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UPLC-QTOF-MS fingerprinting combined with chemometrics to assess the solvent extraction efficiency, phytochemical variation, and antioxidant activities of *Beta vulgaris L.*

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Abstract

Accurate assays of plant antioxidants and other phytochemicals require efficient extraction conditions and enable rigorous assessments of crop varieties and production systems. This study assessed the extraction of phytochemicals and antioxidants from conventionally or organically grown red and golden beets (*Beta vulgaris L.*), using twenty solvent (S1−S20) mixtures containing water, methanol, and ethanol alone or with acids (ascorbic, formic, acetic). Red beetroot extracted with methanol with or without acid had the highest betanin content (2791.0 μg/g and 8222.3 μg/g of fresh weight [FW], respectively) and golden beetroot extracted with methanol/ascorbic acid/water had the highest vulgaxanthin I (193.7 μg/g and 15.0 μg/g of FW, respectively). The radical-scavenging activity and total phenolics in beetroot extracts reflected the different extraction efficiency of each solvent. UHPLC-QTOF-MS was used to identify twenty-seven phytochemicals, including 23 betalains, 2 amino acids, and 2 phenolic acids. Chemometric approaches discriminated the beet varieties and different extracts within one variety based on the composition and abundance of the key phytochemicals. The red beetroot extracted with aqueous ethanol with or without acid (S5, S7, S8, S9), and golden beetroot extracted with methanol-containing solvents (S15 for conventionally and S20 for organically) had the highest levels of phytochemicals, suggesting that these conditions efficiently extract key phytochemicals.

Keywords: Red beet, Golden beet, UPLC-QTOF-MS, Antioxidant activities, Multivariate data analysis

1. Introduction

Epidemiological studies have demonstrated that the excessive production of reactive oxygen species (ROS) induces oxidative stress and increases the risk of chronic diseases such as cancer, diabetes, and cardiovascular diseases [1,2]. These studies also indicate that fruits and vegetables rich in bioactive compounds play a crucial role in the prevention of some chronic diseases and may provide desirable alternatives to some aspects of synthetic medicine due to their better compatibility with and fewer side effects to the human body [3].

Beets (*Beta vulgaris L.*) are common vegetables cultivated worldwide and consumers' increasing interest in the nutritional and heath-beneficial aspects of food has increased interest in this functional food [4]. Beetroots have significant antioxidant activities due to the presence of betalain derivatives and phenolic compounds. These compounds

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remove free radicals and protect the human body against damage from ROS [5]. Betalains are effective antioxidants due to the presence of phenolic hydroxyl groups, aromaticity, and other characteristic functional groups [6]. Betalains consist of red-violet betacyanins and yellow-orange betaxanthins, which are biosynthesized from tyrosine [5]. In addition to betalains, beetroots are rich in nitrate and other phytochemicals and the composition and levels of these compounds vary in different varieties of beets according to their phenotypes and genotypes. For example, red beetroot mainly contains betacyanins, whereas golden beetroot mainly contains betaxanthin.

Betalain levels, composition, and antioxidant activities are influenced by genetics, cultivars, production system, and processing techniques [7,8]. Recently, increasing consumption of organically grown foods has been associated with consumer interest in healthier and safer foods. Compared to conventionally grown foods, the organically grown food production system uses natural manures instead of artificial fertilizers, potentially resulting in more ingestion of health-promoting phytochemicals, allowing organic food to be perceived as healthier and safer than conventional foods [9]. Previous studies demonstrated beetroots grown under conventional and organic cultural practices tended to have different phytochemical contents, but the results were inconsistent or unsubstantiated [10]. Additionally, available studies comparing minerals in organically and conventionally grown beets are rare [11,12]. Therefore, a comprehensive study of mineral content and phytochemical profiles in both production systems is needed.

Comparing phytochemical profiles requires accurate methods for extracting and measuring phytochemicals. Solvent extraction is used for isolation of antioxidants and the extraction yield and resulting antioxidant activity are strongly dependent on the solvent, due to the different antioxidant potentials of compounds with different polarities. For example, the nature of the extraction solvent, polarity, and solvent combination play a crucial role in extracting the hydrophilic betalains [13]. Among various extraction techniques, solid-liquid extraction is commonly performed for extracting natural pigments [14]. Betalains are normally extracted from samples with organic solvents such as methanol, ethanol, acetone, and mixtures of solvents [15–17]; however, the levels of betalains and phytochemicals significantly differ for different extraction conditions. In addition, no specific or appropriate extraction solvent is recommended for optimal recovery of individual or total betalain content from fresh beet samples. Therefore, a comprehensive study for selecting the optimal solvent is required for beet varieties. The present study, using twenty different solvent compositions, was conducted to optimize the phytochemical extraction efficiency from red and golden beetroots grown in conventional and organic production systems. Further, HPLC and UPLC-QTOF-HR-MS were used to quantify and identify the phytochemicals present in the extracts. All the extracts were tested for total phenolics and radical scavenging activities to understand the effect of the production system and extraction efficiency in each beet variety.

2. Material and methods

2.1. Plant materials

Bunches of beetroots (*B. vulgaris L.*) were purchased at the local Farm Patch and HEB grocery stores (College Station, TX, USA). Samples included conventionally and organically grown red and golden beets. The leaves and stems were removed, and the roots were washed and wiped with paper towels. The beetroots were cut into cubes (1.5 × 1.5 × 1.5 cm) and processed in a 12-speed Oster blender (Sunbeam, FL, USA), then a known amount of each sample was weighed in separate 50-mL tubes and the rest of the paste was stored at −20 °C.

2.2. Chemicals

L-ascorbic acid, gallic acid, formic acid, acetic acid, phosphoric acid, sodium carbonate, Folin Ciocalteu (FC) reagent, 2, 2′-azinobis (3-etylbenzothiszoline-6-sulphonic acid) diammonium salt (ABTS), methanol, ethanol, LC-MS and HPLC grade acetonitrile, and crude betanin extracts were obtained from Sigma–Aldrich (St. Louis, MO, USA). All other reagents and solvents were analytical grade. Nanopure water (NANOpure, Barnstead, Dubuque, IA) was used for the entire study.

2.3. Mineral analysis

The cleaned beetroots were cut into pieces and lyophilized (LabConco, Kansas City, MO, USA) and 5 g samples were submitted to the Soil, Water, and Forage Testing Laboratory, Texas A&M University, College Station, TX in triplicate. Micronutrient quantification was conducted by inductively coupled plasma atomic emission spectroscopy (ICP-

AES). First the beet samples were digested with nitric acid 125 °C for 4 h and set aside at room temperature overnight. Then, the samples were diluted with doubled distilled water and analyzed by ICP-AES.

2.4. Extraction of betalains from beetroots

Five grams of frozen beetroot paste was extracted with 10 mL of solvent (see Table S1) in a 50-mL tube by vortexing (30 s), homogenizing (1 min) at room temperature (25 °C) in a dark room, and sonicating for (1 h) in an ice bath in dark conditions. The tubes were centrifuged at 800 $\times g$ for 15 min at 4 °C. The supernatant was collected, and the residue was extracted again with 3 mL of the same solvent. The supernatants were pooled, and the total volume was measured. Two mL of each supernatant was passed through a 0.45-μm membrane filter paper for HPLC and mass spectrometry analysis and the rest of the sample was stored −20 °C for bioassays. All extractions were carried out in triplicate and the results were expressed as means and standard error.

2.5. Purification of betanin by flash chromatography

Pure betanin is not commercially available for quantification of betalains. Therefore, crude beet methanolic extract was loaded on a C18 glass reverse phase flash column (particle size 40–63 μm, 200 × 60 mm), (ACE Glass Inc, Vineland, NJ, USA). The separation of betanin was carried out on a flash chromatography system (Combiflash Rf, Teledyne Isco, Lincoln, NE, USA). Before loading the sample, the C_{18} column was equilibrated with 1 L of 1% aqueous formic acid for 30 min. Then, the separation of betanin was performed by a gradient program using solvent A (1% formic acid) and solvent B (acetonitrile) with a flow rate of 40 mL/min. The betalains were monitored at 480 and 540 nm with a peak width of 15 s and threshold of 0.05 AU. A total of 180 fractions of 15 mL each were collected. All fractions were analyzed by HPLC and fractions 48–56 containing the same peak were pooled and lyophilized to obtain pure compound. The purity and structure of compound (betanin) was confirmed by HPLC and UPLC-HR-MS.

2.6. Quantification of the betalains by HPLC

Agilent 1200 HPLC series (Agilent Technologies, Palo Alto, CA, USA) connected to a photodiode array detector was used for the quantification of betalains. The separation was carried out on a Gemini C_{18} Column (250 mm × 4.6 mm i.d.) (Phenomenex, Torrance, CA, USA) at 30 °C. The mobile phase consisted of 0.3 M phosphoric acid (solvent A) and acetonitrile (solvent B). At a flow rate of 0.8 mL/min, different betalains were monitored at 540, 480, and 420 nm and identified by elution order and UV spectra from published papers [8]. Different concentrations (31.25–500 μg/mL) of purified betanin were injected into the HPLC and a calibration curve was prepared. Samples of 10 μL were injected and the betalain content was expressed as μg betanin equivalent/g of fresh weight [18] using the formula BC (mg/g) = [A(DF)(MW)Vd/$\mathcal{E}$LWd], where A is the absorbance at the λ_{max} of 535 and 477 nm for betacyanins and betaxanthins, respectively, DF is the dilution factor, MW is the molecular weight, Vd is the solution volume (mL), $\mathcal{E}$ is the extinction coefficient, Wd is the beet weight (g), and L is the path length (1 cm) of the cuvette. The MW of betanin is 550 g/mol and $\mathcal{E}$ = 60000L/(mol cm); for betacyanin, $\mathcal{E}$ = 48,000 L/(mol cm).

2.7. Total phenolics and ABTS radical scavenging activity

2.7.1. Estimation of total phenolics

The total phenolic content was assessed using the Folin-Ciocalteu assay according to our previous publication [19]. The absorbance was measured at λ_{max} = 734 nm and total phenolics were expressed as μg gallic acid equivalents/g of fresh weight.

2.7.2. ABTS radical scavenging activity

A slight modification of the published method [19] was used to assess the 2,2′-azinobis-(3-ethylbenzotiazoline-6-sulphonic acid) radical scavenging activity. Results were expressed as μg ascorbic acid equivalents/g of fresh weight.

2.7.3. Identification of phytochemicals by UHPLC-QTOF-MS

Ultra-high-performance liquid chromatography (UHPLC) separation was performed with the 1290 Agilent HPLC LC system (Agilent Technologies, Santa Clara, CA) equipped with a maXis impact mass spectrometer (Bruker Daltonics, Billerica, MA, USA). An Eclipse Plus rapid resolution C_{18} column (1.8 μm, 50 × 2.1 mm) was used for the separation of phytochemicals with a binary mobile phase consisting of 0.2% formic acid in water (A), and 0.2% formic acid in acetonitrile (B) with a flow rate was

0.2 mL/min. The gradient program consisted of 0% B (0–2 min), linear gradient from 0% B to 35% B (2–9 min), then increased to 100% B (9–14 min) and back to 0% B (14–16 min). The post-run equilibration time was 2 min. The total run time was 18 min.

For identification of betalains, quadrupole time-of-flight mass spectrometry (QTOF-MS) by positive electrospray ionization was used. MS and broad band collision induced spectral (bbCID) data were acquired in the *m/z* range of 50–1200. The capillary voltage of the ion source was 4200 V. The nebulizer (nitrogen) gas pressure was 4.0 bar and the drying gas flow rate 12.0 L/min. The drying gas temperature was 250 °C. The transfer time of the source was 120.8 μs and the prepulse storage time 1 μs. The quadrupole MS collision energy and bbCID collision energy were set at 5 and 55 eV, respectively. External instrument calibration was performed with a sodium formate solution containing 1 mM sodium hydroxide in isopropanol with 0.2% formic acid (1:1 v/v). Nine sodium formate clusters were used in the calibration using the high-precision calibration mode. An automated post-run internal mass scale calibration of individual samples was performed by injecting the above calibrant at the end of each run. Calibration of each sample was performed at the end of the run.

2.8. Statistical and chemometric analysis

The significance of variation in the levels of minerals were analyzed by one-way analysis of variance (ANOVA) by employing JMP Pro12 software (SAS, NC, USA). Results were expressed as mean value ± standard error. A probability of 5% or less was accepted as statistically significant. Multivariate data analysis was performed by translating the LC-MS data in csv format and subjecting to the online MetaboAnalyst software 4.0 (https://www.metaboanalyst.ca/).

3. Results and discussion

3.1. Variation in betalains in conventional and organic red and golden beets

Most of the published papers quantified betalains using molar extinction coefficients and UV absorption spectra due to lack of commercially available pure compound [20,21]. Therefore, we purified betanin from red beet methanolic extract using flash chromatography. Fig. S2 (A, B) illustrates HPLC chromatograms of crude red beet extract and the purified betanin, with a purity of 96%. The identity of the purified compound was confirmed by high-resolution tandem mass spectra (Fig S2C); the betanin (*m/z* 551.1386) with the loss of a sugar unit of 162 (551–389) is indicative of glucosylation. After confirmation, pure betanin was used for preparing the calibration graph and quantified in both red and golden beets. The levels of betalains were expressed as betanin equivalents (Table 2).

The conventional red beet extracted with methanol:water:formic acid (S18) had the highest betanin content (260.7 μg/g FW), and the water: ascorbic acid extract (S2) had the lowest betanin (57.7 μg/g FW). The isobetanin was highest in the water:acetic acid (S4, 59.8 μg/g FW) extract, which was 5–9 fold higher than in other water-containing solvents (S1, S2, S3). Interestingly, conventional red beet extracted by aqueous methanol mixed with organic acids (S17, S18, S19) had higher betanin contents than the other methanol solvent extract (S16). This result is in agreement with Narkprasom and others, who demonstrated that organic acids enhanced betalain retention through oxygen scavenging and complexation of metal cations [16]. Another study indicated that slight acidification of the extraction medium enhanced betacyanin stability and avoids oxidation by polyphenol oxidases [22]. In the present study, the highest vulgaxanthin I and valine-betaxanthin content was found in the ethanol:water extract (S6, 121.6 and 24.1 μg/g FW). In organic red beet samples, the highest betanin content was found in the methanolic extract (756.4 μg/g FW), and the aqueous extract had the lowest betanin (37.5 μg/g FW). In comparison, the highest isobetanin was found in the ethanol:water:formic acid (S12) extract (54.5 μg/g FW), followed by methanol:water:formic acid (S18).

In conventionally grown golden beet, the highest vulgaxanthin I content (74.0 μg/g FW) was found in samples extracted by methanol:water:ascorbic acid (S17). The highest valine-betaxanthin was found in the aqueous methanol (S15) extract, with a content of 12.8 μg/g FW. Organic golden beet extracted by aqueous ethanol (S6, 82.6 μg/g FW) had the highest vulgaxanthin I content compared to other solvents. Whereas in aqueous extract, no vulgaxanthin I was detected. Organic golden beet extracted by aqueous ethanol S8 had the highest valine-betaxanthin content (15.8 μg/g FW), suggesting that aqueous ethanol was efficient at extracting betaxanthins.

The total betalains of red and golden beet extracts are shown in Table S3, and the levels in conventionally grown red beet varied from 123.5 to 394.6 μg/g fresh weight (FW). The highest amount of total betalains was found in ethanol: water extract (S6), and the lowest amount was in the water: ascorbic acid extract (S2). Similar concentrations of

Table 1. Identified phytochemicals found in conventionally and organically grown red and golden beetroots.

No.	Identified compounds	RT (min)	Molecular formula	λ_{max} (nm)	Theoretical mass	Experimental mass	Mass error (ppm)	MS/MS fragments	Beet Varieties				Reference
									Con Red	Org Red	Con Golden	Org Golden	
1	Glutamine-isobetaxanthin	1.30	$C_{14}H_{17}N_3O_7$	471	340.1139	340.1172	9.7	321, 277	x	x	x	x	[23]
2	Glutamine-betaxanthin	1.55	$C_{14}H_{17}N_3O_7$	471	340.1139	340.1172	9.7	321, 277	x	x		x	[23]
3	Phenylalanine	3.15	$C_9H_{11}NO_2$	280	166.0863	166.0877	8.4	–	x	x	x		[31]
4	Glutamic acid-betaxanthin	3.50	$C_{14}H_{16}N_2O_8$	470	341.0979	341.1009	8.8	215	x				[23]
5	Hydroxy caffeic acid	3.85	$C_9H_8O_5$	327	197.0445	197.0431	−7.1	–	x				[23]
6	17-Decarboxy-betanidin	4.15	$C_{17}H_{17}N_2O_6^+$	–	345.1081	345.1109	8.1	–	x	x			[23]
7	17-Decarboxy-isobetanidin	4.25	$C_{17}H_{17}N_2O_6^+$	–	345.1081	345.1102	6.1	–	x				[23]
8	γ-Aminobutyric acid-betaxanthin	4.65	$C_{13}H_{16}N_2O_6$	462	297.1081	297.1103	7.4	–	x	x	x	x	[23]
9	Proline-betaxanthin	4.80	$C_{14}H_{16}N_2O_6$	–	309.1081	309.1107	8.4	283, 195	x	x		x	[23]
10	L-tryptophan	5.15	$C_{11}H_{22}N_2O_2$	280	205.0972	205.0986	6.8	–	x	x	x	x	[23]
11	Prebetanin	5.35	$C_{24}H_{26}N_2O_{16}S$	536	631.1076	631.1127	8.1	551, 389	x	x			[23]
12	Betanin	5.45	$C_{24}H_{26}N_2O_{13}$	535	551.1508	551.1511	0.5	389, 150	x	x			[23]
13	2′-O-glucosyl-betanin	5.65	$C_{34}H_{37}N_2O_{15}^+$	535	713.2189	713.2086	−14.4	551, 389	x	x			[23]
14	Isobetanin	5.85	$C_{24}H_{26}N_2O_{13}$	536	551.1508	551.1502	−1.1	389, 150	x	x			[23]
15	Betanidin	6.35	$C_{18}H_{16}N_2O_8$	540	389.0979	389.1016	9.5	343	x				[23]
16	Valine-betaxanthin	6.50	$C_{14}H_{18}N_2O_6$	467	311.1238	311.1263	8.0	–	x	x	x		[23]
17	17-Decarboxy-betanin	6.75	$C_{22}H_{26}N_2O_{11}$	507	507.1609	507.1723	22.5	345	x	x			[23]
18	Syringic acid	6.95	$C_9H_{10}O_5$	216	199.0601	199.0590	−5.5	–	x				[27]
19	Neobetanin	7.05	$C_{24}H_{24}N_2O_{13}$	464	549.1352	549.1389	6.7	387	x	x			[23]
20	17-Decarboxy-neobetanin	7.05	$C_{23}H_{24}N_2O_{11}$	444	505.1453	505.1565	22.2	343, 297	x	x			[23]
21	2-Decarboxy-neobetanin	7.10	$C_{23}H_{24}N_2O_{11}$	445	505.1453	505.1568	22.8	343, 297	x	x			[23]
22	2,17-Bidecarboxy-2,3-dehydro-neobetanin	7.90	$C_{22}H_{22}N_2O_9$	–	459.1398	459.1501	22.4	–	x	x			[23]
23	Leucine-betaxanthin (Vulgaxanthin IV)	8.00	$C_{13}H_{15}N_3O_7$	470	325.1394	325.1419	7.7	–	x			x	[23]
24	Phenylalanine-betaxanthin	8.25	$C_{18}H_{18}N_2O_6$	470	359.1238	359.1261	6.4	313			x		[23]
25	2-Decarboxy-2,3-dehydro-neobetanin	8.60	$C_{23}H_{22}N_2O_{11}$	–	503.1296	503.1336	8.0	341	x	x			[23]
26	6′-feruloyl-betanin	9.40	$C_{34}H_{35}N_2O_{16}^+$	–	727.1981	727.2053	9.9	453, 389		x			[23]
27	6′-feruloyl-isobetanin	9.60	$C_{34}H_{35}N_2O_{16}^+$	–	727.1981	727.2044	8.7	453, 389		x			[23]

Abbreviations: RT, retention time; Con, conventional; Org, organic.

Table 2. Levels of betanin, isobetanin, vulgaxanthin I, and valine-betaxanthin in beetroot extracts.

No.	Conventional Red				Organic Red				Conventional Golden				Organic Golden			
	Betanin	Isobetanin	Vulgaxanthin I	Valine-betaxanthin	Betanin	Isobetanin	Vulgaxanthin I	Valine-betaxanthin	Betanin	Isobetanin	Vulgaxanthin I	Valine-betaxanthin	Betanin	Isobetanin	Vulgaxanthin I	Valine-betaxanthin
S1	197.6 ± 3.7	11.9 ± 0.3	67.8 ± 1.4	13.3 ± 0.5	37.5 ± 1.9	5.6 ± 0.0	14.6 ± 0.8	7.1 ± 0.1	ND	ND	34.5 ± 0.7	7.7 ± 0.1	ND	ND	ND	ND
S2	57.7 ± 1.7	10.0 ± 0.1	46.4 ± 0.5	9.4 ± 0.1	265.3 ± 8.7	11.1 ± 0.3	198.1 ± 25.5	21.5 ± 0.7	ND	ND	46.5 ± 2.8	8.3 ± 0.3	ND	ND	30.5 ± 1.2	ND
S3	159.7 ± 0.5	13.8 ± 0.1	16.2 ± 0.4	10.4 ± 0.2	298.9 ± 9.7	13.4 ± 0.2	59.5 ± 1.0	12.3 ± 0.1	ND	ND	ND	ND	ND	ND	5.9 ± 0.3	ND
S4	256.8 ± 8.4	59.8 ± 2.2	61.3 ± 1.4	10.2 ± 0.1	303.2 ± 13.0	15.1 ± 0.1	19.1 ± 1.7	33.1 ± 1.5	ND	ND	11.1 ± 0.8	9.6 ± 0.7	ND	ND	4.1 ± 0.0	ND
S5	82.2 ± 7.4	9.6 ± 0.3	27.2 ± 0.9	11.6 ± 0.2	297.5 ± 13.5	14.0 ± 0.2	83.1 ± 0.5	20.2 ± 0.2	ND	ND	27.3 ± 7.2	8.3 ± 0.7	ND	ND	18.4 ± 0.1	5.8 ± 0.2
S6	238.9 ± 4.0	10.0 ± 0.3	121.6 ± 0.8	24.1 ± 0.4	356.3 ± 5.5	10.6 ± 0.3	87.8 ± 3.1	21.3 ± 0.5	ND	ND	48.4 ± 0.7	5.3 ± 0.1	ND	ND	82.6 ± 4.6	8.0 ± 0.1
S7	124.9 ± 2.0	10.9 ± 0.1	47.2 ± 0.4	10.2 ± 0.1	346.2 ± 2.8	14.1 ± 0.3	156.3 ± 2.7	23.4 ± 0.5	ND	ND	35.5 ± 0.7	6.7 ± 0.1	ND	ND	36.1 ± 0.7	5.1 ± 1.0
S8	229.8 ± 5.7	11.7 ± 0.5	84.9 ± 2.9	10.9 ± 0.5	331.1 ± 2.4	13.3 ± 0.2	163.9 ± 0.7	22.9 ± 0.2	ND	ND	46.5 ± 2.5	6.7 ± 0.2	ND	ND	27.8 ± 2.4	15.8 ± 0.1
S9	143.9 ± 0.5	12.3 ± 0.1	55.3 ± 0.2	13.1 ± 0.2	235.3 ± 2.2	8.6 ± 0.3	110.7 ± 0.1	22.5 ± 0.2	ND	ND	45.5 ± 1.5	9.0 ± 0.4	ND	ND	53.3 ± 0.3	9.0 ± 1.0
S10	217.7 ± 2.2	18.5 ± 0.1	47.1 ± 0.7	11.0 ± 0.2	279.6 ± 14.5	28.0 ± 1.4	64.3 ± 4.0	15.0 ± 0.9	ND	ND	10.8 ± 0.1	7.5 ± 0.0	ND	ND	9.7 ± 0.0	4.6 ± 0.1
S11	252.5 ± 2.3	15.8 ± 0.3	75.6 ± 0.7	11.1 ± 0.1	161.8 ± 4.3	10.0 ± 0.1	57.4 ± 2.0	11.6 ± 0.2	ND	ND	ND	ND	ND	ND	5.7 ± 0.3	ND
S12	110.4 ± 1.0	15.2 ± 0.3	26.3 ± 1.0	13.1 ± 0.3	330.9 ± 6.8	54.5 ± 1.5	39.1 ± 0.7	20.0 ± 0.6	ND	ND	14.0 ± 1.0	6.1 ± 0.1	ND	ND	15.7 ± 1.4	5.4 ± 0.1
S13	229.0 ± 2.0	13.6 ± 0.3	108.9 ± 0.7	20.2 ± 0.2	756.4 ± 43.4	20.4 ± 1.6	373.7 ± 19.7	11.7 ± 0.7	ND	ND	66.4 ± 0.4	7.8 ± 0.1	ND	ND	69.0 ± 3.0	6.4 ± 0.0
S14	134.5 ± 0.8	9.6 ± 0.1	53.6 ± 0.4	10.5 ± 0.1	235.1 ± 7.1	9.4 ± 0.3	129.7 ± 5.5	16.6 ± 0.5	ND	ND	42.5 ± 1.6	5.6 ± 0.2	ND	ND	42.8 ± 3.4	5.8 ± 0.0
S15	99.5 ± 5.4	6.3 ± 0.3	36.8 ± 2.2	5.9 ± 0.5	506.2 ± 22.2	22.8 ± 0.8	196.3 ± 5.8	22.2 ± 0.5	ND	ND	69.3 ± 2.6	12.8 ± 0.4	ND	ND	76.1 ± 1.8	9.3 ± 0.1
S16	119.9 ± 3.6	6.2 ± 0.1	47.9 ± 0.7	9.2 ± 0.2	327.2 ± 5.8	11.4 ± 0.2	164.2 ± 0.4	18.0 ± 0.3	ND	ND	26.6 ± 1.9	ND	ND	ND	58.8 ± 2.4	7.7 ± 0.3
S17	187.4 ± 2.4	9.5 ± 0.2	61.8 ± 1.5	12.4 ± 0.3	245.9 ± 14.7	16.7 ± 0.4	36.8 ± 2.5	14.0 ± 1.1	ND	ND	74.0 ± 0.4	12.3 ± 0.3	ND	ND	69.7 ± 2.7	8.9 ± 0.3
S18	260.7 ± 5.4	26.0 ± 0.9	36.4 ± 1.8	11.5 ± 0.4	349.0 ± 19.7	46.1 ± 1.3	55.0 ± 2.8	11.7 ± 0.3	ND	ND	13.1 ± 0.5	3.3 ± 0.1	ND	ND	11.7 ± 0.7	5.0 ± 0.1
S19	216.9 ± 2.1	10.6 ± 0.1	50.2 ± 0.8	7.6 ± 0.2	436.5 ± 1.9	14.9 ± 0.1	142.3 ± 2.4	11.7 ± 0.4	ND	ND	ND	ND	ND	ND	7.1 ± 1.1	5.9 ± 0.9
S20	138.2 ± 0.7	10.4 ± 0.2	59.8 ± 0.7	12.0 ± 0.3	752.9 ± 6.2	19.7 ± 1.4	325.8 ± 1.2	12.7 ± 0.3	ND	ND	ND	ND	ND	ND	122.0 ± 3.2	7.9 ± 0.2

Refer to solvent codes in Table S1. Results (μg/g) are expressed as betanin equivalents with three replicates.

total betalains were reported in a previous study of red beets grown in Australia, which had less total betalains, with contents of 0.38–0.65 mg/g FW [4]. In comparison, higher concentrations of total betalains were reported in a previous study of 13 red beet varieties from Poland [5], where the total betalains of red beet were in the range of 1.57–2.70 mg/g FW and the amount significantly differed depending on the beet variety and the extraction solvent.

In organic red beet extracts, we observed higher variation compared to conventional red beet extracts, with the organic beets having 64.8–1162.2 μg/g FW betalain. Similar to the results for conventionally grown red beets, the total betalain concentration was high in the methanolic extract (S13) and the lowest amount was detected in the aqueous extract (S1). Furthermore, the beetroot extracted with water with or without acid gave a lower yield of betalains, compared to the organic solvents. This may be due to the mucilaginous effect of water-soluble pectin [15]. Hence, water may not be a good solvent for the extraction of betalains from organic red beet. Our results were consistent with the literature, as previous studies reported that betalain contents might be influenced by several factors, including varietal, growing season, and climatic and cultivation conditions [5]. Also, the average amount of total betalains were significantly higher in organic red beet extracts than in their conventional counterparts, except for extracts obtained from water (S1) and ethanol: water: acetic acid (S11), which had 4-fold and 1-fold fewer betalains than the conventional red beet extracts. This result indicated that the production system also affects the betalain contents, which could be partly explained by the difference in nitrogen levels [23].

Significant variations of total betalains were also found in golden beet extracts, wherein the total betalains varied from 0.0 to 86.4 μg/g FW for conventionally grown golden beets, and 0.0–129.8 μg/g FW for organic golden beets. Conventional golden beets extracted by methanol: water: ascorbic acid (S17) had the highest total betalains. By contrast, betalain levels were not detectable in the water/acetic acid (S3), ethanol: water:acetic acid or methanol:water:acetic acid mixture (S11 and S19), and methanol:water:formic acid (S20) extracts. In organic golden beet extracts, the highest total betalains were found in the methanol:water:formic acid extract (S20).

In general, total betalains were higher in red beet than in golden beet, with amounts being 3-fold higher in conventionally grown red beets, and 8-fold higher in organically grown cultivars. This finding was consistent with previous research, where total betalain content in red beetroot was significantly higher than that in yellow beetroot, regardless of the extraction solvent [5]. The presence of high amounts of betalains in red beetroot indicates that limited utilization of tyrosine, the precursor of betalain synthesis, occurs in yellow beetroot compared to the red beetroot. In the biosynthetic pathway of betalains, tyrosine is first oxidized to L-dihydroxy phenylalanine (L-DOPA) by reacting with CYP76AD1, AD5, and AD6 L-DOPA is then converted to betalamic acid and cyclo-DOPA, which are required for betalain production. Betalamic acid and cyclo-DOPA are needed for betacyanin production, whereas only betalamic acid along with amino acids or amines are required for betaxanthins. The limited amounts of tyrosine in golden beet may cause the overall low betalain content [24]. To verify this, peak areas were generated for betalain biosynthesis-related metabolites (Fig. S3). Indeed, the levels of tyrosine were much higher in conventionally and organically grown red beets than in golden beets. As a result, the levels of L-DOPA (*m/z* 198) were higher, and the cyclo-DOPA (*m/z* 196) and betalamic acid (*m/z* 212) were higher, ultimately causing higher betalain levels in red beet than golden beets. This was consistent with previously reported findings [8,24], showing that the primary and secondary metabolites within the betalain biosynthesis pathway can be influenced by the variety of vegetable.

3.2. Influence of solvent composition on total phenolics and radical scavenging activities

Ideally, a single extraction will work for all compounds of interest; therefore, we compared the extraction of betalains with extraction of total phenolics and free radical-scavenging compounds, which are also of interest for the health-promoting effects of functional foods such as beetroot. The total phenolics and ABTS free radical scavenging activities for conventional and organic red beets are presented in Fig. 2. The use of diverse solvent ratios and combinations resulted in beet extracts with significantly different total phenolics and ABTS activities. Conventional red beetroot extracted by methanol alone (S13) had the highest phenolics (323.67 μg gallic acid equivalents [GAE]/g FW). A previous study [25] reported that methanol was an effective solvent due to the interactions between the polar sites of the antioxidant molecules and the solvent. The lowest total phenolics value was found in the methanol:water:ascorbic acid extract (S17, 91.64 μg GAE/g FW), which indicated that the ratio of methanol significantly affects the extraction of

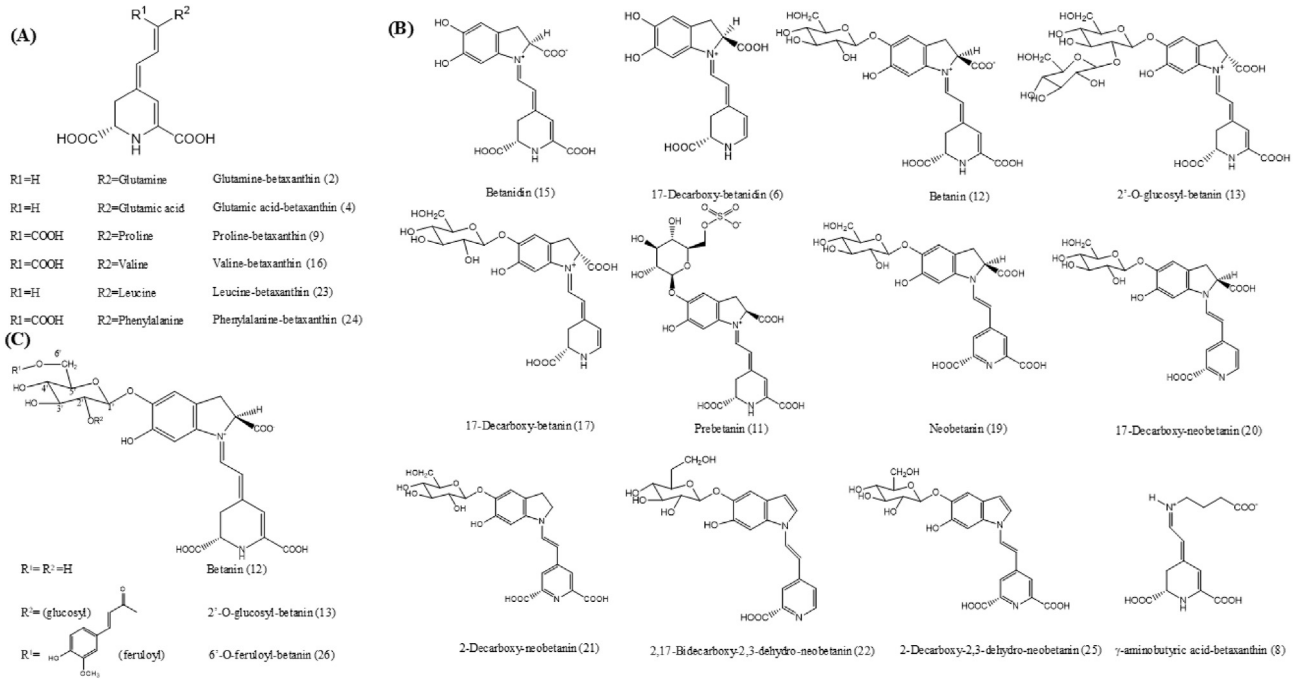

Fig. 1. Structures of (A) Betaxanthins and (B, C) Betacyanins identified in conventional and organic beets. The number in parenthesis of each compound refer to Table 1.

total phenolics. The conventional red beet extracted by nanopure water or ethanol solvents with added acids tended to have higher total phenolics than the sample extracted by solvents added ascorbic acid, suggesting that acidification might increase the recovery of phenolic compounds; however, this trend was not observed in extracts obtained by methanol-containing solvents. In organic red beet extracts, the highest total phenolics was obtained from the ethanol:water (S8) extract (436.69 μg GAE/g FW), and the lowest total phenolics were found in the water:acetic acid (S4) extract (153.64 μg GAE/g FW). Other water-containing solvents (S1, S2, S3) extracts showed moderate amounts of total phenolics.

Among the various extracts of golden beets (Fig. 3), conventionally grown golden beetroot extracted by ethanol (S5) had the highest total phenolics (212.35 μg GAE/g FW), and the lowest amount was found in the methanol:water:formic acid extract (S18, 43.44 μg GAE/g FW). In contrast to conventionally grown red beet, the conventionally grown golden beet extracts of water-containing solvents had high total phenolics. Significant variation in total phenolics was observed in organic golden beet extracts, with the highest value found in the methanol:water:ascorbic acid extract (S15, 123.55 μg GAE/g FW), and the lowest in the water:acetic acid extract (S4, 36.06 μg GAE/g FW). Interestingly, total phenolics in conventional and organic golden beets were higher in the extracts with solvents containing ascorbic acid than those with formic or acetic acid, suggesting that ascorbic acid acidified solvents could be the potential favorite solvent for extracting health-promoting compounds in golden beet.

ABTS assays was performed to evaluate the antioxidant activities of the different beet extracts. The highest and ABTS radical scavenging activities in conventional red beet were found in aqueous methanol:formic acid (S18) extracts, with ascorbic acid (AA) equivalents of 400.76 and 229.83 μg AA/g FW, respectively. The lowest ABTS activities were found in the methanol:ascorbic acid (S17) extract (91.64 and 88.01 μg/AA g FW). In comparison, organic red beet extracted with water: acetic acid (S19) had the highest ABTS activity (302.62 μg/AA g FW). In some of the ethanol-containing solvents, conventional and organic red beet ethanol:water (S6 and S8), ethanol:water:formic acid (S10), and ethanol:water:acetic acid (S11) extracts, high ABTS activity was present. Given their high extraction efficiency and lower toxicity compared with other solvents, aqueous ethanol combined with acid might be considered as a desirable replacement for methanol.

For conventionally grown golden beet, the ethanol:water:formic acid (S10) extract had the highest ABTS activity (and 105.56 μg AA/g FW) and the

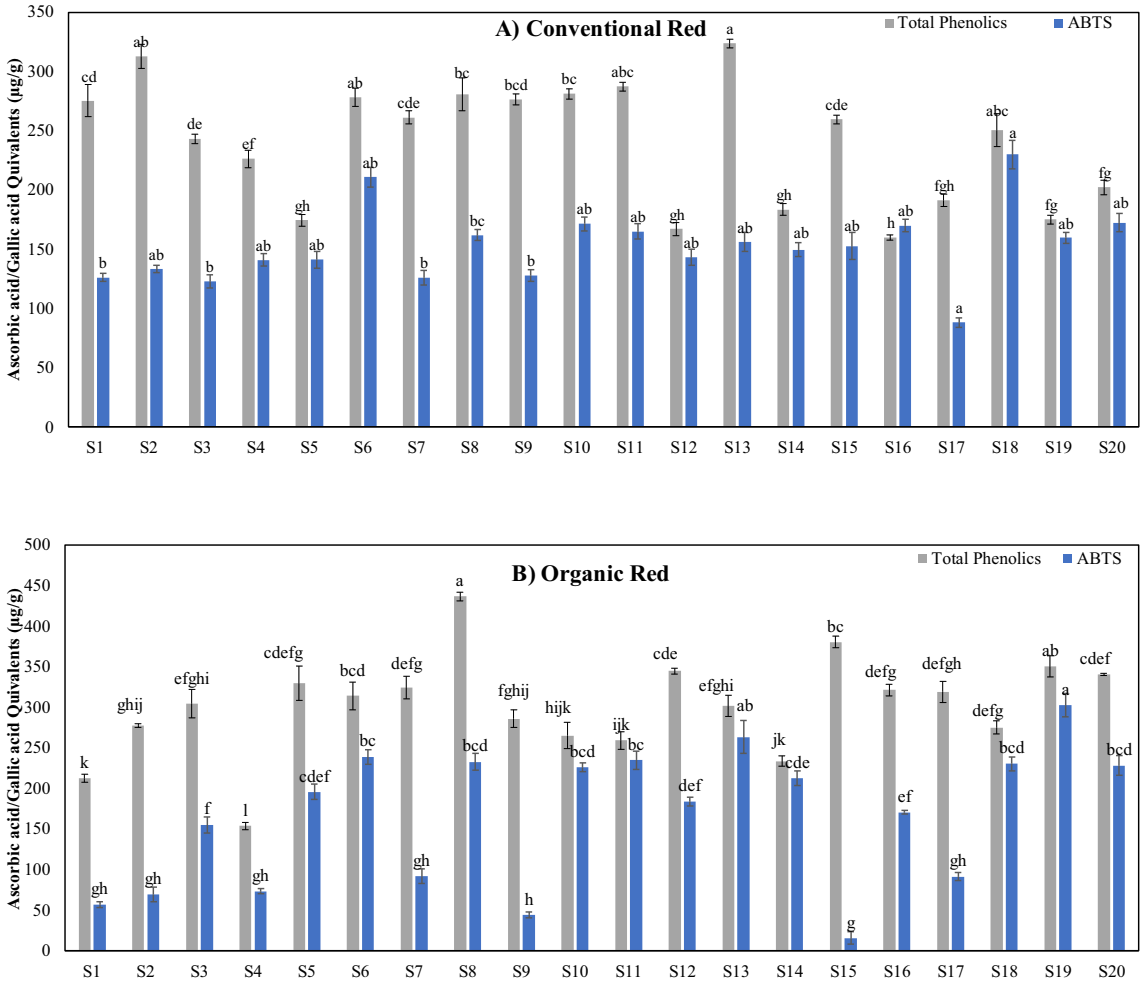

Fig. 2. Total phenolics and ABTS radical scavenging activity of (A) conventional and (B) organic red beetroots extracted with twenty different solvent combinations (see Table S1). Data for total phenolics are expressed as µg gallic acid equivalents per g of fresh weight. Data for ABTS is expressed as µg ascorbic acid equivalents per g of fresh weight. X axis: solvent code, Y axis: ascorbic acid equivalents. Values were expressed as mean ± SE. Different letters indicate significant differences at $P \leq 0.05$.

ethanol:water extract (S6 and S8) had the lowest ABTS activity (24.41 AA/g FW). In organically grown golden beet, the ethanol:water (S8) extract had the highest ABTS activity (98.48 µg AA/g FW) and the water:acetic acid (S4) and methanol (S13) extract (S4 and S13) had the lowest ABTS activity (20.18 AA/g FW), respectively.

The overall ABTS radical scavenging activities of red beetroot extracts were significantly higher than golden beetroot. In our present results, betanin was the predominant betacyanin in red beetroot, whereas vulgaxanthin I was the main betaxanthin in golden beetroot. Betanin contains an aromatic ring with the betalamic acid moiety, a second ring combined with an indoline, which increases the radical scavenging activity. The antiradical activities of betanin and its isomer were further enhanced by the additional hydroxyl group in their benzene rings [26]. In comparison, vulgaxanthin I was the main betalain in golden beet and had minimal antiradical activities due to its structure lacking aromatic resonance, charged, or hydroxyl groups. Therefore, the radical scavenging activities of golden beetroot were significantly lower compared to red beets.

3.3. Identification of phytochemicals from beetroots by UHPLC-QTOF-MS

The individual constituents of the samples obtained by extracting with twenty solvents were assessed by UHPLC-QTOF-MS. The retention time, molecular formula, molecular mass, and MS/MS fragments of individual compounds are presented in Table 1. Twenty-seven phytochemicals including 23 betalains (betacyanins and betaxanthins), 2 amino acids, and 2 phenolic acids were identified

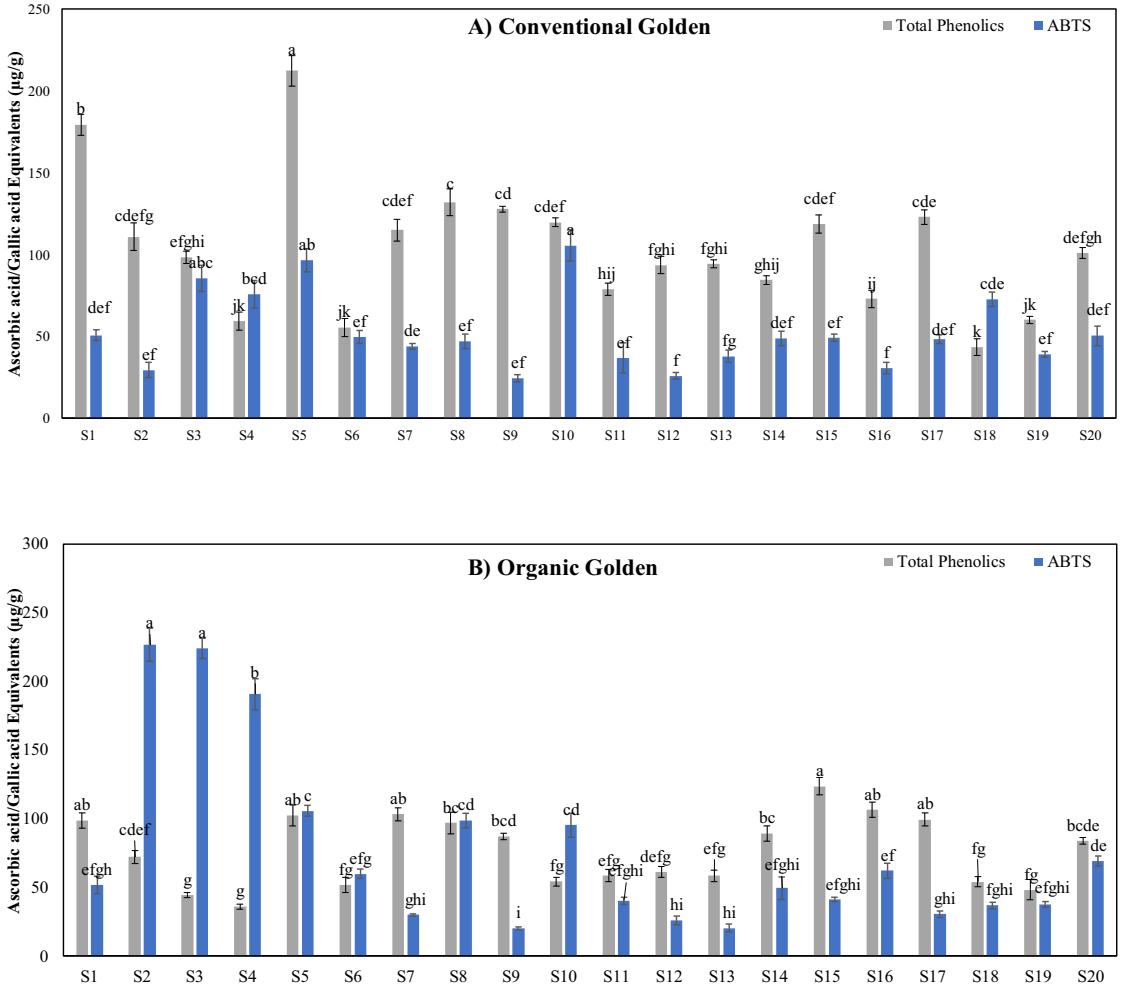

Fig. 3. Total phenolics and ABTS radical scavenging activity of (A) conventionally and (B) organically grown golden beetroots extracted with twenty solvent combinations (see Table S1). Data for total phenolics expressed as µg gallic acid equivalents per g of fresh weight. Data for ABTS expressed as µg ascorbic acid equivalents per g of fresh weight. X axis: solvent code, Y axis: ascorbic acid equivalent values. Values were expressed as mean ± SE. Different letters indicate significant differences at $P \leq 0.05$.

using high-resolution mass spectral data (Table 1). Betalains are condensed products of betalalamic acid and have various substitutions such as glycosylation of one or both hydroxyl groups at position 5 or 6 of the betanidin (Fig. 1). In this study, 14 betacyanins were identified in red beetroots with betanin (*m/z* 551.1508) as the major compound. UPLC chromatograms of red and golden beets at 480 and 540 nm are presented in Fig. S1 (A–D). Red beet shows clear LC peaks at 480 nm for vulgaxanthin I (*m/z* 340.11541), betanin (*m/z* 551.15005), isobetanin (*m/z* 551.14947) and valin-betaxanthin (*m/z* 325.13834). We note that 17-decarboxy-betanidin, 17-decarboxy-isobetanidin, and betanin eluted at 4.15 (1), 4.25 (2), and 5.45 (3) min respectively (Table 1), which does not correspond to the values found in a previous study [23], which reported separation of these compounds at 14.9 (1), 19.8 (2), and 21.4 (3) min. Moreover, we identified these compounds using photoarray detector data and accurate mass data; therefore, we are confident in their identities. This phenomenon was probably due to the polarity of compounds and retention time does not correlate. In addition, UV–visible absorption peaks and their high-resolution mass spectra are shown in Fig. S1 (E-H). Interestingly, only vulgaxanthin I and valin-betaxanthin peaks were observed in golden beets.

Compared to the organic red beet, conventional red beet extracts had more types of betalains, which included glucosylated betacyanins such as 2′-O-glucosyl-betanin (*m/z* 713.2086), 6′-feruloyl betanin (*m/z* 727.2053), and 6′-feruloyl-isobetanin (*m/z* 727.2044). The decarboxylated and dehydrogenated betacyanin compounds 17-decarboxy-(iso) betanidin (*m/z* 345.1109), 17-decarboxy-betanin (*m/z* 507.1723), 2,17-decarboxy-neobetanin (*m/z* 505),

2,17-bidecarboxy-2,3-dehydro-neobetanin (*m/z* 459), and 2-decarboxy-2,3-dehydro-neobetanin (*m/z* 503.1336). Betanidin (*m/z* 389) and 17-decarboxy-isobetanidin (*m/z* 345) were not detected in the organic beetroot extracts. Consistent with previous research, betacyanins were not detected at significant levels in conventional and organic golden beetroots [8].

In contrast to betacyanins, betaxanthins react with free amino acids and form new compounds with amino acid adducts. In golden beet extracts, we identified betaxanthins with glutamine, valine, leucine, and phenylalanine adducts. These betaxanthin derivatives were also present in conventional and organic red beet extracts. In addition, amino acids such as phenylalanine (*m/z* 166.0877) and L-tryptophan (*m/z* 205.0986), were identified based on their molecular mass and MS/MS fragmentation. Further, hydroxy caffeic acid (*m/z* 197.0431) and syringic acid (*m/z* 199.0590) were found in four beet cultivars. Previous studies identified these two phenolic acids in beetroot samples [23,27].

3.4. Chemometrics approach to identify the influence of extraction solvent on phytochemical profiles

In the present study, multivariate statistical analysis was applied to build an intuitive overview of the beet extract results. The abundances of phytochemicals identified by LC-MS are presented as a heatmap in Fig. 4. Significant variation in phytochemicals was detected in conventional red beetroot extracted with the 20 solvents. Among these, beets extracted by water:ascorbic acid (S2), ethanol (S5), different concentration of ethanol with ascorbic acid (S7 and S9), and methanol:water:formic acid (S20) had higher amounts of betacyanin derivatives, namely betanidin, 6′-feruloyl-betanin and its isomer, prebetanin, betanin, 2′-glucosyl-betanin, 2,17-bidecarboxy-2,3-dehydro-neobetanin, 17-decarboxy-neobetanin, neobetanin, 17-decarboxy-betanidin, and amino acid-adducted betaxanthins including valine-betaxanthin, leucine-betaxanthin, proline-betaxanthin, phenylalanine-betaxanthin and glutamic acid-betaxanthin.

Higher levels of betanin in red beetroot was found in aqueous ethanol:ascorbic acid extracts (S7 and S9). Another main compound, isobetanin, was highest in water:formic acid (S3) extracts, with the mass area 1- to 430-fold higher than the conventional red beet extracted with other water-containing solvents (S1−3). In addition, amino acids (phenylalanine, L-tryptophan), and the syringic acid were also identified in those extracts. Two decarboxylated betacyanins, 17-decarboxy-betanin and 2-decarboxy-neobetanin, were found at the highest levels in the aqueous ethanol:formic acid (S10) extract, with an area 1 to 3-fold higher than the ethanol:acetic acid (S11) extract. Another compound, 2-decarboxy-2,3-dehydro-neobetanin had a significantly higher area in the aqueous ethanol (S8) extract. The water:ethanol solvent system affects the stability of betanin, since ethanol has a high electron density on the oxygen atom, leading to nucleophilic attack on the oxygen atom, and ultimately causing decarboxylation [28].

Conventional red beet extracted by water or aqueous combined with ascorbic acid solvents (S2, S7, S9) yielded larger quantities of phytochemicals compared to the extracts obtained from formic:-acetic acid (S3, S4) or aqueous ethanol (S6), indicating ascorbic acid with water or ethanol might be an effective solvent combination for obtaining large quantities of betalains. The overall different abundance of betalains in beet extracts may be attributed to their interaction with the extracting solvents, which altered the linear symmetry restrictions of the betanin molecule and thus changed their characteristics [29]. Similarly, in organic red beet extracts, water and ascorbic mixture (S2), ethanol with or without ascorbic acid (S5, S7, and S9), and methanol:water:formic acid (S20) solvents were efficient in obtaining various betalain compounds such as decarboxylated betacyanins, namely 2-decarboxy-neobetanin, 17-decarboxy-neobetanin, 17-decarboxy-betanidin, 2-decarboxy-2,3-dehydro-neobetanin, and 2,17-bidecarboxy-2,3-dehydro-neobetanin. In water/formic acid (S3) extract, and ethanol:water:acid (S10, S12) extracts, only one betacyanin with relatively high levels was found. For instance, isobetanin was identified in water:formic acid extract (S3) with a mass area 4-fold higher than aqueous extract (S1), and 17-fold higher than methnol-water-formic acid red extract (S20). In other solvent combinations (S1, S8, S13, S15−19), the levels of phytochemicals were negligible. However, betalains including betanidin and 17-decarboxy-isobetanidin, glutamic acid-betaxanthin, leucine-betaxanthin, and phenolic acids, hydroxy caffeic acid and syringic acid were not detected in all organic red beet extracts, suggesting that those compounds might be considered as biomarkers to discriminate conventional and organic red beets.

The conventional golden beet samples extracted by methanol (S13), aqueous ethanol and ascorbic acid mixtures (S7 and S9) were rich in L-tryptophan and valine-betaxanthin. Phenylalanine was detected in extracts obtained by water with or without

formic/acetic acid (S1, S3, and S4) extracts, aqueous methanol with the ascorbic acid mixture (S15 and S17) were detected. Similar to conventional red beet, γ-aminobutyric acid-betaxanthin was also detected in S15 and S17 extracts. Glutamine-isobetaxanthin was only detected in ethanol, water, and acetic acid extract (S11) with high levels. In other extracts, this compound was not detected. Proline-betaxanthin (*m/z* 309) was detected in ethanol (S5) and aqueous methanol (S14) extracts. Other solvents (S2, S6, S8, S18–20) also lacked detectable amounts of those compounds.

In organic golden beet, extracted with methanol:water:formic acid (S20), leucine-betaxanthin, glutamine-betaxanthin, and γ-aminobutyric acid-betaxanthin exhibited the highest mass areas, with the levels 19- to 91-fold, 1- to 49-fold, and 14-fold higher than in aqueous ethanolic or methanolic extracts, respectively. The glutamine-betaxanthin (*m/z* 340) was only detected the

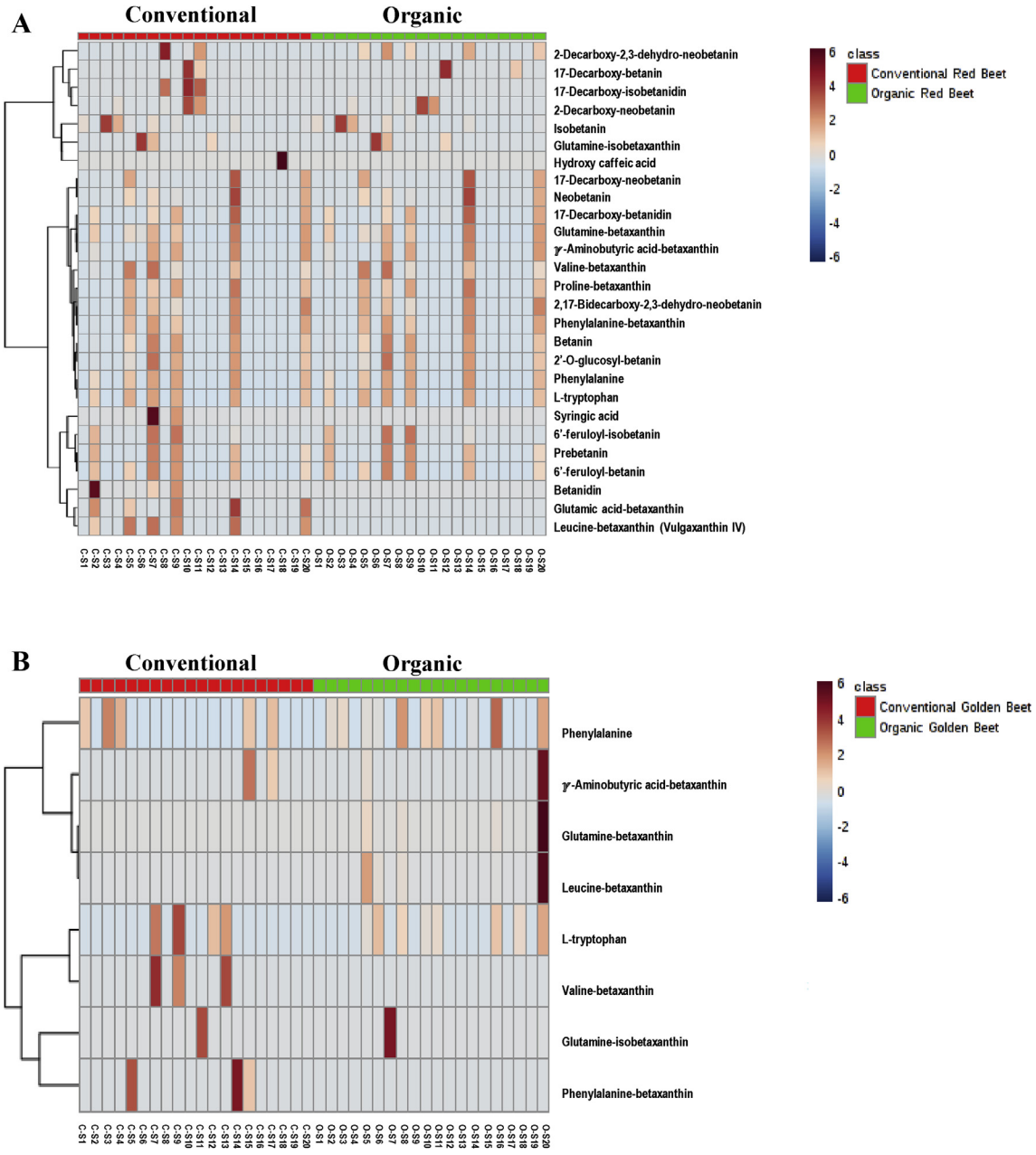

Fig. 4. Heatmap of metabolites from conventionally and organically grown (A) red beetroots and (B) golden beetroots extracted by 20 solvents (see Table S1) based on Pearson distances, Ward clustering and standardized by auto-scale features. O: organic, C: conventional beetroots. Colored squares represent the abundance of each compound with blue indicating the lowest values and red the highest values. The relative intensities should be compared within each solvent.

ethanol:water:ascorbic acid (S7) extract. However, few amino acids were found in aqueous ethanol and methanol extracts (S6 and S16). The other extracts contained only low levels of those phytochemicals.

3.5. Mineral contents in conventional and organic beets

In the present study, we measured the levels of minerals in red and golden beets from conventional and organic production systems (Table S2). Significantly higher levels of minerals (N, P, Mg, Na, Fe, Mn, and B) were found in conventional red beets, whereas organic beets had higher levels of K, Ca, and Cu, but the difference in Zn was non-significant. In golden beets, the conventionally produced beets had a higher accumulation of all minerals except P and N, which were higher in the organic beets. There are no comprehensive data correlating the effects of conventional and organic production systems on mineral levels of beetroots [11,12]. One study found that Mg and Cu were higher in organically grown beets than in conventionally grown beets. However, variation in the levels of P and K were dependent on the growing locations [11]. Pfiffner and coauthors [30] found K was higher in conventional beets, but the P, Ca, and Mg contents fluctuated each year. Therefore, genetic, agronomic, and environmental factors significantly influence the content and composition of minerals.

4. Conclusion

The present study indicated that solvent combination significantly influences the composition and content of phytochemicals in beetroot samples. UHPLC-QTOF-MS analysis combined with chemometrics identified 27 phytochemicals, of which 27, 21, 6, and 6 phytochemicals were found in conventional red, organic red, conventional golden, and organic golden beetroots, respectively. The levels of these compounds differed based on each solvent composition, and total betalains were higher in red beetroot extracts than in the golden beetroot extract. Distinct compounds identified from heatmap analysis might be considered as potential biomarkers to discriminate beet extracts obtained from various solvents or raw beets from different production systems. Conventional red beetroot extracted by methanol (S13) showed the highest total phenolics, and methanol:water:formic acid extract (S18) had the highest ABTS scavenging activity. In organic red beetroot, aqueous ethanol extract with or without acid (S8 and S12) presented the highest total phenolics, and aqueous methanol with acid (S19) had the highest ABTS value. Conventional and organic golden beetroot extracted by solvents with ascorbic acids had higher antioxidant activities than other extracts. This provides useful information for optimizing methods for identification of phytochemicals and biomarkers.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.38212/2224-6614.1056.

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