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Determination of Saponins in Leaves of Four Swiss Chard (*Beta vulgaris* L.) Cultivars by UHPLC-CAD/QTOF-MS/MS

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Key words: *Beta vulgaris*, Swiss chard, Amaranthaceae, triterpene saponins, oleanolic acid, hederagenin, UHPLC-MS

Swiss chard is a vegetable valued not only for the taste of its leaves but also because of its health-promoting properties. To date, nothing is known regarding the occurrence of saponins in the Swiss chard plant, even though they could be at least partially responsible for the nutraceutical activities of this plant. This research aimed to describe saponins from the leaves of four Swiss chard (*Beta vulgaris* L.) cultivars. Saponin structures were analyzed by UHPLC-CAD/QTOF-MS/MS. Based on the fragmentation patterns, we tentatively identified 16 triterpene saponins in *B. vulgaris*, including two that had not been detected previously. The observed compounds were glycosides of five different, tentatively identified aglycones, *i.e.*, oleanolic acid, hederagenin, gypsogenin, akebonoic acid, and serjanic acid. Moreover, the structure of four saponins detected in Swiss chard leaves included dioxolane-type and six acetal-type substituents. Eleven, eight, eleven, and eight saponins were observed in saponin fractions obtained from Rhubarb, Bulls Blood, Perpetual Spinach, and White Silver cultivars, respectively. Furthermore, the content of all identified triterpene derivatives in the investigated cultivars was estimated using a method based on the UHPLC coupled with QTOF-MS/MS and charged aerosol detector (CAD). The analyzed cultivars differed in the total and individual saponin content. The total saponin content ranged from 125.53 to 397.09 μg/g DW.

INTRODUCTION

Swiss chard (*Beta vulgaris* L.) is a plant grown strictly for its edible leaves. This vegetable is valued not only for the taste of its leaves but also because of its health-promoting properties [Ivanović *et al.*, 2019]. Swiss chard was used in folk medicine to treat diabetes, kidney diseases, and the immune system [Hashem *et al.*, 2016; Ninfali *et al.,* 2013]. In contemporary studies, extracts from its leaves have been demonstrated to reveal anticancer, anti-inflammatory, and antioxidant properties [Ninfali *et al.*, 2013; Pyo *et al.*, 2004]. Previous works have reported that Swiss chard contains flavonoids, such as vitexin, vitexin 2-*O*-rhamnoside, and vitexin 2-*O*-xyloside [Hashem *et al.*, 2016; Ninfali *et al.*, 2013], while its leaves to contain many phenolic acids, such as syringic acid, 4-hydroxybenzoic acid, caffeic acid, chlorogenic acid, and vanillic acid, along with two aldehydes: 2,5-dihydroxybenzaldehyde and 2,4,5-trihydroxybenzaldehyde [Gennari *et al.*, 2011].

Swiss chard belongs to the Amaranthaceae family that includes many economically important plants utilized as herbal medicines or vegetables. The Amaranthaceae plants' phytochemical composition includes essential oils, betalains, phenolic compounds, and triterpene saponins [Mroczek, 2015]. From the various groups of secondary metabolites present in Amaranthaceae plants, triterpene saponins appear to be one of the most significant from the nutraceutical point of view, yet they are still undervalued.

Triterpene saponins are amphiphilic compounds characterized by their structure containing a triterpenoid aglycone, also called sapogenin, which consists of a pentacyclic C_{30} skeleton and one or more sugar chains [Sparg *et al.*, 2004]. The carbohydrate portion consists of one or more sugar moieties, and the most common monosaccharides include hexoses (D-glucose, D-galactose), 6-deoxyhexoses (L-furanose, L-quinovose, L-rhamnose), pentoses (L-arabinose, D-xylose), and uronic acids (mainly *d*-glucuronic acid). Saponin can also consist of non-carbohydrate acetyl, organic acid type, aminoacyl, or sulfuric substituents [Arslan & Cenzano, 2020; Mroczek, 2015]. Saponins are categorized according to the number of sugar chains in their structure as mono, di- or tridesmosidic. Monodesmosidic saponins have a single sugar chain, often attached through an ether linkage at C-3 of the aglycone, while bisdesmosidic saponins have an additional sugar chain attached through an ester linkage at

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C-28. Tridesmosidic saponins have three sugar chains and seldomly occur. The structural complexity of saponins, arising from the variable structure of the aglycone and the number and structure of the sugars or functional groups, results in many physical, chemical, and biological properties of this diverse group of compounds [Sparg *et al.*, 2004].

Saponins are associated with a wide range of other biological properties, *i.e.*, plant defence against pathogens [Sparg *et al.*, 2004], deterrence to insects [Tava & Odoardi, 1996], antifungal properties [Osbourn *et al.*, 1996], and low digestibility in ruminants [Troszyńska, 2004]. Widely distributed in medicinal herbs and edible plants, triterpene saponins are also interesting from a pharmaceutical point of view. Various studies have reported beneficial antimicrobial, anti-inflammatory, immunomodulatory, hepatoprotective, antidiabetic, hypolipidemic, anticancer, and adjuvant effects of saponins [Kirk *et al.*, 2004; Podolak *et al.*, 2010]. Due to their abilities as surfactants and antifungal agents, saponins or saponin-containing plant extracts can be used for industrial applications, such as in cosmetic or food industries.

Although these compounds have significant biological importance, little is known about their distribution in plants from the Amaranthaceae [Mroczek, 2015]. Triterpene saponins have recently been extensively studied in sugar beet roots and sugar pomace due to a floc problem during sugar production [Yoshikawa *et al.*, 1996]. They were also detected in red beet cultivars – Red Sphere [Mroczek *et al.*, 2012; Spórna-Kucab *et al.*, 2020], Rocket, Wodan [Mroczek *et al.*, 2012], Chrobry, and Nochowski [Mikołajczyk-Bator *et al.*, 2016a]. Recently, saponins have been qualitatively characterized in the leaves of the red beet cv. Wodan [Mroczek *et al.*, 2019]. What is worth noticing is that, in both *Beta vulgaris* groups, *i.e.*, sugar and red beet, glycosides of simple sugars moieties were reported along with saponins bearing dioxolane-type and acetal-type substituents [Mikołajczyk-Bator *et al.*, 2016a; Yoshikawa *et al.*, 1996, 1998].

To date, nothing is known regarding the occurrence of saponins in the Swiss chard plant, even though they could be at least partially responsible for its pharmacological and nutraceutical activities due to the broad spectrum of their biological activities. Thus, the aim of this study was a qualitative and quantitative analysis of saponins in the leaves of four cultivar of Swiss chard: Lukullus, Rhubarb, White Silver, and Perpetual Spinach.

Consequently, appropriate methods needed to be established for the qualitative and quantitative analysis of saponins in complex plant matrices. Mass spectrometry (MS), as an extremely sensitive and specific analytical technique, providing qualitative and quantitative data, is an essential tool for saponin characterization [Foubert *et al.*, 2010; Ge *et al.*, 2017; Gómez-Caravaca *et al.*, 2011; Kowalczyk *et al.*, 2011; Mikołajczyk-Bator *et al.*, 2016a, 2016b]. In the current study, we used ultra-performance liquid chromatography (UHPLC) coupled with quadrupole-time-of-flight mass spectrometry (QTOF-MS/MS) that has recently been widely applied to characterize natural compound structures, including saponins [Alara *et al.*, 2018; Mikołajczyk-Bator *et al.*, 2016a; Onlom *et al.*, 2017].

Although the mass spectrometry is considered a universal detection method, the mass detector's response, particularly with electrospray ionization, is highly dependent on the investigated compounds' chemical properties. Furthermore, significant matrix effects can affect the electrospray ionization of analytes, leading to inaccurate measurements. Thus, quantitative analyses employing mass spectrometry detection with electrospray ionization generally require an individual reference standard for each investigated analyte and careful investigation of the matrix effects, which are unavoidable if raw extracts are analyzed. The requirement for authentic standards is often difficult to fulfill in investigations of chemically diverse groups of compounds, especially in the phytochemical and environmental analyses. In the case of saponins, the inherent chemical diversity often practically precludes obtaining analytical standards for all possible congeners.

Therefore, various types of the so-called universal detectors are employed that generate responses independently of the investigated analytes' chemical properties and are less prone to matrix effects. Thus, the second detector utilized in this study was a charged aerosol detector (CAD), which is suitable for detecting semi-volatile and non-volatile analytes and has been used to analyze saponins for over a decade [Baker & Regg, 2018; Vehovec & Obreza, 2010; Wu *et al.*, 2019]. The use of a universal detector enabled the estimation of saponin contents without costly and time-consuming purification of individual saponins for reference standards.

Such a multi-detector approach allowed for the rapid, simultaneous characterization of saponins present in plant material based on their fragmentation pathways and subsequent estimation of detected compounds' content.

MATERIALS AND METHODS

Chemicals

Analytical-grade methanol, LC-MS-grade acetonitrile, diethyl ether, and acetic acid were purchased from J.T. Baker (Phillipsburg, NJ, USA); formic acid was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Ultrapure water was obtained using a Merck Millipore (Burlington, MA, USA) system.

Sample preparation

Plants of Swiss chard cv. Rhubarb, Bulls Blood, Perpetual Spinach, and White Silver were cultivated in the field in the standard growing conditions in temperate climatic region with fertile, neutral soil in the central Mazovia region in Poland (coordinates 52°31'45.6"N 21°38'58.3"E) during the 2015 vegetational season. Ten plants were randomly collected per cultivar. Aerial parts were lyophilized, finely powdered, and used immediately for the successive extraction. The dried and finely powdered leaves (1 g) were defatted with diethyl ether *via* ultrasonic-assisted extraction for 1 h at room temperature. Defatted material was then extracted with 80% (*v/v*) aqueous methanol (3×50 mL) *via* ultrasonic-assisted extraction for 1 h at room temperature. This method was based on the method optimized in our previous studies [Mroczek *et al.*, 2012, 2019]. The extracts were collected and concentrated under a nitrogen atmosphere (extract mass: 32–49 mg) and subsequently redissolved in water.

The solution was applied to 1 g LiChroprep RP-18 SPE cartridges (40–63 μm, Merck, Germany), previously conditioned with water. The saponin fraction was monitored by TLC on silica gel 60 F-254 plates (Merck, Darmstadt, Germany) with an ethyl acetate/acetic acid/water (7:2:2, *v/v/v*) solvent system. The spots were visualized by spraying with the Liebermann-Burchard reagent and subsequent heating at 130°C. The column was first washed with water and then with 40% (*v/v*) methanol to remove sugars and phenolics. Saponins were eluted with 80% (*v/v*) methanol and dried under a vacuum.

UHPLC–CAD/QTOF-MS/MS analysis

Samples obtained after SPE fractionation were suspended in distilled water and diluted in the 1:1 (*v/v*) ratio with the internal standard solution (digoxin Sigma-Aldrich D6003, CAS 20830–75–5, final concentration 20 pmol/μL). Analyses were carried out using a UHPLC Thermo Ultimate 3000RS system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Thermo Corona Veo RS charged aerosol detector. Separations were performed using a Waters BEH C18 column (1.7 μm, 100×2.1 mm; Waters, Milford, MA, USA). A mobile phase consisting of 0.5% (*v/v*) formic acid in acetonitrile (B) and 0.5% (*v/v*) formic acid in water (A) was used for the separation. The elution profile was isocratic at 24% B, 0–1 min; linear from 25 to 55% B, 1–22 min; isocratic at 55% B, 22–24 min; and linear from 55 to 24% B, 24–30 min. The column was maintained at 30°C with a constant flow rate of 0.4 mL/min.

The eluate from the column was split in a ratio of 3 to 1 between the CAD and the ion source of the QTOF mass spectrometer (Bruker Impact II HD; Bruker, Billerica, MA, USA). The following instrumental parameters were used for QTOF-MS/MS analysis: capillary voltage, 2.8 kV; nebulizer pressure, 0.7 bar; drying gas flow, 6 L/min; drying gas temperature, 200°C; ion energy 4 eV; collision RF 700.0 Vpp; transfer time 100.0 μs; and pre-pulse storage 10.0 μs. Negative ions were acquired over the range of *m/z* 100–1500 with 5 Hz scanning frequency. MS/MS spectra were obtained using automated data-dependent acquisition, in which two of the most intense precursor ions were fragmented by collision-induced dissociation (CID, Ar collision gas). Collision energies were automatically selected from the pre-defined list based on the *m/z* of fragmented ions and ramped between 75 and 125% of the selected value. The QTOF analyzers' internal mass calibration was based on the sodium formate clusters, injected in the 10 mM solution in 50% (*v/v*) 2-propanol to the ion source through 20 μL directly before every analysis.

Tentative identifications of saponins were carried out using high-resolution measurements of *m/z* ratios with errors not exceeding 5 ppm, and chemical formulas were calculated on this basis. Due to the presence of isobaric compounds with identical formulas in the analyzed samples, additional identification data were obtained from the MS/MS fragmentation spectra.

For each tentatively identified saponin, extracted ion chromatograms corresponding to either the deprotonated molecule or the formic acid adduct ions were created with 0.01 Da width. Signals from QTOF-MS/MS and CAD detectors were aligned (-3.6 s delay of CAD vs. QTOF-MS/MS signal), and for each chromatographic peak detected on the extracted ion chromatograms, a corresponding peak (if present) on the signal from the CAD detector was manually integrated. The CAD response was calibrated in the concentration range from 5 to 35 pmol/μL using a series of dilutions from 1 mg/mL stock solutions of 3,28-diglucoside of medicagenic acid (medicoside G) and soyasaponin Bb. The calibrated ratio between peak area and internal standard peak area was linear in the utilized range of concentrations. All measurements were performed at least in triplicate. The results were expressed as μg of saponin per g of dry weight (DW) of leaves.

Statistical analysis

Microsoft Excel 2010 was used for statistical analysis. The resulting data were presented as means ± standard deviations. Means were compared by the analysis of variance (ANOVA) with Tukey's post-hoc test adopting the standard criterion of significance at $p \leq 0.05$.

RESULTS AND DISCUSSION

Characterization of saponin structures

After the leaves of four different Swiss chard cultivars were extracted under the optimized conditions, the SPE fractionation on RP-18 cartridges was carried out with the step gradient of methanol and water. SPE 80% (*v/v*) methanol fractions of saponins were analyzed by UHPLC-CAD/QTOF-MS/MS to identify their components. Figure 1 shows a chromatogram of leaves of Swiss chard (*Beta vulgaris* L.) cultivars with numerous peaks observed. Eleven, eight, eleven, and eight compounds, which could be identified as saponins due to their molecular mass, were observed in extracts from Rhubarb, Bulls Blood, Perpetual Spinach, and White Silver cultivars, respectively.

The compounds detected in this work were tentatively characterized using MS data, together with the interpretation of the observed MS/MS spectra. Table 1 shows the list of all compounds tentatively characterized through UHPLC-QTOF-MS/MS experiments along with their retention times (t_R), the accurately detected mass of each saponin and the MS/MS fragment ions, and the bibliographic references used in the identification process.

Based on the analysis of the MS^2 spectra, it could be concluded that Swiss chard saponins are derivatives of several different aglycones: oleanolic acid (*m/z* 455), hederagenin (*m/z* 471), gypsogenin (*m/z* 469), akebonoic acid (*m/z* 439), and serjanic acid (*m/z* 499) (Figure 2). Except for serjanic acid, the remaining sapogenins were previously identified in the red beet and sugar beet [Mikołajczyk-Bator *et al.*, 2016a,b; Mroczek *et al.*, 2012, 2019; Murakami *et al.*, 1999; Spórna-Kucab *et al.*, 2020; Yoshikawa *et al.*, 1995, 1996, 1998]. Nevertheless, all detected aglycones, including highly oxidized serjanic and akebonoic acids, were previously found in glycoside forms of other representatives of the Amaranthaceae family. Specifically, serjanic acid derivatives were detected in *Chenopodium quinoa* [Gómez-Caravaca *et al.*, 2011], while akebonoic acid was categorized as an aglycone of saponins in *Chenopodium quiona* [Gómez-Caravaca *et al.*, 2011],

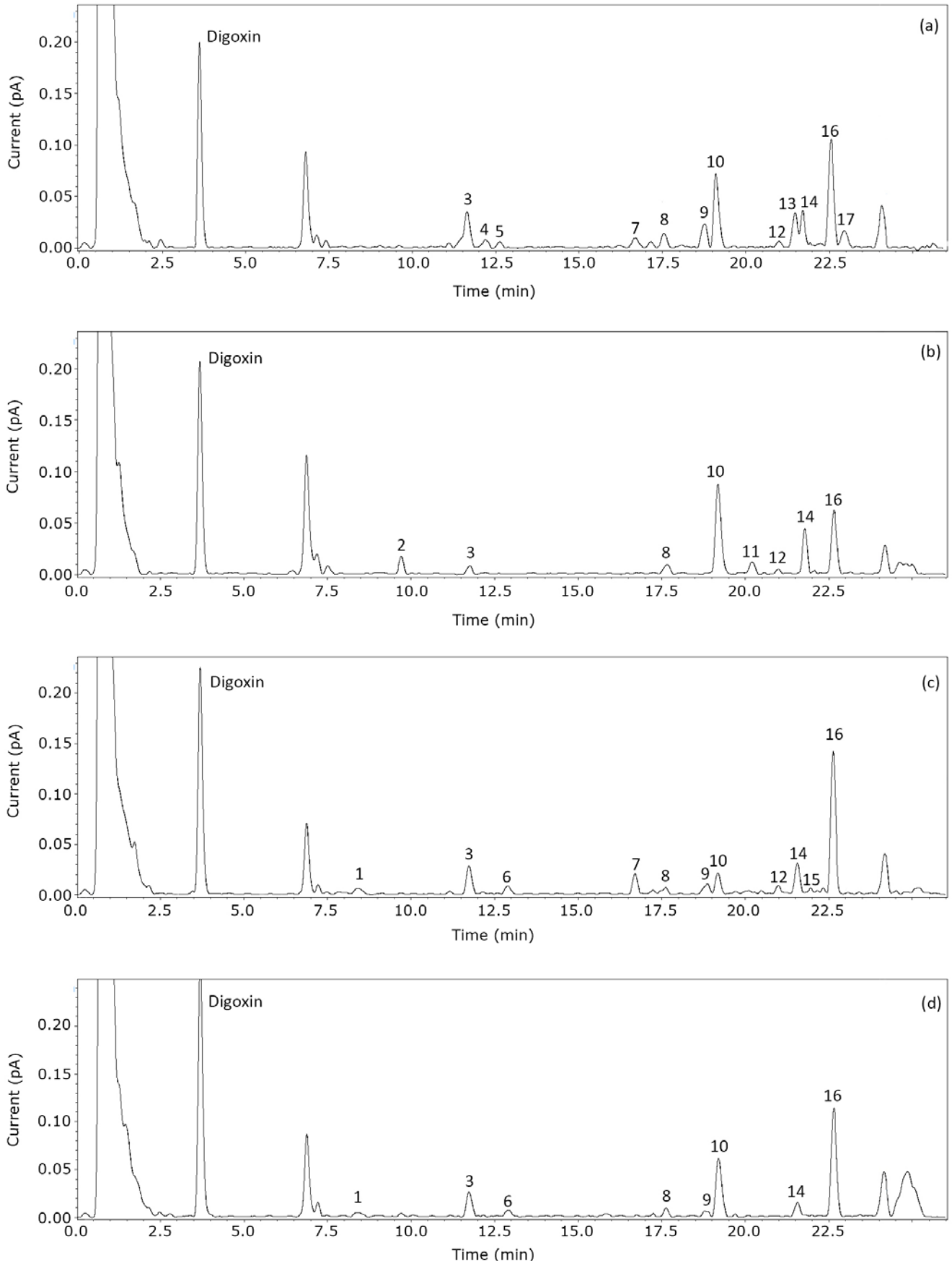

FIGURE 1. UHPLC-CAD chromatogram of saponin fractions from leaves of Swiss chard (*Beta vulgaris* L.) cultivars Rhubarb (a), Bulls Blood (b), Perpetual Spinach (c), and White Silver (d).

TABLE 1. Spectral characteristic of saponins detected by UHPLC-QTOF-MS/MS in Swiss chard leaves and their tentative identification.

Nr	t_R (min)	Precursor ion (*m/z*)[a]	Calculated formula	Error (ppm)	Isotopic fit (mSigma)	Primary product ions (*m/z*)	Tentative identification	Reference
1	8.4	1087.4914	$C_{53}H_{83}O_{23}$	4.8	21.8	925 [M-Hex-H]$^-$, 793 [M-Hex-Pen-H]$^-$, 731 [M-Hex-CO_2-hPen-H]$^-$, 455 [M-Hex-Hex-Pen-UrA-H]$^-$	Hex-Hex-Pen-UrA-oleanolic acid	Mroczek *et al.* [2012]; Mikołajczyk-Bator *et al.* [2016a]
2	9.1	1117.5019	$C_{53}H_{81}O_{25}$	4.8	72.8	835 [M-Act-$C_4H_8O_4$-H]$^-$, 793 [M-Act-Hex-H]$^-$, 731 [M-Act-hHex-CO_2-H]$^-$, 631 [M-Act-Hex-Hex-H]$^-$ 455 [M-Act-Hex-Hex-UrA-H]$^-$	Act-Hex-Hex-UrA-oleanolic acid	Yoshikawa *et al*. [1998]; Mikołajczyk-Bator *et al*. [2016b]
3	11.7	955.4489	$C_{47}H_{71}O_{20}$	5.7	16.3	835 [M-$C_3H_4O_5$-H]$^-$, 793 [M-Act-H]$^-$, 631 [M-Act-Hex-H]$^-$, 455 [M-Act-Hex-UrA-H]$^-$	Act-Hex-UrA-oleanolic acid	Yoshikawa *et al*. [1996]
4	12.5	837.3891	$C_{42}H_7O_{16}$	4.4	2.4	717 [M-$C_3H_4O_5$-H]$^-$, 675 [M-$C_3H_4O_5$-C_2H_2O-H]$^-$, 499 [M-$C_3H_4O_5$-C_2H_2O-UrA-H]$^-$	Act-UrA-serjanic acid	
5	12.9	925.4391	$C_{46}H_{69}O_{19}$	5.1	57.9	745 [M-Hex-H_2O-H]$^-$, 569 [M-Hex-CO_2-hPen-H]$^-$, 551 [M-Hex-CO_2-H_2O-hPen-H]$^-$	Pen-Hex-UrA-oleanolic acid	Yoshikawa *et al*. [1996]; Mikołajczyk-Bator *et al*. [2016a]
6	16.8	807.3795	$C_{41}H_{59}O_{16}$	3.8	17.5	647 [M-Diox-H]$^-$, 471 [M-Diox-UrA-H]$^-$	Diox-UrA-hederagenin	
7	17.6	809.3954	$C_{41}H_{61}O_{16}$	1.4	15.8	689 [M-$C_3H_4O_5$-H]$^-$, 647 [M-$C_3H_4O_5$-C_2H_2O-H]$^-$, 471 [M-$C_3H_4O_5$-C_2H_2O-UrA-H]$^-$	Act-UrA-hederagenin	Yoshikawa *et al*. [1998]; Mikołajczyk-Bator *et al*. [2016a]
8	18.7	779.3845	$C_{40}H_{59}O_{15}$	1.9	47.7	647 [M-Pen-H]$^-$, 471 [M-Pen-UrA-H]$^-$	Pen-UrA-hederagenin	Mikołajczyk-Bator *et al*. [2016a]
9	19.1	925.4779	$C_{46}H_{63}O_{19}$	2.5	18.2	763 [M-dAct-H]$^-$, 569 [M-dAct-HexA-H]$^-$, 631 [M-dAct-Pen-H]$^-$	Act-Pen-UrA-oleanolic acid	Mroczek *et al*. [2012]
10	20.1	925.4419	$C_{47}H_{73}O_{18}$	2.2	17.6	793 [M-Pen-H] $^-$, 745 [M-Hex-H_2O-H]$^-$, 731 [M-UrA-H_2O-H]$^-$, 455 [M-Pen-Hex-UrA-H]$^-$	Pen-Hex-UrA-oleanolic acid	Mroczek *et al*. [2012]
11	20.9	807.3815	$C_{41}H_{59}O_{16}$	-0.8	9.0	627 [M- $C_3H_4O_5$-C_2H_2O-H-H]$^-$, 469 [M-$C_3H_4O_5$-C_2H_2O-UrA-H]$^-$	Act-UrA-gypsogenin	Mikołajczyk-Bator *et al*. [2016a]
12	21.5	809.3945	$C_{41}H_{61}O_{16}$	2.5	15.9	689 [M-$C_3H_4O_5$-H]$^-$, 647 [M-$C_3H_4O_5$-C_2H_2O-H]$^-$, 471 [M-$C_3H_4O_5$-C_2H_2O-UrA-H]$^-$	Act-UrA-hederagenin	Yoshikawa *et al*. [1998]; Mikołajczyk-Bator *et al*. [2016a]
13	21.7	763.4267	$C_{41}H_{63}O_{13}$	1.0	18.3	631 [M-Pen-H]$^-$, 455 [M-Pen-UrA-H]$^-$	Pen-UrA-oleanolic acid	Mroczek *et al*. [2012]; Mikołajczyk-Bator *et al*. [2016a]
14	19.6	777.3683	$C_{40}H_{57}O_{15}$	2.6	43.4	657 [M-$C_3H_4O_5$-H]$^-$, 615 [M-$C_3H_4O_5$-C_2H_2O-H-H]$^-$, 439 [M-$C_3H_4O_5$-C_2H_2O-UrA-H]$^-$	Act-UrA-akebonoic acid	Mikołajczyk-Bator *et al*. [2016a,b]
15	22.5	793.4007	$C_{41}H_{61}O_{15}$	1.1	9.4	673 [M-$C_3H_4O_5$-H]$^-$, 631 [M-$C_3H_4O_5$-C_2H_2O-H]$^-$, 455 [M-$C_3H_4O_5$-C_2H_2O-UrA-H]$^-$	Act-UrA-oleanolic acid	Yoshikawa *et al*. [1998], Mikołajczyk-Bator *et al*. [2016b]
16	22.9	791.3838	$C_{41}H_{59}O_{15}$	2.7	5.7	631 [M-Diox-H]$^-$, 455 [M-Diox-UrA-H]$^-$	Diox-UrA-oleanolic acid	Yoshikawa *et al.* [1996], Mikołajczyk-Bator *et al.* [2016b]

Act – acetal substituent, aAct – deoxyactetal, Diox – dioxolane substituent Hex – hexose, hHex – hydrated hexose, Pen – pentose, UrA – uronic acid.

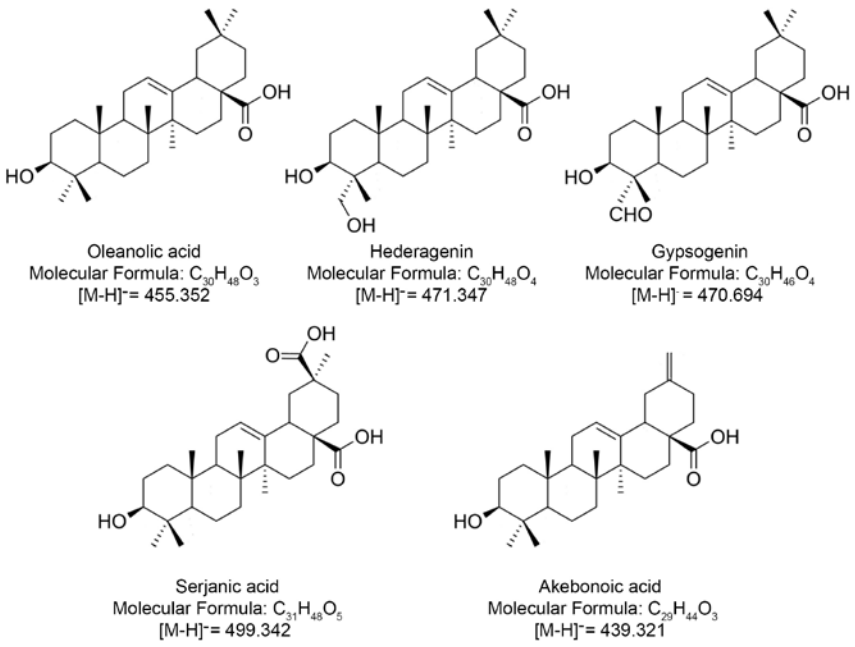

FIGURE 2. Chemical structures of saponin aglycones present in the leaves of Swiss chard *(B. vulgaris* L.).

Amaranthus hypochondriacus [Kohda *et al.*, 1991], and *Pfaffia glomerata* [Nakamura *et al.*, 2010].

Specifically, the majority of the saponins in the leaves contained oleanolic acid as an aglycone, including 5 (Rhubarb), 4 (Bulls Blood), 5 (Perpetual Spinach), and 6 (White Silver) in each cultivar. The number of hederagenin derivatives was 3 (Rhubarb), 1 (Bulls Blood), 3 (Perpetual Spinach), and 2 (White Silver). Only one gypsogenin derivative was detected in cv. Rhubarb, Bulls Blood, and Perpetual Spinach. Serjanic acid was present in the structure of one saponin of cv. Rhubarb and akebonoic acid in one compound of cv. Perpetual Spinach.

The differences in triterpene saponins' chemical structures are due to various aglycones, the saccharide units' composition, and the varied linkages of sugar moieties. The saccharide moieties detected in saponins from Swiss chard cultivars were composed of uronic acid (UrA), pentose (Pen), and hexose (Hex). However, different monosaccharide epimers cannot be differentiated using mass spectrometric data alone. Similarly, the determination of the number of sugar chains and their composition usually needs additional analytical steps.

Additionally, acetal (Act)- and dioxolane (Diox)-type dicarboxylic acids linked to the sugar moieties were detected in Swiss chard leaves. Acetal and dioxolane-type substituents were previously detected in sugar beet and red beet saponins and were linked by 1→2 (Glc→GlcA), 1→3 (Xyl→GlcA), and 3→3 (Act→GlcA) glycosidic bonds [Mikołajczyk-Bator *et al.*, 2016a; Murakami *et al.*, 1999; Yoshikawa *et al.*, 1995, 1996, 1998]. Moreover, an acetal type substituent was described in the saponin isolated from the *Chenopodium album* plant [Mroczek, 2015]. The presence of this type of residues in a carbohydrate chain, presumed to be derived by oxidative degradation of pentose and hexose units, seems to be unique for Amaranthaceae saponins.

The structures of 16 saponins of Swiss chard leaves tentatively proposed based on the detailed fragmentation patterns are shown in Table 1, whereas selected fragmentation spectra are presented in Figure 3.

The compound with the smallest molecular mass detected in all saponin fractions was **13** (t_R=21.7 min), which exhibited a deprotonated ion at *m/z* 763.4016. The fragmentation pattern of this compound indicated the presence of pentose (product ion at *m/z* 631 [M–132–H]⁻) and uronic acid (product ion at *m/z* 455 [M–132–176–H]⁻) in the structure. According to data found in the literature, in triterpene saponins, uronic acid is typically attached to the C-3 hydroxyl group of the aglycone [Mikołajczyk-Bator *et al.*, 2016b]. However, based on the MS/MS data alone, it is impossible to reliably detect whether hexuronic acid is attached to C-3 hydroxyl or C-28 carbonyl of the aglycone. Nevertheless, the C-3 linkage of hexuronic acid is also evident from the known structures of various *Beta vulgaris* saponins, such as betavulgarosides (Figure 4). Therefore, this compound and other saponins described in this study are presumably derivatives of C-3 glucuronides of the ascribed triterpene acids. Furthermore, an analogous sugar chain consisting of pentose and uronic acid also occurred in the glycoside of hederagenin **8** (t_R=18.7 min), which was present in all cultivars except Bulls Blood. Such a derivative of hederagenin was not described in *Beta vulgaris* plants before.

The MS^2 spectrum of saponin **15** (t_R=22.5 min), which was present in all cultivars and exhibited a deprotonated precursor ion at *m/z* 793.4016, yielded product ions at *m/z* 673 [M–120–H]⁻ and *m/z* 631 [M–120–42–H]⁻, which indicated the presence of an acetal moiety composed of tartaraldehyde and glycolic acid in the oleanolic glucuronide structure. Additionally, a substituent having such a structure was also present in the sugar chain of the derivatives of serjanic acid

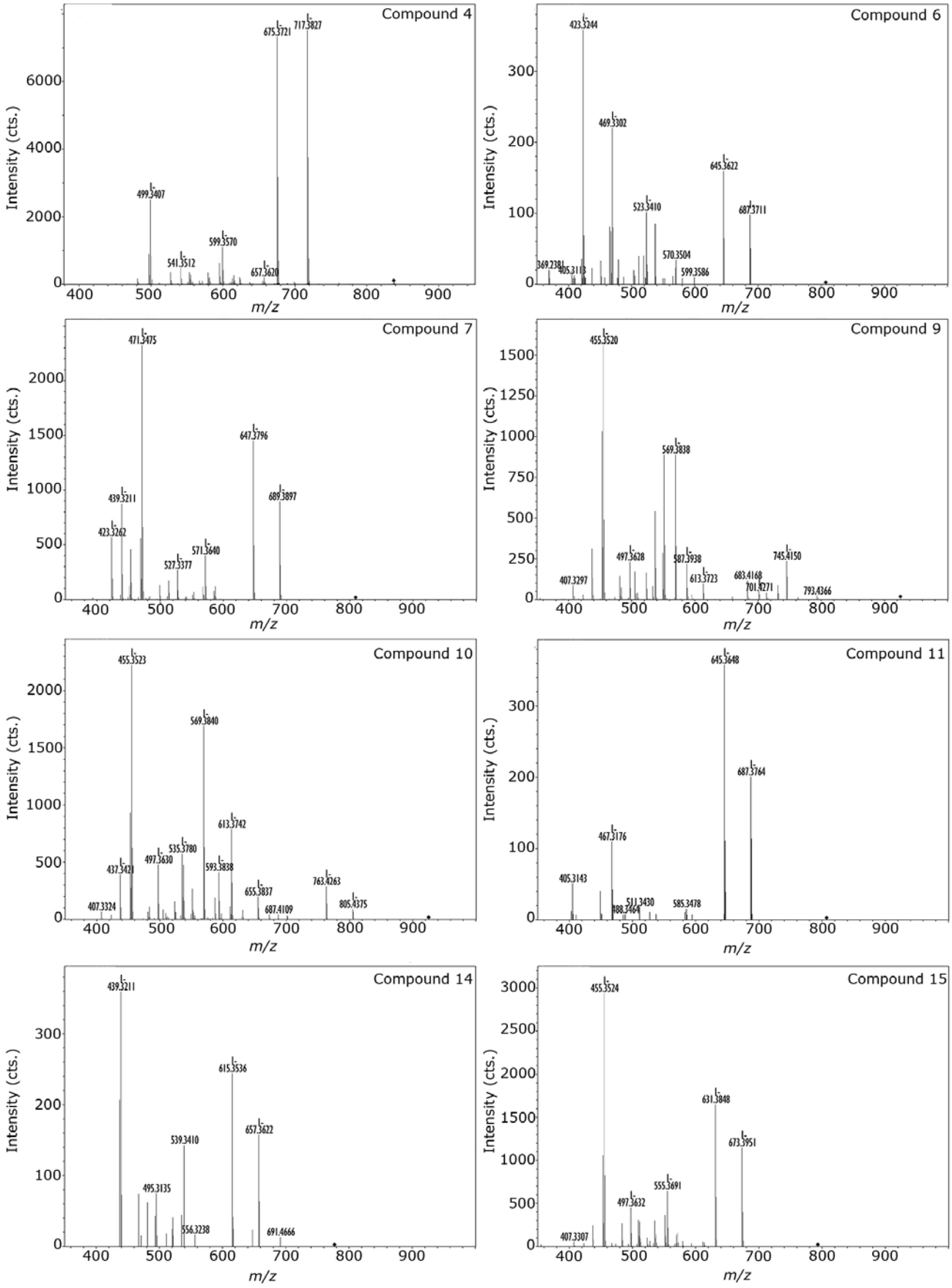

FIGURE 3. MS/MS spectra of compounds **4**, **6**, **7**, **9**, **10**, **11**, **14**, and **15** detected in leaves of Swiss chard (*B. vulgaris* L.) cultivars, tentatively identified as saponins.

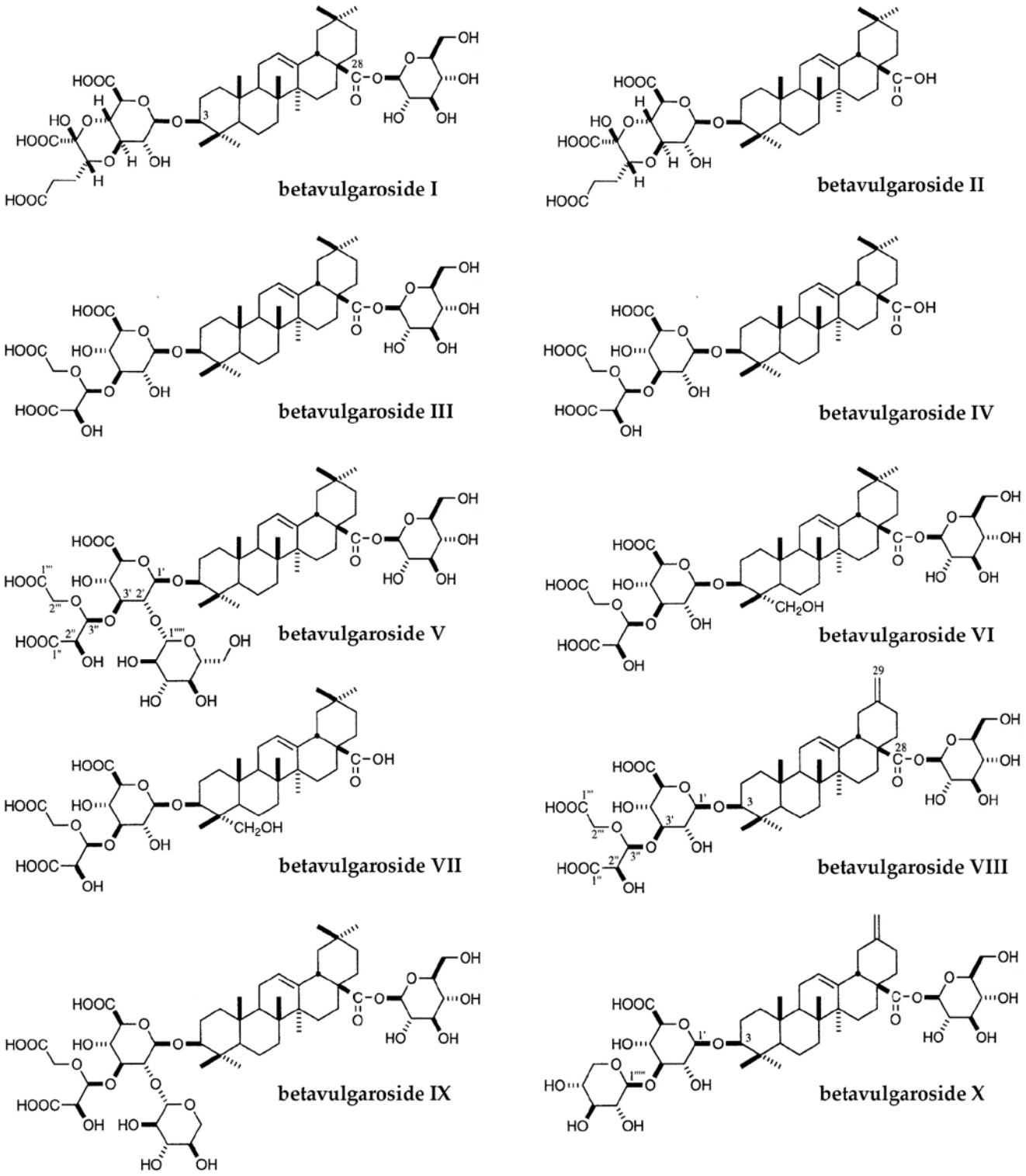

FIGURE 4. Chemical structures of betavulgarosides I–X [adopted from Murakami *et al.*, 1999; Yoshikawa *et al.,* 1995, 1996, 1998].

4 (t_R=12.5 min, detected only in the Rhubarb cultivar), gypsogenin derivative **11** (t_R=20.9 min, present in all cultivars except White Silver), akebonoic acid derivative **14** (t_R=19.6 min, present only in Perpetual Spinach), hederagenin derivative **7** (t_R=17.6 min, which was present in all cultivars except Rhubarb), and **12** (t_R=21.5 min, was present only in Rhubarb). It should be noted that the acetal group described in this study has also been identified in saponins of sugar beet and red beet roots [Mikołajczyk-Bator *et al.*, 2016a,b; Mroczek *et al.*, 2019; Yoshikawa *et al.*, 1995, 1996]. For saponin **3**, fragmentation of the precursor ion at *m/z* 955.4512 led to product ions at *m/z* 835, 793, and 631, resulting in an abundant aglycone ion at *m/z* 455 due to the loss of the acetal moiety, hexose, and uronic acid. According to the MS/MS spectra, saponin **3** is a triglycoside of oleanolic acid differing from compound **15** by the presence of additional hexose.

Another unusual substituent found in the *Beta vulgaris* species is dioxolane [Mikołajczyk-Bator *et al.*, 2016a; Murakami *et al.*, 1999; Yoshikawa *et al.*, 1995, 1996, 1998]. In Swiss chard leaves, this moiety was present in saponins **6** ($t_{R=}$16.8 min) and **16** (t_R= 22.9 min). The MS/MS fragmentation of both saponins showed some similarity. Concerning saponin **16** with the precursor $[M–H]^-$ ion at *m/z* 791.3859, MS/MS data showed the loss of 160 Da, forming a fragment at *m/z* 631, followed by the loss of an uronic acid residue. The formation of the 160 Da fragment was due to the cleavage of the dioxolane substituent. Similarly, the fragmentation of the deprotonated ion of saponin **6** at 807.3795 *m/z* clearly showed the loss of a dioxolane substituent (160 Da). As for saponin **16**, it showed a fragment at *m/z* 455, which is characteristic of oleanolic acid, and the fragmentation of **6** had shown a fragment ion at *m/z* 471, which corresponds to a hederagenin aglycone. Saponin **16** was previously detected in sugar beet [Yoshikawa *et al.*, 1996] and red beet [Mikołajczyk-Bator *et al.*, 2016a], while saponin **6** was detected in *Beta vulgaris* for the first time.

Saponins **9** (t_R=19.1 min) and **10** (t_R=20.1 min) were identified with similarity in the appearance of peaks exhibiting pseudomolecular ions $[M–H]^-$ at *m/z* 925.4439 and 925.4761. Saponin **10** showed fragments formed at *m/z* 745 $[M–Hex–H_2O–H]^-$, 569 $[M–Hex–CO_2–hPen–H]^-$, *m/z* 551 $[M–Hex–CO_2–H_2O–hPen–H]^-$ and *m/z* 455 $[M–Hex–CO_2–H_2O–hPen–H–UrA–H]^-$, which were due to the loss of hexose, pentose and, uronic acid from the oleanolic acid derivative. For saponin **9**, fragmentation of the precursor ion led to product ions at *m/z* 763, 569, and 631, resulting in an abundant aglycone ion at *m/z* 455 due to the loss of the deoxy-acetal moiety, pentose, and uronic acid. According to the MS/MS spectra, saponin **9** was a triglycoside of oleanolic acid differing from compound **11** by the presence of acetal instead of hexose.

The fragmentation of compound **1** (t_R=8.4 min), differing from compound **10** by 162 Da, resulted in the formation of product ions at *m/z* 925 $[M–Hex–H]^-$, *m/z* 793 $[M–Hex–Pen–H]^-$, and *m/z* 455 $[M–Hex–Hex–Pen–UrA–H]^-$ corresponding to the loss of two hexose, pentose, and uronic acid units. The sugar chain based on sugar moieties appears similar to that of the saponin with the same molecular mass described in the red beet roots [Mroczek *et al.*, 2012; Mikołajczyk-Bator *et al.*, 2016a].

The MS/MS spectrum of saponin **2** (t_R=9.1min) showed fragment ions at *m/z* 835, 925, 631, and 455 $[M–162–H]^-$, indicating the loss of an acetal moiety, two glucose units, and uronic acid, respectively. Therefore, based on this data, it can be speculated that saponin **2** has the same structure as betavulgaroside V present in the sugar beet [Yoshikawa *et al.*, 1998]. As described in the literature, betavulgaroside V is an oleanolic acid bidesmoside with a branched carbohydrate chain composed of glucose, an acetal substituent, and glucuronic acid attached to the C-3 hydroxyl group of the aglycone and another glucose attached in the C-28 position [Yoshikawa *et al.*, 1998].

Triterpenoid saponins were detected in *Beta vulgaris* for the first time by the Yoshikawa and Murakami teams and named betavulgarosides (Figure 4). The structures of these substances were determined by spectral methods and by chemical correlation [Yoshikawa *et al.*, 1995, 1996, 1998]. Among the substances tentatively identified in the present study, compounds **6**, **3**, **15**, **2**, **12**, and **1** have the same summary formulae and, based on MS/MS data, also similar structural features as betavulgarosides II, III, IV, V, VII, and IX, respectively. It cannot be excluded that these betavulgarosides are present in Swiss chard plants; however, this needs to be confirmed in additional analyses. Nevertheless, the structural similarity between the saponins from Swiss chard leaf and the sugar beet is in line with what could be expected considering both plants' close affinity – they are derived from a common ancestor – the wild beet [Biancardi *et al*., 2016].

Quantitative analysis

Because of structural features of dioxolane- and acetal-type substituents, *B. vulgaris* saponins are relatively difficult to separate using reversed-phase chromatography. The calculations of pKa for carboxylic groups of a dioxolane substituent carried out using Perkin-Elmer ChemDraw software (v. 19.1) indicate that the lowest acidic pKa is at 2.05. In the typical conditions used in LC-MS, the addition of 0.1% formic acid decreases the mobile phase's pH to approximately 2.7. This value is not low enough to suppress the dissociation of all carboxylic groups and results in very broad and often overlapping chromatographic peaks of dioxolane derivatives. Such difficulties were never reported in the literature addressing the *B. vulgaris* saponins, and they were, apparently, not perceived as a problem in the previously published qualitative and qualitative analyses of *B. vulgaris* saponins [Mikołajczyk-Bator *et al.*, 2016a; Mroczek *et al.*, 2012, 2019]. However, chromatograms presented in all these studies (for example, see Figure 1A, peaks 27–32 in Mikołajczyk-Bator *et al.* [2016a]) clearly demonstrate that resolving these issues is crucial for any reliable quantitative method (peak broadening results in increased detection limits) and that good separation of the analytes is often critical for many detection methods, including mass spectrometric detection. Various approaches can yield improved chromatographic resolution and peak shape for dioxolane- and acetal-containing saponins. A recent LC-MS quantitative study of saponins from roots of *Achyranthes* sp., including betavulgarosides, used an ion-pairing reagent, dihexylammonium acetate (DHAA), to increase chromatographic resolution [Kawahara *et al.*, 2016]. While this approach enabled significant improvement of the chromatographic resolution and excellent peak shapes, from our experience, DHAA is a relatively challenging to purge persistent contaminant of chromatographic systems that severely affects the performance and sensitivity during the positive-mode electrospray ionization analyses. For this reason, mobile phases containing DHAA may only be suitable for dedicated LC-MS systems.

Another possibility for improving the chromatography of betavulgarosides and related compounds is the acidification of the mobile phase. To this end, an acid with lower pKa (for example, trifluoroacetic acid – TFA) or a much higher formic acid concentration must be used. Our study used the latter approach with 0.5% (*v/v*) formic acid, even though

it required a decrease in capillary voltage to prevent excessive capillary current and arcing. The alternative application of 0.05% (*v/v*) TFA as a mobile phase additive was also tested (data not shown). However, it offered a better chromatographic resolution at the cost of significant signal suppression in the negative electrospray mode, to the point where obtaining high-quality MS/MS spectra, a crucial component of the proposed method, became impossible.

The method we propose uses two detectors to collect qualitative and quantitative data on Swiss chard saponins. This approach allows correlating the identification and relative quantification of constituents in complex mixtures of botanical origin [Baker *et al.*, 2018]. It can, by no means, replace classic LC-MS assays based on the individual reference standards. Instead, it can be considered as a viable alternative to the total saponin content (TSC) measurement carried out using other methodologies, such as the spectrophotometric assay [Le *et al.*, 2018], the macro lens-coupled smartphone assay [León-Roque *et al.*, 2019], or the UHPLC-UV assay [Wu *et al.*, 2019]. In turn, the approach we propose offers more reliable quantitation of individual saponin peaks (thus avoiding false-positive signals of spectrophotometric and foam-formation methods) as well as detection that is not dependent on the particular structural features of the investigated analytes. Structure-dependent detection is the weak point of the UHPLC-UV-QTOF method [Wu *et al.*, 2019], which is very similar to our methodology in general concept and application. However, it uses a calibration compound with a specific structure and a specific number of chromophores contributing to the absorbance at 210 nm used for quantitation. Any structural deviation resulting in a different number of chromophores (for example, an additional carboxyl group due to esterification) will change the molar absorbance coefficient and invalidate calibration for that specific compound. Similarly, saponins without chromophores, such as selected steroidal saponins or pseudoginsenoside F11, will produce no signal at all. Non-specific absorbance at 210 nm also restricts the choice of mobile phases for chromatography, practically excluding those with higher UV cut-off, such as methanol or acetone.

The CAD we applied in this study is considered a universal detector. However, like with all aerosol evaporative detectors, its response varies as a function of mobile phase composition [Hutchinson *et al.*, 2010]. Thus, in an ideal isocratic separation, all the analytes at the same concentration should produce identical responses. However, in gradient elution, the higher is the percentage of the organic solvent, the higher signal will be obtained for the analytes. Besides the isocratic elution, which is often not feasible for very complex samples, there are two ways of correcting this discrepancy. One possibility is to provide the detector with a constant mobile phase concentration throughout the analysis, utilizing the secondary pump running the reverse gradient through a separate, identical column. Both columns' outflows are mixed before the detector, providing the constant concentration of the mobile phase [Baker *et al.*, 2018; Górecki *et al.*, 2006]. While relatively simple in application and providing nearly perfect results [Baker *et al.*, 2018], this approach requires additional, careful equipment set up and uses significant volumes of solvents. As an alternative, the so-called 3-D calibration can compensate for signal changes during the elution [Hutchinson *et al.*, 2010].

TABLE 2. Details of the UHPLC-CAD/QTOF-MS/MS method validation.

Parameter	Medicoside G	Soyasaponin Bb
Range [μM]	5–35	5–35
Regression equation (*n=9*)	y = 0.065x+ 0.185	y = 0.076x+ 0.261
R^2	0.995	0.992
LOD [μM]	2.4 (1.8 μg/g DW)	3.6 (3.4 μg/g DW)
LOQ [μM]	7.3	10.69
Intra-day precision peak area ratio RSD% (*n=5*)		
7.5 μM	1.0	2.5
32.5 μM	0.8	2.4
Intra-day accuracy % (*n=5*)		
7.5 μM	91.9 (76.5)*	105.7 (92.8)*
32.5 μM	96.6 (80.3)*	102.0 (116.2)*
Inter-day precision peak area ratio RSD% (*n=10*)		
7.5 μM	5.1	6.7
32.5 μM	4.4	5.6
Inter-day accuracy % (*n=10*)		
7.5 μM	93.4	90.3
32.5 μM	91.6	99.4

* Cross-calibration result, LOD – Limit of Detection, LOQ – Limit of Quantitation, RSD – relative standard deviation, DW- dry weight.

We applied a slight modification of the latter approach. Using two calibration standards, medicoside G and soyasaponin Bb, eluting at two different t_R in the gradient (5.6 min and 21.8 min, respectively), we obtained a set of calibrations applicable to estimate several analytes eluting between these two points. Furthermore, elution during the separation was switched to isocratic with 55% acetonitrile at 22 min. Therefore the concentration of compounds eluting after that time could be estimated using soyasaponin Bb calibration with acceptable accuracy. Cross-calibration, estimation of medicoside G with soyasaponin Bb curve, and *vice versa* produced errors generally not exceeding 25% (Table 2), which in our view were acceptable. Our method's main disadvantage is the relatively narrow linear dynamic range, slightly under one order of magnitude. However, the obtained limits of detection were at acceptable levels of approx. 1–3 μg/g DW.

Nevertheless, the method's main limitation is that the detector's response is determined based on a single calibrant, which might lead to errors if the detector's response to the analyte is significantly different from the response toward the calibrant. Furthermore, because CAD is a 1D detector, it is difficult to assess the purity of integrated peaks in co-elution cases, although this can be aided by analyzing the corresponding MS signals. Needless to say, the method cannot be applied directly to raw extracts and requires some clean-up and fractionation before the UHPLC-CAD/QTOF-MS/MS analysis.

TABLE 3. The contents of individual and total saponins in Swiss chard (*B. vulgaris* L.) cultivars (μg/g DW).

No	Rhubarb	Bulls Blood	Perpetual Spinach	White Silver
1	nd	nd	6.02±0.85^{a}	2.95±0.39^{b}
2	nd	19.83±2.71	nd	nd
3	17.20±2.37^{a}	5.25±1.20^{c}	18.18±1.39^{a}	12.9±1.26^{b}
4	4.68±0.87	nd	nd	nd
5	nd	nd	18.88±3.88^{a}	5.47±2.92^{b}
6	11.01±1.90^{b}	nd	26.69±2.34^{a}	nd
7	5.90±0.97^{a}	4.99±0.26^{b}	3.10±0.39^{c}	3.46±1.10^{bc}
8	18.17±0.30^{a}	nd	19.59±2.99^{a}	3.24±0.27^{b}
9	30.57±4.48^{b}	63.11±3.97^{a}	29.61±2.52^{b}	34.58±2.20^{b}
10	nd	17.71±1.26	nd	nd
11	3.19±0.51^{b}	2.69±0.28^{b}	6.26±1.01^{a}	nd
12	10.57±1.46	nd	nd	nd
13	9.54±0.26^{a}	23.04±1.60^{b}	52.57±3.29^{c}	5.86±1.13^{a}
14	nd	nd	17.44±2.21	nd
15	77.76±6.22^{b}	42.39±4.21^{c}	178.92±24.36^{a}	57.07±2.96^{bc}
16	13.52±1.67	nd	nd	nd
TOTAL	202.11	159.18	397.09	125.53

Values are expressed as the means ± standard deviations of three independent samples. Results in rows not sharing a common letter are significantly different ($p \leq 0.05$); nd – not detected. The numbers (1–16) correspond to the numbers of compounds named in Table 1.

The developed method was then applied to quantify 16 individual saponins present in the leaves of Swiss chard cultivars. The quantitative data revealed differences in saponins' content thereof (Table 3). Specifically, relatively the high content of individual saponins was in the Perpetual Spinach cultivar's leaves, with a total content of 397.09 μg/mg DW. This content was approximately 2 and 2.5 times higher than that in cv. Rhubarb and White Silver leaves and approximately three times higher than in cv. Bulls Blood leaves.

All cultivars contained relatively large amounts of saponin **15**, which predominated in leaves of cultivars Rhubarb, Perpetual Spinach, and White Silver, accounting for 37, 45, and 46% of total saponin content, respectively. Moreover, saponin **15** was the second most abundant saponin in the Bulls Blood cultivar (27% of total saponin content). Saponin **9** was predominant in the Bulls Blood cultivar (40% of total saponin content) and was the second most abundant saponin in leaves of Rhubarb, Perpetual Spinach, and White Silver (15, 16, and 27% of total saponin contents, respectively). The significant accumulation of these two specific triterpene saponins suggests their biological function, but further research is necessary to resolve this issue.

The quantitative analysis of saponins in Swiss chard leaves showed significant differences between their contents in different cultivars. Our previous study demonstrated divergences in the saponin content in leaves of different red beet cultivars [Mroczek *et al.*, 2019]. In contrast to Swiss chard cultivars, showing strong divergences in saponin profiles, red beet leaves of all analyzed cultivars contained the same number of saponin, although in different proportions. The selection of new varieties of Swiss chard, aiming at improving such crop features like pest resistance, storage stability of the collected plant material, taste, or the content of betalains, influences possibly the content of saponins in different plant varieties indirectly or directly, *e.g.*, the antimicrobial activities of Swiss chard saponins can determine its resistance. The different saponin content can also impact the nutraceutical effects of these widely consumed plants. However, extensive studies are required to assess their biological bioactivity and beneficial health properties.

CONCLUSIONS

The UHPLC-CAD/QTOF-MS/MS method has been established for the simultaneous qualitative and quantitative analyses of saponins in Swiss chard leaves. For the qualitative analysis, a total of 16 compounds were tentatively characterized in different Swiss chard cultivars based on MS data and MS/MS fragmentation patterns. In turn, for the quantitative analysis, all saponins detected in plant material were separated and simultaneously determined. The results show that various cultivars differ in the total content of saponins, yet the major compounds are common for all tested cultivars. Specific composition patterns and content of individual saponins may also reflect their function, as saponins' biological activity is often associated with specific chemical constituents. However, further studies are required to provide the total structures of Swiss chard saponins and to elucidate their biological properties within the frame of structure-function relationship studies.

To the best of our knowledge, this work presents the first study of saponins in the Swiss chard plant. The data obtained indicate that Swiss chard is a vital source of these bioactive phytochemicals. As the relative distribution and composition of saponins vary between different cultivars, the developed method allows a complete analysis of Swiss chard saponins and can be of great significance for future investigations and applications. Furthermore, this analytical approach could be validated to determine and quantitate saponins in different Amaranthaceae family species.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1. Alara, L.R., Abdurahman, N.H., Ukaegbu, C.I., Azhari, N.H., Kabbashi, N.A. (2018). Metabolic profiling of flavonoids, saponins, alkaloids, and terpenoids in the extract from *Vernonia cinerea* leaf using LC-Q-TOF-MS. *Journal of Liquid Chromatography & Related Technologies*, *41*, 722–731. https://doi.org/10.1080/10826076.2018.1511995
2. Arslan, I., Cenzano, A.M. (2020). Triterpene saponins in cancer therapy: a review of mode of action. *Revista Brasileira de Farmacognosia, 30*, 1–6. https://doi.org/10.1007/s43450-020-00033-5
3. Baker, T.R., Regg, B.T. (2018). A multi-detector chromatographic approach for characterization and quantitation of botanical constituents to enable *in silico* safety assessments. *Analytical and Bioanalytical Chemistry*, *410*, 5143–5154. https://doi.org/10.1007/s00216-018-1163-y
4. Biancardi, E., Panella, L.W., Lewellen, R.T. (2012). History and current importance. In Biancardi, E., Panella, L.W., Lewellen, R.T. (Eds.), *Beta maritima*. *The Origin of Beets*, 1st edition, Springer, New York, USA, pp. 1–41. https://doi.org/10.1007/978-1-4614-0842-0
5. Foubert, K., Cuyckens, F., Vleeschouwer, K., Theunis, M., Vlietinck, A., Pieters, L., Apers, S. (2010). Rapid quantification of 14 saponins of *Maesa lanceolata* by UPLC–MS/MS. *Talanta*, *81*, 1258–1263. https://doi.org/10.1016/j.talanta.2010.02.018
6. Ge, Y-W., Zhu, S., Yoshimatsu, K., Komatsu, K. (2017). MS/MS similarity networking accelerated target profiling of triterpene saponins in *Eleutherococcus senticosus* leaves. *Food Chemistry*, *227*, 444–452. https://doi.org/10.1016/j.foodchem.2017.01.119
7. Gennari, L., Felletti, M., Blasa, M., Angelino, D., Celeghini, C., Corallini, A., Ninfali, P. (2011). Total extract of *Beta vulgaris* var. cicla seeds *versus* its purified phenolic components: antioxidant activities and antiproliferative effects against colon cancer cells. *Phytochemical Analysis*, *22*, 272–279. https://doi.org/10.1002/pca.1276
8. Gómez-Caravaca, A.M., Segura-Carretero, A., Fernández-Gutiérrez, A., Caboni, M.F. (2011). Simultaneous determination of phenolic compounds and saponins in qinoa (*Chenopodium quinoa* Willd) by a liquid chromatography-diode array detection-electrospray ionization-time-of-flight mass spectrometry methodology. *Journal of Agricultural and Food Chemistry*, *59*, 10815–10825. https://doi.org/10.1021/jf202224j
9. Górecki, T., Lynen, F., Szucs, R., Sandra, P. (2006). Universal response in liquid chromatography using charged aerosol detection. *Analytical Chemistry*, *78*, 3186–3192. https://doi.org/10.1021/ac060078j
10. Hashem, A.N., Soliman, M.S., Hamed, M.A., Swilam, N.F., Lindequist, U., Nawwar, M.A. (2016). *Beta vulgaris* subspecies cicla var. flavescens (Swiss chard): flavonoids, hepatoprotective and hypolipidemic activities. *Pharmazie*, *71,* 227–232.
11. Hutchinson, J.P., Li, J., Farrell, W., Groeber, E., Szucs, R., Dicinoski, G., Haddad, P.R. (2010). Universal response model for a corona charged aerosol detector. *Journal of Chromatography A*, *1217*, 7418–7427. https://doi.org/10.1016/j.chroma.2010.09.056
12. Ivanović, L., Milašević, I., Topalović, A., Đurović, D., Mugoša, B., Knežević, M., Vrvić, M. (2019). Nutritional and phytochemical content of Swiss chard from Montenegro, under different fertilization and irrigation treatments. *British Food Journal*, *121*, 411–425. https://doi.org/10.1108/BFJ-03-2018-0142
13. Kawahara, Y., Hoshino, T., Morimoto, H., Shinizu, T., Narukawa, Y., Fuchino, H., Kawahara, N., Kiuchi, F. (2016). LC-MS-based quantification method for *Achyranthes* root saponins. *Journal of Natural Medicines*, *70*, 102–106. https://doi.org/10.1007/s11418-015-0942-4
14. Kirk, D.D., Rempel, R., Pinkhasov, J., Walmsley, A.M. (2004). Application of *Quillaja saponaria* extracts as oral adjuvants for plant-made vaccines. *Expert Opinion on Biological Therapy*, *4*, 947−958. https://doi.org/10.1517/14712598.4.6.947
15. Kohda, H., Tanaka, S., Yamaoka, Y., Ohhara, Y. (1991). Saponins from *Amaranthus hypochondriacus*. *Chemical and Pharmaceutical Bulletin*, *39*, 2609–2612. https://doi.org/10.1248/cpb.39.2609
16. Kowalczyk, M., Pecio, Ł., Stochmal, A., Oleszek, W. (2011). Qualitative and quantitative analysis of steroidal saponins in crude extract and bark powder of *Yucca schidigera* Roezl. *Journal of Agricultural and Food Chemistry*, *59*, 8058–8064. https://doi.org/10.1021/jf2022397
17. Le, A.V., Parks, S.E., Nguyen, M.H., Roach, P.D. (2018). Improving the vanillin-sulphuric acid method for quantifying total saponins. *Technologies*, *6*, art. no. 84. https://doi.org/10.3390/technologies6030084
18. León-Roque, N., Aguilar-Tuesta, S., Quispe-Neyra, J., Mamani-Navarro, W., Alfaro-Cruz, S., Condezo-Hoyos, L. (2019). A green analytical assay for the quantitation of the total saponins in quinoa (*Chenopodium quinoa* Willd.) based on macro lens-coupled smartphone. *Talanta*, *204*, 576–585. https://doi.org/10.1016/j.talanta.2019.06.014
19. Mikołajczyk-Bator, K., Błaszczyk, A., Czyżniejewski, M., Kachlicki, P. (2016a). Characterization and identification of triterpene saponins in the roots of red beet (*Beta vulgaris* L.) using two HPLC-MS systems. *Food Chemistry*, *192*, 979–990. https://doi.org/10.1016/j.foodchem.2015.07.111
20. Mikołajczyk-Bator, K., Błaszczyk, A., Czyżniejewski, M., Kachlicki, P. (2016b). Identification of saponins from sugar beet (*Beta vulgaris*) by low and high-resolution HPLC–MS/MS. *Journal of Chromatography B, 1029–1030*, 36–47. https://doi.org/10.1016/j.jchromb.2016.06.038
21. Mroczek, A. (2015). Phytochemistry and bioactivity of triterpene saponins from Amaranthaceae family. *Phytochemistry Reviews*, *14*, 577–605. https://doi.org/10.1007/s11101-015-9394-4
22. Mroczek, A., Kapusta, I., Janda, B., Janiszowska, W. (2012). Triterpene saponin content in the roots of red beet (*Beta vulgaris* L.) cultivars. *Journal of Agricultural and Food Chemistry*, *60*, 12397–12402. https://doi.org/10.1021/jf303952x
23. Mroczek, A., Kapusta, I., Janda, B., Stochmal, A., Janiszowska, W. (2019). MS/MS and UPLC-MS profiling of triterpenoid saponins from leaves and roots of four red beet (*Beta vulgaris* L.) cultivars. *Phytochemistry Letters*, *30*, 333–337. https://doi.org/10.1016/j.phytol.2019.02.015
24. Murakami, T., Matsuda, H., Inadzuki, M., Hirano, K., Yoshikawa, M. (1999). Medical foodstuffs. XVI. Sugar beet. (3): abso-

lute stereostructures of betavulgarosides II and IV hypoglycemic saponins having a unique substituent, from the root of *Beta vulgaris* L. *Chemical and Pharmaceutical Bulletin, 47*, 1717–1724. https://doi.org/10.1248/cpb.47.1717

25. Nakamura, S. Chen, G., Nakashima, S., Matsuda, H., Pei, Y., Yoshikawa, M. (2010). Brazilian natural medicines. IV. New noroleanane-type triterpene and ecdysterone-typesterol glycosides and melanogenesis inhibitors from the roots of Pfaffia-glomerata. *Chemical and Pharmaceutical Bulletin*, *58*, 690–695. https://doi.org/10.1248/cpb.58.690
26. Ninfali, P., Angelino, D. (2013). Nutritional and functional potential of *Beta vulgaris* cicla and rubra. *Fitoterapia*, *89*, 188–199. https://doi.org/10.1016/j.fitote.2013.06.004
27. Onlom, C., Nuengchamnong, N., Phrompittayarat, W., Putalun, W., Waranuch, N., Ingkaninan, K. (2017). Quantification of saponins in *Asparagus racemosus* by HPLC-Q-TOF-MS/MS. *Natural Product Communications, 12*(1), 7–10. https://doi.org/10.1177/1934578X1701200103
28. Osbourn, A.E. (1996). Preformed antimicrobial compounds and plant defense against fungal attack. *Plant Cell*, 8, 1821–1831. https://doi.org/10.1105/tpc.8.10.1821
29. Podolak, J., Galanty, A., Sobolewska, D. (2010). Saponins as cytotoxic agents: a review. *Phytochememistry Reviews*, *9*, 425–474. https://doi.org/10.1007/s11101-010-9183-z
30. Pyo, Y.H., Lee, T.C., Logendra, L., Rosen, R.T. (2004). Antioxidant activity and phenolic compounds of Swiss chard (*Beta vulgaris* subspecies *cycla*) extracts. *Food Chemistry*, *85*, 19–26. https://doi.org/10.1016/S0308-8146(03)00294-2
31. Sparg, S.G., Light, M.E., Van Staden, J. (2004). Biological activities and distribution of plant saponins. *The Journal of Ethnopharmacology*, *94*, 219–243. https://doi.org/10.1016/j.jep.2004.05.016
32. Spórna-Kucab, A.E., Wybraniec, S. (2020). High-speed countercurrent chromatography in separation and identification of saponins from *Beta vulgaris* L. cultivar Red Sphere. *Polish Journal of Food and Nutrition Sciences*, *70*(1), 67–74. https://doi.org/10.31883/pjfns/116416
33. Tava, A., Odoardi, M. (1996). Saponins from *Medicago* spp.: Chemical characterization and biological activity against insects. In G. Waller, K. Yamasaki (Eds.), *Saponins Used in Food and Agriculture*, Plenum Press, New York, US, pp. 97–109. https://doi.org/10.1007/978-1-4613-0413-5_9
34. Troszyńska, A. (2004). Non-nutrient bioactive substances in food of plant origin causing bitterness and astirgency. *Polish Journal of Food and Nutrition Sciences, 54*, 65–73.
35. Vehovec, T., Obreza, A. (2010). Review of operating principle and applications of the charged aerosol detector. *Journal of Chromatography A, 1217*, 1549–1556. https://doi.org/10.1016/j.chroma.2010.01.007
36. Wu, X., Jia, L., Wu, J., Liu, Y., Kang, H., Liu, X., Li, P., He, P., Tu, Y., Li, B. (2019). Simultaneous determination and quantification of triterpene saponins from *Camellia sinensis* seeds using UPLC--PDA-QTOF-MS/MS. *Molecules*, *24*, art. no. 3794. https://doi.org/10.3390/molecules24203794
37. Yoshikawa, M., Murakami, T., Kadoya, M., Matsuda, H., Yamahara, J., Muraoka, O., Murakami, N. (1995). Betavulgarosides I, II, III, IV and V hypoglycemic glucuronide saponins from the roots and leaves of *Beta vulgaris* L. (sugar beet). *Chemical Pharmaceutical Bulletin*, *41*, 1621–1626. https://doi.org/10.3987/COM-95-7111
38. Yoshikawa, M., Murakami, T., Kadoya, M., Yamahara, J., Matsuda, H. (1996). Medical foodstuffs. III. Sugar beet. (1): hypoglycemic oleanolic acid oligoglycosides, betavulgarosides I, II, III, and IV, from the root of *Beta vulgaris* L. (Chenopodiaceae). *Chemical and Pharmaceutical Bulletin*, *44*, 1212–1217. https://doi.org/10.1248/cpb.44.1212
39. Yoshikawa, M., Murakami, T., Kadoya, M., Yamahara, J., Matsuda, H. (1998) Medical foodstuffs. XV. Sugar beet. (2): structures of betavulgarosides V, VI, VII, VIII, IX and X from the roots and leaves of sugar beet (*Beta vulgaris* L., Chenopodiaceae). *Chemical and Pharmaceutical Bulletin*, *46*, 1758–1763. https://doi.org/10.1248/cpb.46.1758