

# Cultivar- and Growth-Stage-Dependent Variability of Saponins in Roots and Leaves of *Beta vulgaris* L. Characterized by Liquid Chromatography Coupled with Mass Spectrometry

Anna Tekieli<sup>1†</sup>, Kamila Frącek<sup>1†</sup>, Giorgia Perelli<sup>2†</sup>, Szymon Janecki<sup>1</sup>, Aneta Spórna-Kucab<sup>1\*</sup>

<sup>1</sup>Department of Chemical Technology and Environmental Analysis, Faculty of Chemical Engineering and Technology, Cracow University of Technology, Warszawska 24, 31-155 Kraków, Poland

<sup>2</sup>Department of Agriculture and Forest Sciences (DAFNE), University of Tuscia, Via San Camillo de Lellis, 01100 Viterbo, Italy

*Beta vulgaris* L. is an important food crop and a rich source of bioactive triterpene saponins. This study evaluated cultivar- and growth-stage-dependent variability of saponins in leaves and roots of three beet cultivars (Round Dark Red, Cylindra, and Snow Ball) and in leaves of Swiss chard (Rhubarb Chard), harvested at seven harvest dates between June and September 2024. Saponins were identified and quantified using liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI-MS/MS). A total of 32 triterpene saponins representing oleanane-type, akebonoic acid-, hederagenin-, and gypso-genin-derived aglycones were detected. Pronounced organ-dependent differences were observed. Total saponin content ranged from 386 to 10,414 mg/kg fresh weight (FW) in leaves and from 1,170 to 23,298 mg/kg FW in roots. Two-way analysis of variance confirmed highly significant effects of cultivar, harvest time, and their interaction on total saponin levels in both leaves and roots (all  $p < 0.0001$ ). Roots exhibited a broader content range and a pronounced mid-season maximum, whereas leaf saponin levels generally peaked in the mid-to-late season in a genotype-dependent pattern. Major saponins (Act-UrA-akebonoic acid and betavulgarosides II, III, IV, and VII) predominated in the quantitative profile and exhibited coordinated seasonal variation. Multivariate analyses (principal component analysis and hierarchical cluster analysis) clearly separated samples according to plant organ and further resolved cultivar- and season-related patterns. Overall, saponin accumulation in *B. vulgaris* is strongly regulated by organ type, genotype, and growth stage, emphasizing the importance of cultivar selection and harvest timing for maximizing bioactive potential.

**Keywords:** oleanane-type aglycones, plant metabolomics, seasonal variation, secondary metabolites, triterpene glycosides

## INTRODUCTION

*Beta vulgaris* L. is a plant of considerable economic and nutritional importance, comprising four main cultivated forms: red beet, Swiss chard, fodder beet, and sugar beet [Mroczek, 2015]. It is a rich source of bioactive compounds, including polyphenols, betalains, and triterpene saponins, which are increasingly

recognized for their health-promoting and disease-preventive properties [Biswas & Dwivedi, 2019]. Among these compounds, triterpene saponins have attracted considerable attention owing to the broad range of their biological activities, including antiviral, anticancer, antimicrobial, anti-inflammatory, antihypertensive, and antidiabetic effects [Jolly *et al.*, 2024; Kaur *et al.*, 2024].

### \*Corresponding Author:

e-mail: [aneta.sporna-kucab@pk.edu.pl](mailto:aneta.sporna-kucab@pk.edu.pl) (Prof. A. Spórna-Kucab)

†These authors had equal contribution to this work

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Saponins are composed of a sugar moiety (glycone) and an aglycone, also referred to as sapogenin. Their classification is primarily based on the structure of the aglycone – which may have either a triterpenoid or steroidal skeleton – as well as on the number and type of sugar units present in the glycone [Vincken *et al.*, 2007]. The glycone consists of a linear or branched oligosaccharide chain, typically containing hexoses and pentoses such as glucose, galactose, arabinose, rhamnose, or xylose, and may also include uronic acids, most commonly glucuronic acid [Jolly *et al.*, 2024].

The saponin composition and content in plants exhibit substantial variability and depend on multiple factors, including species, plant organ, environmental conditions, and developmental stage. Environmental stressors, such as soil salinity, drought, nutrient deficiencies, and elevated temperatures, can markedly reduce saponin levels [Kaur *et al.*, 2024; Sharma *et al.*, 2022]. In addition, saponin accumulation is strongly species- and growth-stage-dependent: in tea plants, saponin levels increase during early maturation and later stabilize, whereas in yam and *Lycium barbarum* peak accumulation occurs during tuber development or fruiting stages [Kregiel *et al.*, 2017; Yu & He, 2018]. This highlights the need to consider the biological and environmental context when interpreting saponin variability.

Previous research on *B. vulgaris* saponins has primarily focused on their composition in mature plants and across different cultivars, whereas information on their variation across different growth stages is currently lacking [Mikołajczyk-Bator *et al.*, 2016a, 2024; Mroczek *et al.*, 2012, 2019, 2021]. The aim of the present study was to investigate organ-, cultivar-, and growth-stage-dependent variation in saponin content in *B. vulgaris* using liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS), with particular emphasis on seasonal changes in total and individual saponins.

## MATERIALS AND METHODS

### ■ Solvents and reference compounds

Respective standards from a previous study on *B. vulgaris* (Red Sphere cultivar) were used for saponin identification [Spórna-Kucab & Wybraniec, 2020], along with extracts of red, yellow, and white *B. vulgaris* cultivars – Ceryl, Chrobry, Forono, Tytus, and Boldor [Spórna-Kucab *et al.*, 2022]. Oleanolic acid standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Liquid chromatography–mass spectrometry (LC–MS)–grade acetonitrile and formic acid (purity  $\geq 98\%$ ) were obtained from Sigma-Aldrich. Acetone was purchased from Avantor Performance Materials Poland S.A. (Gliwice, Poland). All chemicals and solvents were of analytical grade and used as received. Water utilized throughout the experiments with a resistivity of 18.0 m $\Omega$ /cm at 295 K was deionized through a Milli-Q purification system (Merck Millipore, Burlington, MA, USA).

### ■ Plant materials

Roots and leaves of *Beta vulgaris* L. cultivars Round Dark Red, Cylindra, and Snow Ball, as well as leaves of Swiss chard (cv. Rhubarb

Chard), were collected from plants grown under standard agronomic conditions in a temperate climate zone on fertile, neutral soil in Zręcin, southeastern Poland, without the application of fertilizers. Seeds of Rhubarb Chard and the white cultivar Snow Ball were purchased from Torseed (Toruń, Poland), while seeds of the red cultivars Cylindra and Round Dark Red were obtained from Legutko (Jutrosin, Poland).

Plants were harvested at two-week intervals from June to September 2024. At each harvest date, plant material was collected from multiple individual plants *per* cultivar, constituting independent biological replicates. Immediately after harvesting, fresh roots and leaves were washed, weighed, and subjected directly to extraction.

### ■ Preparation of plant extracts

The roots and leaves of beets were individually blended in a household blender. A 100-g portion of each sample was extracted by maceration with 400 mL of 50% (v/v) acetone for 30 min at room temperature. The extraction was performed three times using a fresh portion of the solvent for each plant part. After each extraction, the resulting extracts were combined, partially concentrated at 25°C under reduced pressure, and then freeze-dried. Finally, the freeze-dried extracts were weighed and used for further studies on the quantitative and qualitative profile of saponins by the LC–ESI–MS/MS system. All extraction and analytical steps were carried out identically for all biological replicates.

### ■ LC–ESI–MS/MS – instrumentation and conditions

Analyses were conducted by means of high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry on an LCMS-8030 system (Shimadzu, Kyoto, Japan), equipped with a DGU-20A5R degasser, CBM-20A controller, Nexera LC-20ADXR binary pump, and SIL-20ACXR autosampler, operated *via* LabSolutions software (version 5.60 SP1, Shimadzu). Chromatographic separation was performed on a Kinetex C<sub>18</sub> column (150×4.6 mm, 5.0  $\mu$ m) with a matching guard column (Phenomenex, Torrance, CA, USA), maintained at 40°C. The flow rate was set to 0.5 mL/min. A binary mobile phase consisting of 2% (v/v) aqueous formic acid (solvent A) and acetonitrile (solvent B) was used. For saponin analysis, the gradient program was as follows: 38–60% B for 15 min, 60–99% B for 1 min, 99% B for 2 min, followed by re-equilibration to 38% B; the total run time was 19 min. For oleanolic acid, the gradient consisted of 20–99% B for 7 min, 99% B for 8 min, and re-equilibration to 20% B, giving a total run time of 19 min.

Tandem mass spectrometry (MS/MS) analysis was performed on a triple-quadrupole mass spectrometer with an electrospray ion source. The following ESI–MS/MS parameters were applied: curved desolvation line (CDL) and heat-block temperatures of 230°C, nebulizing gas flow of 1.5 L/min, electrospray voltage of 4.5 kV, and capillary temperature of 250°C, with nitrogen used as nebulizing and drying gas. Collision-induced dissociation (CID) was carried out with argon as the collision gas, using a collision

energy of 35 V. Data were acquired in a negative-ion mode using full-scan ( $m/z$  100–2,000) and selected-ion monitoring (SIM).

### ■ Analysis of saponins

Prior to instrumental analysis of saponins, 10 mg of each freeze-dried extract were dissolved in 200  $\mu$ L of demineralized water and centrifuged at 3,000 $\times$ g for 5 min using microcentrifuge type 320 (UNIPAN, Warsaw, Poland). Subsequently, 150  $\mu$ L of the supernatant were collected for further analysis. Aliquots of 15  $\mu$ L of each sample and 5  $\mu$ L of the standard solution were injected into the LC–ESI–MS/MS system.

Each extract, originating from an independent biological replicate, was analyzed in three independent technical replicates. Saponin identification was based on characteristic MS/MS fragmentation patterns, accurate mass data, and comparison with data previously reported for *B. vulgaris* triterpene saponins [Tekieli *et al.*, 2026]. Diagnostic aglycone fragment ions and neutral losses of sugar moieties were used for structural assignment. Individual saponins as well as the total saponin content were quantified using an external calibration curve prepared with oleanolic acid in the concentration range of 1.25–20.0  $\mu$ g/mL. Oleanolic acid was selected as the calibration standard because it represents the aglycone of the major oleanane-type saponins reported in *B. vulgaris*, including betavulgarosides, and due to the limited availability of individual saponin reference standards.

The calibration curve, constructed based on MS peak area vs. concentration, exhibited excellent linearity ( $R^2=0.9996$ ). The content of saponins was expressed as mg of saponin *per* kg of fresh weight of root or leaf material (mg/kg FW). All samples were processed under identical analytical conditions to ensure comparability.

### ■ Statistical analysis

All quantitative results are presented as mean and standard deviation (SD) calculated from two independent biological replicates (material collected from different individual plants for each cultivar, organ, and harvest date). Each biological replicate was independently extracted and analyzed in three technical LC–ESI–MS/MS replicates; technical replicates were averaged prior to statistical analysis.

The effects of cultivar, harvest time, and their interaction on the total saponin content and on the contents of individual saponins were evaluated using two-way analysis of variance (ANOVA), performed separately for leaves and roots. When significant main effects or interactions were detected, post hoc multiple comparisons were conducted using Tukey's honestly significant difference (HSD) test. A significance level of  $p \leq 0.05$  was applied. Normality of residuals was verified using the Shapiro-Wilk test, and homogeneity of variances was assessed prior to ANOVA.

Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were applied as exploratory multivariate tools to visualize relationships among samples based on the full set of 32 quantified saponins. Statistical analyses were conducted

using RStudio (version 2023.13.0+386, Posit Software, PBC, Boston, MA, USA).

## RESULTS AND DISCUSSION

### ■ Identification of triterpene saponins in *B. vulgaris*

In the present study, LC–ESI–MS/MS analysis of extracts from four *B. vulgaris* cultivars (Round Dark Red, Cylindra, Rhubarb Chard, and Snow Ball) revealed a total of 32 triterpene saponins, reflecting substantial diversity in aglycone structures and sugar moieties, including uronic acid (UrA), pentoses (Pen), hexoses (Hex), and substituted acetal (Act) and dioxolane (Diox) residues. The detected saponins comprised oleanane-type, akebonoic acid-based, hederagenin- and gypsogenin-derived aglycones. Because closely related oleanane-type aglycones may generate highly similar MS/MS fragmentation patterns, aglycone assignments based solely on LC–ESI–MS/MS data were discussed at the oleanane-type level unless supported by authentic standards or previously reported nuclear magnetic resonance (NMR) data, particularly in the case of features consistent with GOTCAB-type (glycosides of oleanane-type triterpenoid carboxylic acid bisdesmosides) structures [Arslan, 2017, 2020; Arslan & Cenzano, 2021]. Most compounds were identified using precursor ion masses, MS/MS spectra, and reference standards, while the remaining features were tentatively annotated based on literature fragmentation data. Detailed analytical parameters are summarized in **Table 1**. Representative chemical structures of the dominant and structurally confirmed saponins identified in the investigated cultivars are shown in **Figure 1**.

### ■ Oleanane-type saponins

Oleanane-type saponins predominated in the saponin profile and represented the most structurally diverse group, comprising 20 detected compounds (**Table 1**). Oleanane-type cores were detected predominantly in glycosylated form, as indicated by a characteristic neutral loss of 176 Da corresponding to a uronic acid residue [Mroczek *et al.*, 2012, 2019; Spórna-Kucab & Wybraniec, 2020; Wen *et al.*, 2023]. The simplest oleanane-type saponins (**31** and **32**;  $m/z$  631) showed diagnostic fragments at  $m/z$  455 and a neutral loss of 176 Da, which was consistent with a uronic acid–substituted oleanane core, and were confirmed by comparison with reference compounds previously isolated from *B. vulgaris*.

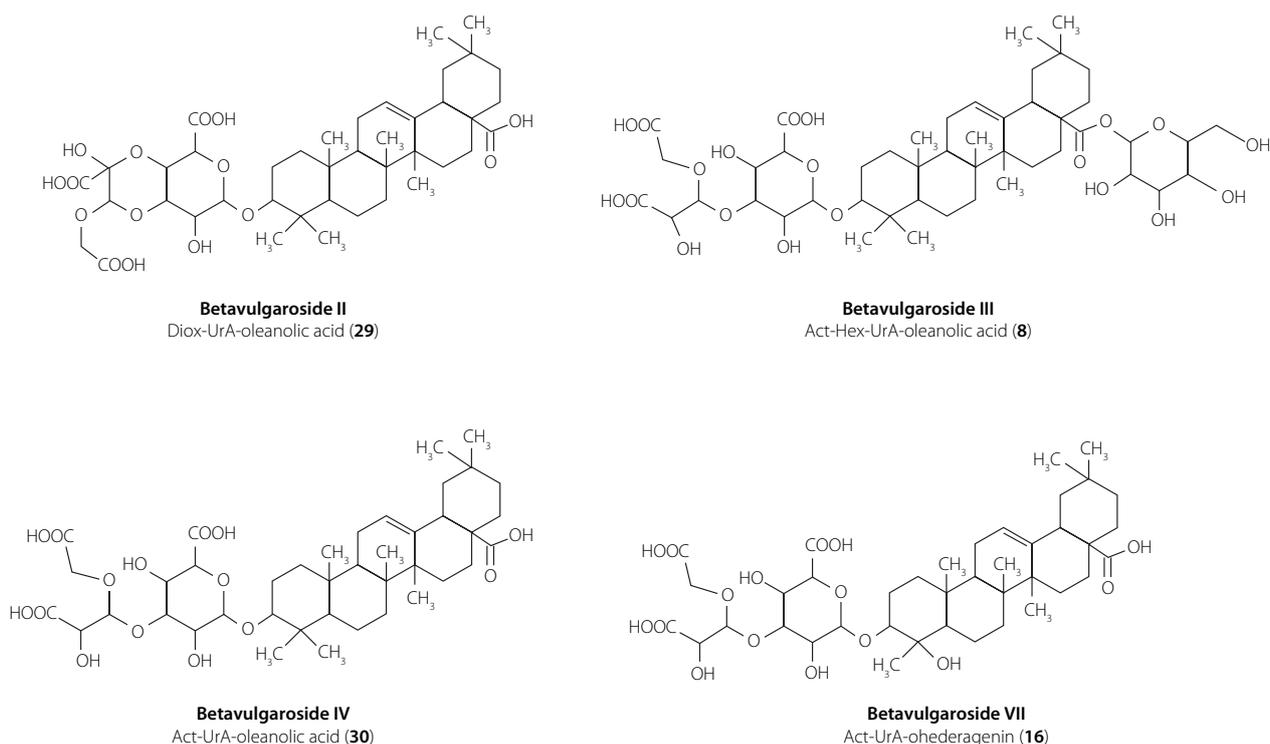
The structural diversity of oleanane-type saponins is largely generated by modifications of the core unit composed of an oleanane-type aglycone and uronic acid ( $m/z$  631), which varies through the attachment of additional sugar residues. Accordingly, several structural variants were identified, including mono- and diglycosylated derivatives bearing pentose, hexose, dioxolane, or acetal substituents (saponins **5** and **26–30**). Selected structures were confirmed using reference standards (saponins **26–30**) or literature data (saponin **5**).

Oleanane-type saponins bearing two sugar groups were observed in several configurations, including Hex–Pen (saponins **12**

**Table 1.** Chromatographic and mass-spectrometric data of identified saponins in the *Beta vulgaris* L. extracts.

No.	Saponin	Trivial name	Molecular formula	t <sub>R</sub> (min)	m/z [M-H] <sup>-</sup>	m/z from MS <sup>2</sup> of [M-H] <sup>-</sup>
1	Hex-Hex-Hex-UrA-oleane-type		C <sub>53</sub> H <sub>82</sub> O <sub>25</sub>	5.59	1,117	955; 793; 631; 455
2	Diox-Hex-UrA-hederagenin		C <sub>47</sub> H <sub>70</sub> O <sub>21</sub>	6.20	969	925; 867; 849; 809; 647; 471
3	Act-Hex-Hex-UrA-oleane-type	Betavulgaroside V	C <sub>53</sub> H <sub>82</sub> O <sub>25</sub>	6.30	1,117	997; 955; 835; 793; 455
4	Act-Hex-UrA-akebonoic acid	Betavulgaroside VIII	C <sub>46</sub> H <sub>68</sub> O <sub>20</sub>	6.36	939	819; 777; 657; 615; 553; 439
5	Hex-UrA-oleane-type		C <sub>42</sub> H <sub>66</sub> O <sub>14</sub>	6.80	793	673; 631; 569; 455
6	Act-Hex-Pen-UrA-oleane-type	Betavulgaroside IX	C <sub>52</sub> H <sub>80</sub> O <sub>24</sub>	6.80	1087	967; 925; 805; 763; 455
7	Act-Hex-Hex-UrA-oleane-type	Betavulgaroside V	C <sub>53</sub> H <sub>82</sub> O <sub>25</sub>	7.00	1,117	997; 955; 835; 793; 455
8	Act-Hex-UrA-oleane-type	Betavulgaroside III	C <sub>47</sub> H <sub>72</sub> O <sub>20</sub>	7.80	955	835; 793; 673; 631; 569; 455
9	Hex-Pen-UrA-oleane-type		C <sub>47</sub> H <sub>74</sub> O <sub>18</sub>	7.84	925	805; 763; 701; 569; 455
10	Hex-UrA-hederagenin		C <sub>42</sub> H <sub>66</sub> O <sub>15</sub>	8.00	809	749; 689; 647; 629; 585; 471
11	Diox-Hex-UrA-oleane-type	Betavulgaroside I	C <sub>47</sub> H <sub>70</sub> O <sub>20</sub>	8.00	953	909; 851; 793; 631; 455
12	Hex-Pen-UrA-oleane-type		C <sub>47</sub> H <sub>74</sub> O <sub>18</sub>	8.30	925	805; 763; 701; 569; 455
13	Act-Hex-UrA-akebonoic acid	Betavulgaroside VIII	C <sub>46</sub> H <sub>68</sub> O <sub>20</sub>	9.24	939	819; 777; 657; 615; 553; 439
14	Hex-Pen-UrA-akebonoic acid	Betavulgaroside X	C <sub>46</sub> H <sub>70</sub> O <sub>18</sub>	10.00	909	789; 747; 685; 597; 553; 439
15	Pen-UrA-hederagenin		C <sub>41</sub> H <sub>64</sub> O <sub>14</sub>	10.20	779	647; 629; 585; 567; 471
16	Act-UrA-hederagenin	Betavulgaroside VII	C <sub>41</sub> H <sub>62</sub> O <sub>16</sub>	10.40	809	689; 647; 471
17	Act-Hex-UrA-oleane-type	Betavulgaroside III	C <sub>47</sub> H <sub>72</sub> O <sub>20</sub>	10.60	955	835; 793; 673; 631; 569; 455
18	UrA-hederagenin		C <sub>36</sub> H <sub>56</sub> O <sub>10</sub>	10.70	647	615; 535; 471
19	Act-Hex-UrA-oleane-type	Betavulgaroside III	C <sub>47</sub> H <sub>72</sub> O <sub>20</sub>	11.40	955	835; 793; 673; 631; 569; 455
20	Pen-UrA-akebonoic acid		C <sub>40</sub> H <sub>60</sub> O <sub>13</sub>	11.74	747	615; 535; 439
21	Act-Pen-UrA-oleane-type		C <sub>46</sub> H <sub>70</sub> O <sub>19</sub>	11.84	925	763; 569; 631; 455
22	Act-UrA-akebonoic acid		C <sub>40</sub> H <sub>58</sub> O <sub>15</sub>	11.91	777	657; 615; 439
23	Act-UrA-gypsogenin		C <sub>41</sub> H <sub>60</sub> O <sub>16</sub>	11.95	807	687; 645; 469
24	Act-Pen-UrA-oleane-type		C <sub>46</sub> H <sub>70</sub> O <sub>19</sub>	12.27	925	763; 569; 631; 455
25	UrA-akebonoic acid		C <sub>35</sub> H <sub>52</sub> O <sub>9</sub>	12.70	615	539; 493; 439
26	Pen-UrA-oleane-type		C <sub>41</sub> H <sub>64</sub> O <sub>13</sub>	13.80	763	631; 587; 455
27	Act-UrA-oleane-type	Betavulgaroside IV	C <sub>41</sub> H <sub>62</sub> O <sub>15</sub>	13.81	793	673; 631; 455
28	Pen-UrA-oleane-type		C <sub>41</sub> H <sub>64</sub> O <sub>13</sub>	14.24	763	631; 587; 455
29	Diox-UrA-oleane-type	Betavulgaroside II	C <sub>41</sub> H <sub>60</sub> O <sub>15</sub>	14.50	791	747; 631; 455
30	Act-UrA-oleane-type	Betavulgaroside IV	C <sub>41</sub> H <sub>62</sub> O <sub>15</sub>	14.55	793	673; 631; 455
31	UrA-oleane-type		C <sub>36</sub> H <sub>56</sub> O <sub>9</sub>	14.60	631	587; 455
32	UrA-oleane-type		C <sub>36</sub> H <sub>56</sub> O <sub>9</sub>	15.50	631	587; 455

Aglycone annotations are based on LC-ESI-MS/MS data. Due to the well-recognized similarity of fragmentation patterns among closely related oleanane-type saponins – including structures consistent with GOTCAB saponins (oleanane-type triterpenoid carboxylic acid 3,28-bisdesmosides) – compounds yielding characteristic oleanane-type fragments are reported at the oleanane-type level unless supported by authentic standards or literature NMR data. For defined betavulgarosides (I–X), names and aglycone identities are discussed in the text based on previously reported NMR evidence. t<sub>R</sub>, Retention time; m/z, mass-to-charge ratio.



**Figure 1.** Chemical structures of the dominant saponins in *Beta vulgaris* L. cultivars: Round Dark Red, Cylindra, Snow Ball, and Rhubarb Chard.

and **19**), Diox–Hex (saponin **11**), Act–Pen (saponins **21** and **24**), and Act–Hex (saponins **8**, **17**, and **19**). Saponins **8**, **11**, **17**, and **19** were confirmed using authentic standards or previously reported NMR data [Yoshikawa *et al.*, 1996], whereas saponins **12** and **19** were confirmed by comparison with reference compounds, and saponins **21** and **24** were assigned based on literature data [Mroczek *et al.*, 2021]. These saponins have been previously reported in *B. vulgaris* tissues [Mroczek *et al.*, 2012, 2019, 2021; Mikołajczyk-Bator *et al.*, 2016a, 2024].

Triglycosylated oleanane-type saponins were also detected, including Act–Hex–Hex (saponins **3** and **7**), Hex–Hex–Hex (saponin **1**), and Act–Hex–Pen (saponin **6**). An acetal substituent derived from a hexose unit was confirmed for saponins **3**, **6**, and **7** using reference standards, whereas saponin **1** was tentatively identified based on literature MS data [Mroczek *et al.*, 2012, 2019]. Saponins **3** and **7** (betavulgaroside V) are widely distributed in *B. vulgaris* cultivars and have also been reported in *Achyranthes fauriei* [Edelmann *et al.*, 2020a,b; Ida *et al.*, 1995; Mroczek *et al.*, 2012, 2019, 2021; Mikołajczyk-Bator *et al.*, 2016, 2024; Yoshikawa *et al.*, 1998].

#### ■ Saponins with akebonoic acid aglycone

In addition to oleanane-type saponins, *B. vulgaris* contained triterpene saponins with akebonoic acid as the aglycone, identified by a diagnostic fragment ion at  $m/z$  439 under negative ESI conditions (Table 1). The simplest derivative, saponin **25** ( $m/z$  615), was assigned as an akebonoic acid–uronic acid conjugate based on a characteristic neutral loss of 176 Da from the C-3 position of the aglycone and represents a compound not previously reported in *B. vulgaris*.

Single-glycosylated akebonoic acid derivatives were detected as saponin **20** ( $m/z$  747; pentose substituent), tentatively assigned based on its MS/MS fragmentation pattern, and saponin **22** ( $m/z$  777; acetal substituent), which was unambiguously identified by comparison with reference standards. More complex derivatives included saponin **14** ( $m/z$  909; betavulgaroside X), identified based on sequential sugar losses and previously reported NMR data [Yoshikawa *et al.*, 1998], as well as saponins **4** and **13** ( $m/z$  939; betavulgaroside VIII), both confirmed using reference standards and previously reported in *B. vulgaris* and related beet cultivars [Edelmann *et al.*, 2020a,b; Mikołajczyk-Bator *et al.*, 2016; Mroczek *et al.*, 2021; Yoshikawa *et al.*, 1998].

#### ■ Hederagenin and gypsogenin aglycones of saponins

*B. vulgaris* contained a minor group of triterpene saponins with hederagenin and gypsogenin aglycones (Table 1), detected at much lower levels than oleanane- and akebonoic acid-based derivatives and characterized by diagnostic ions at  $m/z$  471 and 469, respectively. Saponin **18** ( $m/z$  647) was identified as a hederagenin monoglycoside bearing a uronic acid residue based on a neutral loss of 176 Da and has not been previously reported in *B. vulgaris*. Compounds **10**, **15**, and **16** ( $m/z$  779 and 809) were identified as hederagenin-based saponins containing a uronic acid residue and additional hexose and/or pentose units or an acetal substituent, as confirmed by a diagnostic fragment at  $m/z$  471. Saponin **16** (betavulgaroside VII) is the most thoroughly characterized saponin in this group, with its structure elucidated by NMR [Yoshikawa *et al.*, 1998] and reported across numerous *B. vulgaris* cultivars [Mikołajczyk-Bator *et al.*, 2016a,b,

2024]. Saponins **15** and **16** were confirmed using reference standards, whereas saponin **10** was assigned based on literature data [Mroczek *et al.*, 2012; Mikołajczyk-Bator *et al.*, 2016a,b, 2024].

Saponin **2** ( $m/z$  969) was identified as a hederagenin-based saponin bearing a uronic acid, a hexose, and a dioxolane substituent and has previously been reported only in sugar beet cultivars [Mikołajczyk-Bator *et al.*, 2016b]. Saponin **23** ( $m/z$  807) was the sole gypsogenin-based saponin detected, as confirmed by a diagnostic fragment at  $m/z$  469, and was identified by comparison with reference standards; this compound has been previously reported in sugar beet and Swiss chard cultivars [Mikołajczyk-Bator *et al.*, 2016b; Mroczek *et al.*, 2021].

### ■ Analytical precision and variability assessment

Instrumental precision and extraction repeatability were evaluated using three independent samples prepared from the same homogeneous plant material and subjected to the complete extraction procedure. Each resulting extract was analyzed three times by LC–ESI-MS/MS to assess instrumental variability.

The relative standard deviation (RSD) obtained from repeated LC–ESI-MS/MS analyses of the same extract ( $n=3$ ) ranged from 2.7% to 4.3%, indicating satisfactory instrumental precision. Extraction repeatability was assessed by comparing the results of three independent extractions performed on identical plant material. The RSD values calculated between these extractions ranged from 2.3% to 9%, which was considered acceptable given the complexity of plant matrices. Biological variability was assessed based on the two independent biological replicates collected for each cultivar, organ, and harvest date and expressed as biological RSD (%). The biological RSD values exhibited a relatively broad range. For individual saponins, biological RSD values generally ranged from approximately 5% to 16%, with the majority of compounds showing variability between 7% and 14%, depending on cultivar, organ, and harvest time.

The relative standard deviation (RSD) values for total saponins (calculated as the sum of individual saponins for each biological replicate) most commonly fell within the range of approximately 8–11%, although slightly higher values were occasionally recorded at specific harvest dates or for particular cultivars (Tables S1–S8 in Supplementary Materials).

The reduced variability of the total saponin content reflects the cumulative nature of this parameter, whereby variability in individual saponins partially compensates when summed at the level of biological replicates. In cases where a given saponin was not detected in either biological replicate (mean value equal to zero), calculation of RSD was not applicable and was therefore omitted.

### ■ Saponin quantitative analysis

The total saponin content in leaves and roots of *B. vulgaris* cultivars harvested at different times is shown in Figure 2, and the relative proportions of the 32 identified compounds in Figure 3. Additionally, the contents of individual saponins in the fresh weight of the plant materials are presented in Tables S1–S8. Quantitative

LC–ESI-MS/MS analysis demonstrated that saponin accumulation in *Beta vulgaris* L. was influenced by plant organ, harvest date and cultivar. Two-way ANOVA showed highly significant effects of cultivar, harvest time, and their interaction on total saponin content in leaves ( $f=137.73$  for cultivar,  $f=68.26$  for time,  $f=25.50$  for cultivar  $\times$  time; all  $p<0.0001$ ) and in roots ( $f=77.45$ ,  $69.36$ ,  $31.56$ , respectively; all  $p<0.0001$ ). Similarly strong effects were observed for most individual saponins (typically  $p<0.0001$ ). Detailed two-way ANOVA outputs for individual saponins and total saponins in leaves and roots are provided in Table S9 in Supplementary Materials.

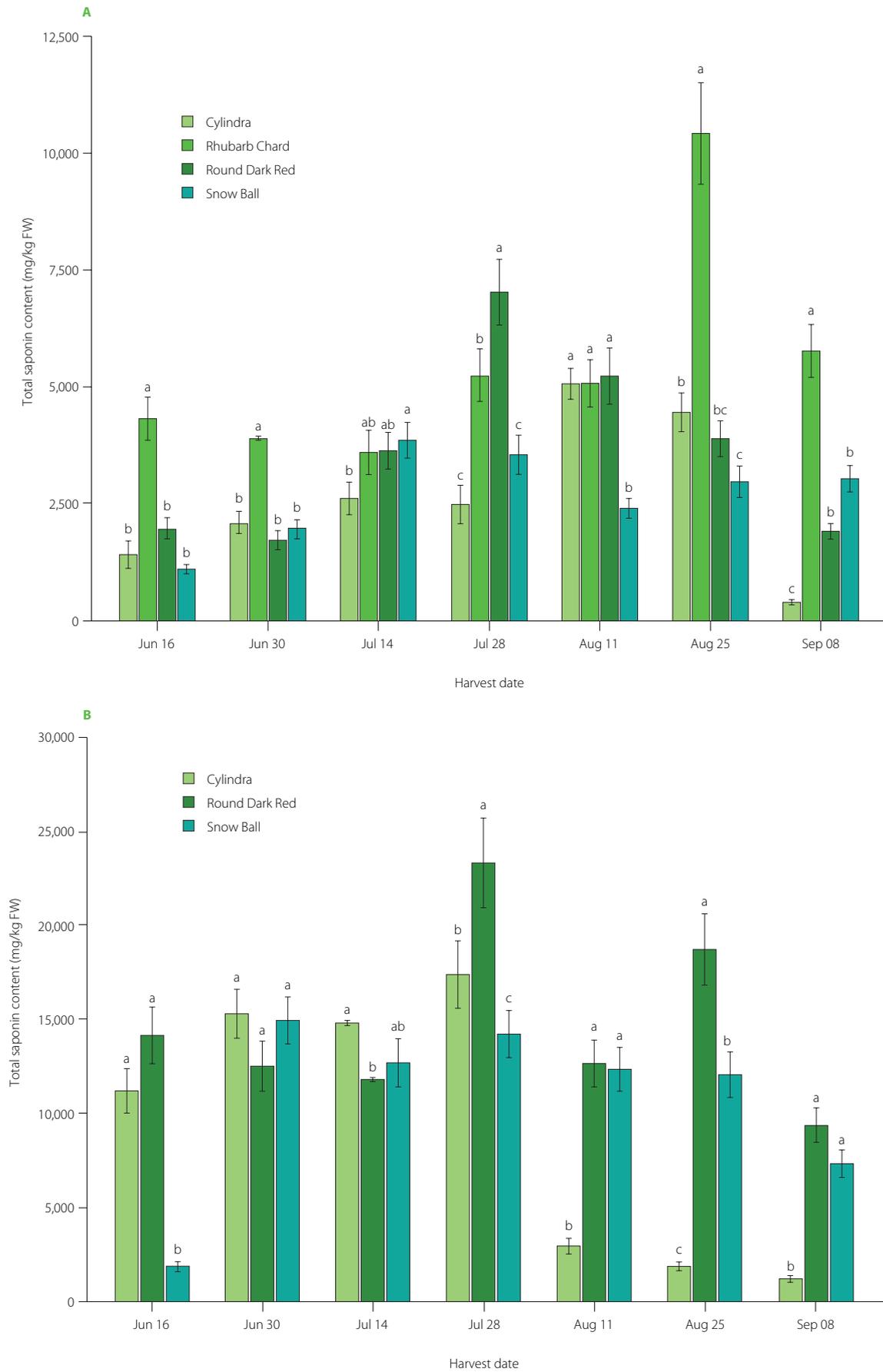
### ■ Organ-dependent quantitative trends in total saponin content

Across cultivars and harvest dates, roots accumulated higher total saponin levels than leaves, with a much wider range of values (Figure 2 and Figure 3). Total saponin content ranged from 386 to 10,414 mg/kg FW in leaves and from 1,170 to 23,298 mg/kg FW in roots. These values are consistent with those reported previously [Spórna-Kucab *et al.*, 2022], where up to 20,810 mg/kg FW were found in beetroot peels of various cultivars. Although Mroczek *et al.* [2019] reported higher total saponin levels in leaves for some cultivars, their study also demonstrated that, depending on genotype, roots may accumulate equal or even higher concentrations. Beyond the overall differences between organs, distinct temporal patterns were also evident. Seasonal dynamics were organ-specific: total saponin content in leaves peaked in mid-to-late season (depending on cultivar), whereas roots exhibited a pronounced mid-season maximum followed by a decline (Figure 2 and Figure 3). This pattern likely reflects developmental regulation of secondary metabolism and tissue-specific biosynthesis, with preferential accumulation in protective outer tissues. Indeed, Spórna-Kucab *et al.* [2022] demonstrated significantly higher saponin concentrations in beet peel compared to the flesh, supporting their enhanced deposition in tissues directly exposed to environmental stressors.

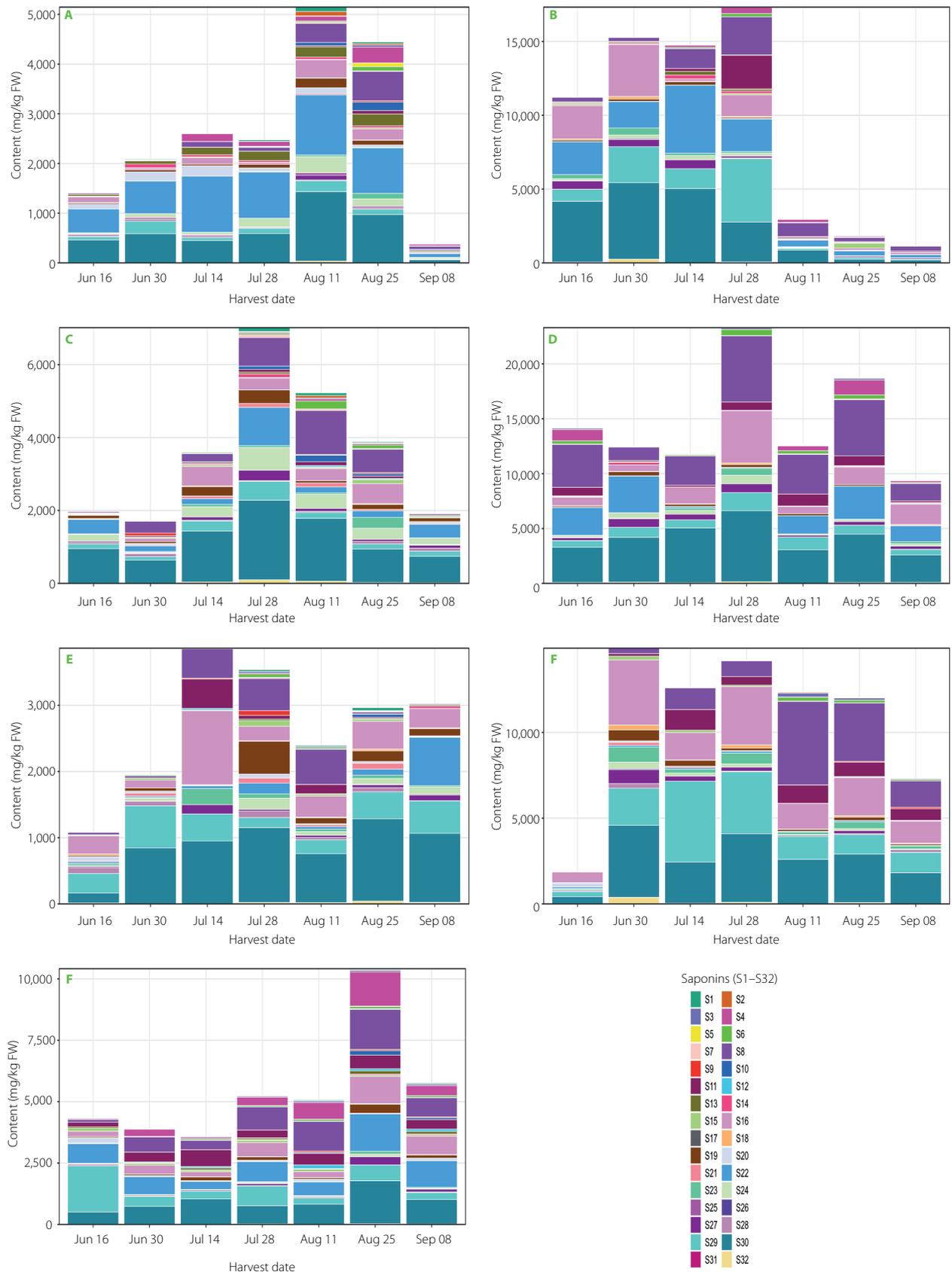
### ■ Cultivar-specific patterns of total saponin accumulation

In leaves, genotype-related differences were pronounced and strongly dependent on harvest date. The cultivar Rhubarb Chard showed the highest total saponin content, reaching 10,414 mg/kg FW on 25 August. The highest value for the cultivar Round Dark Red was recorded on 28 July (7,010 mg/kg FW), for the cultivar Cylindra on 11 August (5,060 mg/kg FW), and for the cultivar Snow Ball on 14 July (3,861 mg/kg FW) (Figure 2 and Figure 3). These results are consistent with Mroczek *et al.* [2019], who demonstrated significant variability in saponin content among *B. vulgaris* genotypes.

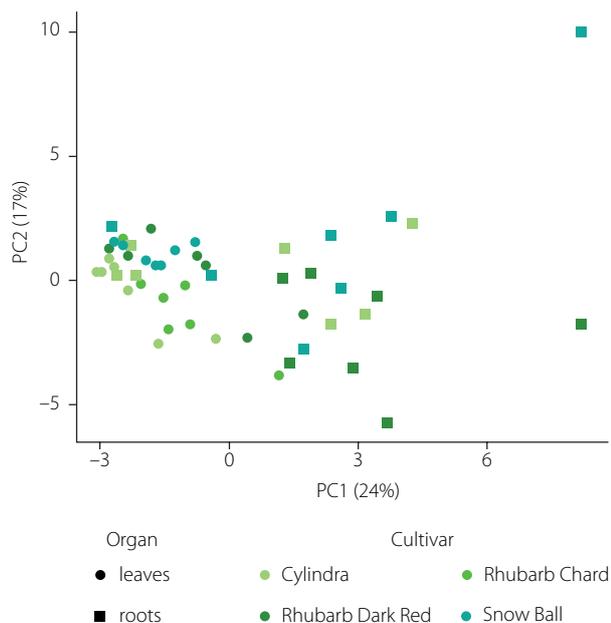
Root tissues also displayed clear genotype-dependent accumulation patterns. The greatest accumulation capacity was observed for the cultivar Round Dark Red, which reached 23,290 mg/kg FW on 28 July. The cultivar Cylindra exhibited a pronounced mid-season maximum (17,330 mg/kg FW on 28 July), followed by a sharp decline to 1,170 mg/kg FW on 8 September.



**Figure 2.** Total saponin content in leaves (A) and roots (B) of four *Beta vulgaris* L. cultivars across seven harvest dates. Data are expressed as mean and standard deviation. Different lowercase letters indicate significant differences among cultivars within each harvest date (Tukey's HSD test,  $p < 0.05$ ). FW, fresh weight.



**Figure 3.** Stacked bar plots showing the total content and relative proportions of 32 saponins in leaves and roots of four *Beta vulgaris* L. cultivars collected across seven harvest dates. Panels: (A) *Cylindra* leaves, (B) *Cylindra* roots, (C) *Round Dark Red* leaves, (D) *Round Dark Red* roots, (E) *Snow Ball* leaves, (F) *Snow Ball* roots, and (G) *Rhubarb Chard* leaves. Bar height represents total saponin content, and colors indicate individual saponins S1–S32, which are listed in [Table 1](#). FW, fresh weight.



**Figure 4.** Principal component analysis (PCA) score plot of saponin profiles in leaves (circles) and roots (squares) of four *Beta vulgaris* L. cultivars collected across seven harvest dates. PC1 explains 24% and PC2 explains 17% of the total variance. Colors indicate cultivars.

The cultivar Snow Ball showed elevated early-to-midseason total saponin content (14,910 mg/kg FW on 30 June), gradually decreasing to 7,310 mg/kg FW by 8 September (Figure 2 and Figure 3). These trends are consistent with previous reports; Spórna-Kucab *et al.* [2022] documented approximately 20,810 mg/kg in the peel of yellow beet cultivar Boldor, compared with only 500 mg/kg in the flesh of white Snow Ball, indicating strong tissue-specific distribution. Likewise, Mikołajczyk-Bator *et al.* [2024] reported significantly higher saponin content in root skins than in the flesh. Such findings support the mid-season maxima observed here and likely reflect preferential accumulation in outer protective tissues, followed by dilution or metabolic redistribution at later developmental stages.

#### ■ Quantitative variability of individual saponins

Individual saponins showed pronounced heterogeneity in abundance as well as in temporal and cultivar-dependent patterns (Figure 3, Tables S1–S8). Many low-abundance compounds were detected only intermittently, whereas a limited number of high-abundance saponins dominated the overall profile, including in particular, betavulgarosides II (29), III (8), IV (30), VII (16) and Act-UrA-akebonoic acid saponin (22). These major compounds exhibited coordinated seasonal variation in both temporal occurrence and abundance, including cultivar-dependent maxima. Two-way ANOVA showed highly significant effects of cultivar, harvest date, and their interaction on these and most other saponins ( $p < 0.0001$ ), supporting the idea of a differentiated biosynthetic network (Table S9). This aligns with Mroczek *et al.* [2019], who described various accumulation rates of specific saponins and spatially differentiated biosynthesis across organs.

#### ■ Principal component and hierarchical clustering analyses

PCA performed on the dataset comprising 32 saponins clearly separated the samples according to plant organ (Figure 4). The first principal component (PC1), explaining 24% of the total variance, distinctly discriminated root samples (positive PC1 values) from leaf samples (negative PC1 values), while the second principal component (PC2), accounting for 17% of the variance, further differentiated samples according to harvest date.

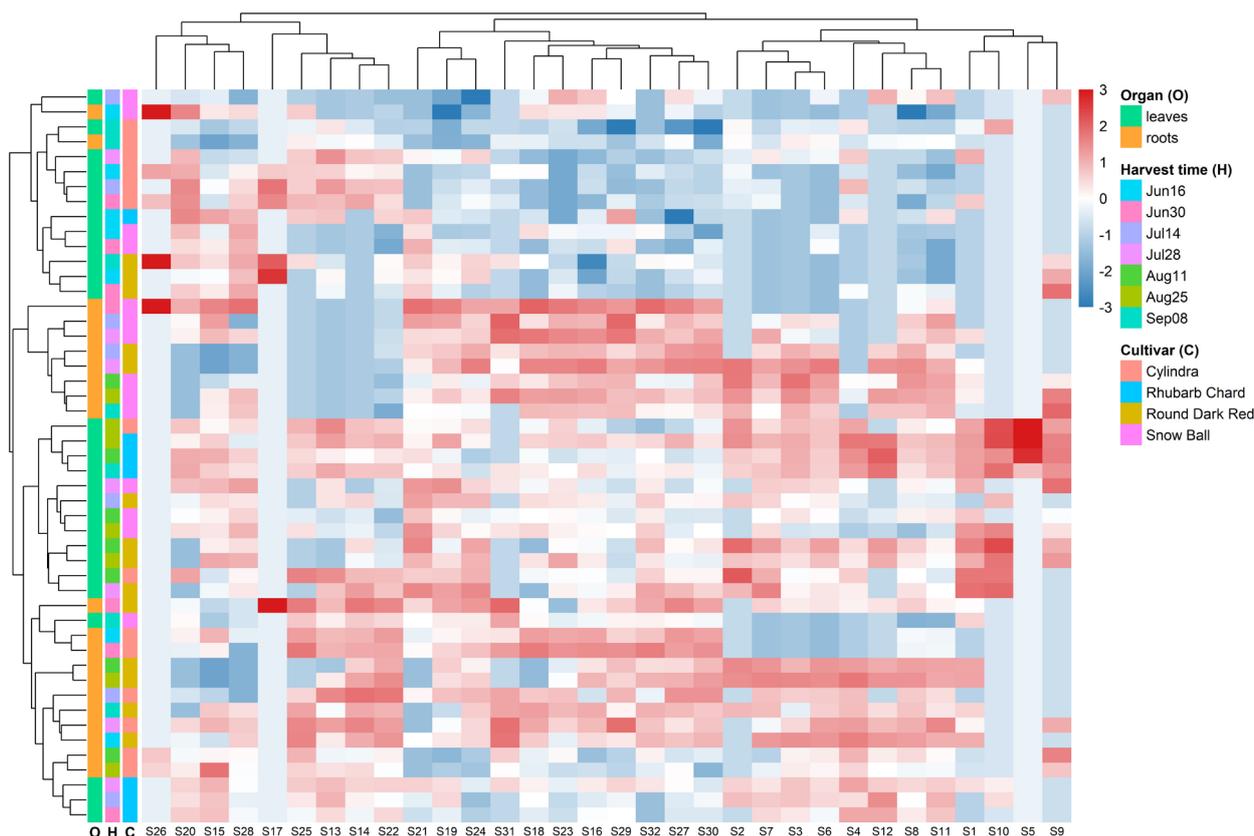
The separation along PC1 and PC2 was primarily driven by four high-loading saponins, corresponding to betavulgaroside III (8), betavulgaroside VII (16), Act-UrA-akebonoic acid saponin (22), and betavulgarosides II (29) and IV (30), previously described in beet [Mroczek *et al.*, 2019]. Their consistent contribution across samples indicates that these compounds constitute the principal sources of variability within the dataset. Hierarchical clustering analysis (heatmap visualization) fully supported the PCA results. Samples were grouped primarily according to plant organ (root vs. leaf), followed by harvest date and cultivar, revealing a clear underlying structure in saponin distribution patterns (Figure 5).

#### ■ Biological significance of triterpene saponins identified in *B. vulgaris*

The triterpene saponins identified in *B. vulgaris* constitute a structurally diverse and biologically relevant group of secondary metabolites with well-established functional and pharmacological importance. The predominance of oleanane-type saponins observed in this study suggests that this compound class may play a central role in determining the biological and functional properties of beet-derived tissues and products. In particular, the presence of NMR-confirmed betavulgarosides [Yoshikawa *et al.*, 1996, 1998], previously associated with anti-inflammatory, hepatoprotective, antimicrobial, antioxidant, and anticancer activities, underscores their potential contribution to the health-promoting effects of *B. vulgaris* [Castellano *et al.*, 2022; Günther & Bednarczyk-Cwynar, 2025; Gupta, 2022; Lisiak *et al.*, 2021; Liu *et al.*, 2019; Sen, 2020; Yoon & Choi, 2010].

The biological activity of oleanolic acid-based saponins is strongly influenced by their glycosylation patterns. The presence of uronic acid residues together with additional hexose or pentose units modulates key physicochemical properties, including solubility, membrane interactions, and bioavailability [Francis *et al.*, 2002]. The predominance of glycosylated oleanane-type cores detected in the present study is consistent with earlier reports indicating that biologically-active forms of oleanolic acid occur predominantly as saponins rather than as free aglycones in plant tissues [De Tommasi *et al.*, 1991; Kinjo *et al.*, 1999].

Beyond oleanane-type compounds, the identification of akebonoic acid-based saponins further expands the biological relevance of the saponin profile of *B. vulgaris*. Akebonoic acid, a 30-noroleanane triterpenoid, has been reported to exhibit pronounced anticancer, antibacterial, and antidiabetic activities [Dirir *et al.*, 2021; Wang *et al.*, 2014]. Although the biological functions of its glycosylated derivatives remain insufficiently



**Figure 5.** Hierarchical clustering and heatmap of 32 saponins detected in leaves and roots of different *Beta vulgaris* L. cultivars across seven harvest dates. The heatmap presents normalized relative liquid chromatography–mass spectrometry signal intensities (z-scores) of individual saponins across all samples (red = higher relative abundance, blue = lower relative abundance). Rows correspond to samples and columns to individual saponins. Colored side bars indicate harvest date (time), plant organ (leaves or roots), and cultivar. Dendrograms represent hierarchical clustering of samples (left) and saponins (top), illustrating similarities in saponin profiles and co-variation patterns among compounds.

characterized, their detection in beet tissues suggests that *B. vulgaris* may represent a valuable source of structurally-unique saponins with largely unexplored bioactivity. The occurrence of simple monoglycosidic derivatives may additionally reflect early biosynthetic intermediates or compounds with enhanced membrane-disrupting potential [Böttger & Melzig, 2013].

Minor saponins containing hederagenin and gypsogenin aglycones further contribute to the functional complexity of the saponin mixture in *B. vulgaris*. Both aglycones are known for their cytotoxic, antimicrobial, antifungal, and antiviral properties [Cheng *et al.*, 2018; Ciftci *et al.*, 2018; Engholm *et al.*, 2017; Favel *et al.*, 1994; Krasteva *et al.*, 2014; Su *et al.*, 2024; Sun *et al.*, 2023; Xie *et al.*, 2023; Zeng *et al.*, 2018]. Despite their lower abundance compared to oleanane- and akebonoic acid–based saponins, these compounds may still be biologically-relevant due to potential additive or synergistic interactions within complex saponin assemblies.

Taken together, the diversity of aglycone scaffolds and glycosylation patterns observed in *B. vulgaris* highlights this species as a rich source of triterpene saponins with multifaceted biological potential. The tissue-specific and season-dependent variability

in saponin composition demonstrated in this study may have important implications for the nutritional value, functional characteristics, and potential health benefits of beet-derived products. Nevertheless, further investigations employing purified compounds and targeted bioassays are required to establish direct links between saponin composition, concentration, and biological activity.

## CONCLUSIONS

This study demonstrates that saponin accumulation in *Beta vulgaris* L. is strongly dependent on plant organ, cultivar, and harvest time, with significant interaction effects between these factors. Roots accumulated higher total saponin levels than leaves and exhibited a pronounced mid-season maximum followed by a decline, whereas leaf saponin contents generally peaked in mid-to-late season depending on genotype.

Quantitative variability was largely driven by a limited group of dominant saponins, which showed coordinated seasonal and cultivar-dependent dynamics. Multivariate analyses confirmed that plant organ represents the primary source of variance in the dataset, with additional structuring according to harvest date and genotype.

Overall, the results indicate that saponin biosynthesis in *B. vulgaris* is developmentally-regulated and spatially-differentiated between organs. From a practical perspective, both cultivar selection and optimized harvest timing are critical parameters for maximizing total saponin yield and shaping the qualitative composition of beet-derived plant material.

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## CONFLICT OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## SUPPLEMENTARY MATERIALS

The following are available online at <https://journal.pan.olsztyn.pl/Cultivar-and-Growth-Stage-Dependent-Variability-of-Saponins-in-Roots-and-Leaves-of,218708,0,2.html>. **Table S1.**

Content of saponins 1–8 in leaves of *Beta vulgaris* L. cultivars at different harvest times (mg/kg FW). **Table S2.** Content of saponins 9–16 in leaves of *Beta vulgaris* L. cultivars at different harvest times (mg/kg FW). **Table S3.** Content of saponins 17–24 in leaves of *Beta vulgaris* L. cultivars at different harvest times (mg/kg FW).

**Table S4.** Content of saponins 25–32 in leaves of *Beta vulgaris* L. cultivars at different harvest times (mg/kg FW). **Table S5.** Content of saponins 1–8 in roots of *Beta vulgaris* L. cultivars at different harvest times (mg/kg FW). **Table S6.** Content of saponins 9–16 in roots of *Beta vulgaris* L. cultivars at different harvest times (mg/kg FW). **Table S7.** Content of saponins 17–24 in roots of *Beta vulgaris* L. cultivars at different harvest times (mg/kg FW).

**Table S8.** Content of saponins 25–32 in roots of *Beta vulgaris* L. cultivars at different harvest times (mg/kg FW). **Table S9.** Summary of two-way ANOVA results showing the effects of cultivar, harvest date, and their interaction on individual saponins (S1–S32) and total saponin content in leaves and roots of *Beta vulgaris* L.

## ORCID IDs

S. Janecki  
A. Spórna-Kucab  
A. Tękieli

<https://orcid.org/0009-0002-3596-9471>

<https://orcid.org/0000-0002-6653-3702>

<https://orcid.org/0000-0003-1147-9136>

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