Patil, B.S. Extraction efficiency and validation of an HPLC method for flavonoid analysis in peppers. *Food Chem.* **2012**, *130*, 751–758. [CrossRef]

26. Shapla, U.M.; Solayman, M.; Alam, N.; Khalil, M.; Gan, S.H. 5-Hydroxymethylfurfural (HMF) levels in honey and other food products: Effects on bees and human health. *Chem. Cent. J.* **2018**, *12*, 35. [CrossRef] [PubMed]

27. Brantsen, J.F.; Hermann, D.; Ravisankar, S.; Awika, J.M. Effect of tannins on microwave-assisted extractability and color properties of sorghum 3-deoxyanthocyanins. *Food Res. Int.* **2021**, *148*, 110612. [CrossRef] [PubMed]

28. Kayodé, A.P.P.; Bara, C.A.; Dalodé-Vieira, G.; Linnemann, A.R.; Mout, M.J.R. Extraction of antioxidant pigments from dye sorghum leaf sheaths. *LWT Food Sci. Technol.* **2012**, *46*, 49–55. [CrossRef]

29. Przybylska-Balcerek, A.; Frankowski, J.; Stuper-Szablewska, K. The influence of weather conditions on bioactive compound content in sorghum grain. *Eur. Food Res. Technol.* **2020**, *246*, 13–22. [CrossRef]

30. Di Meo, F.; Lemaur, V.; Cornil, J.; Lazzaroni, R.; Duroux, J.-L.; Olivier, Y.; Trouillas, P. Free Radical Scavenging by Natural Polyphenols: Atom versus Electron Transfer. *J. Phys. Chem. A* **2013**, *117*, 2082–2092. [CrossRef]

31. Zhao, J.; Ou, S.; Ding, S.; Wang, Y.; Wang, Y. Effect of activated charcoal treatment of alkaline hydrolysates from sugarcane bagasse on purification of p-coumaric acid. *Chem. Eng. Res. Des.* **2011**, *89*, 2176–2181. [CrossRef]

32. Nuutila, A.M.; Kammiovirta, K.; Oksman-Caldentey, K.-M. Comparison of methods for the hydrolysis of flavonoids and phenolic acids from onion and spinach for HPLC analysis. *Food Chem.* **2002**, *76*, 519–525. [CrossRef]

33. Wu, G.; Johnson, S.K.; Fang, Z. Sorghum non-extractable polyphenols: Chemistry, extraction and bioactivity. In *Non-Extractable Polyphenols and Carotenoids: Importance in Human Nutrition and Health*; Saura-Calixto, F., Pérez-Jiménez, J., Eds.; Royal Society of Chemistry: London, UK, 2018; pp. 326–344.

34. Ofosu, F.K.; Elahi, F.; Daliri, E.B.M.; Yeon, S.-J.; Ham, H.J.; Kim, J.-H.; Han, S.-I.; Oh, D.-W. Flavonoids in Decorticated Sorghum Grains Exert Antioxidant, Antidiabetic and Antiobesity Activities. *Molecules* **2020**, *25*, 2854. [CrossRef] [PubMed]

35. Pontieri, P.; Pepe, G.; Campiglia, P.; Mercial, F.; Basilicata, M.G.; Smolensky, D.; Calcagnile, M.; Troisi, J.; Romano, R.; Del Giudice, F.; et al. Comparison of Content in Phenolic Compounds and Antioxidant Capacity in Grains of White, Red, and Black Sorghum Varieties Grown in the Mediterranean Area. *ACS Food Sci. Technol.* **2021**, *1*, 1109–1119. [CrossRef]

36. Mizuno, H.; Yazawa, T.; Kasuga, S.; Sawada, Y.; Ogata, J.; Ando, Y.; Kanamori, H.; Yonemaru, J.-I.; Wu, J.; Hirai, M.Y.; et al. Expression level of a flavonoid 3'-hydroxylase gene determines pathogen-induced color variation in sorghum. *BMC Res. Notes* **2014**, *7*, 761. [CrossRef]

37. Petti, C.; Kushwaha, R.; Tateno, K.; Harman-Ware, A.E.; Crocker, M.; Awika, J.; DeBolt, S. Mutagenesis Breeding for Increased 3-Deoxyanthocyanidin Accumulation in Leaves of *Sorghum bicolor* (L.) Moench: A Source of Natural Food Pigment. *J. Agric. Food Chem.* **2014**, *62*, 1227–1232. [CrossRef] [PubMed]

38. Xiong, Y.; Zhang, P.; Warner, R.D.; Fang, Z. In vitro and cellular antioxidant activities of 3-deoxyanthocyanidin colourants. *Food Biosci.* **2021**, *42*, 101171. [CrossRef]

39. Oboh, G.; Adewuni, T.M.; Ademosun, A.O.; Olasehinde, T.A. Sorghum stem extract modulates Na^+/K^+-ATPase, ecto-5'-nucleotidase, and acetylcholinesterase activities. *Comp. Clin. Path.* **2016**, *25*, 749–756. [CrossRef]

40. Parkes, R.; McGee, D.; McDonnell, A.; Gillespie, E.; Touzet, N. Rapid screening of phenolic compounds in extracts of photosynthetic organisms separated using a C18 monolithic column based HPLC-UV method. *J. Chromatogr. B* **2022**, *1213*, 123521. [CrossRef] [PubMed]