

## Characterization of Triterpene Saponin Composition of White, Yellow and Red Beetroot (*Beta vulgaris* L.)

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*Beta vulgaris* L. is an important source of bioactive saponins – a group of secondary metabolites – that have spurred a growing interest due to their health-promoting properties. This study aimed to gain information on triterpene saponin profile of the peel and flesh of white, yellow and red beet of six cultivars – Snow Ball, Boldor, Ceryl, Chrobry, Forono and Tytus – harvested in Poland, in the same region. Twenty four saponins with oleanolic acid, hederagenin, akebonoic acid and gypsogenin as aglycons were identified and quantified by liquid chromatography/tandem mass spectrometry (LC-ESI-MS/MS). Among them, betavulgaroside I, II, III and IV were the major compounds, but the quantitative profile of saponins was found to be dependent on beet cultivar and root part, respectively. The highest content of saponins was found in the peel of yellow *B. vulgaris* Boldor (20812 mg/kg fresh weight, fw), while the lowest saponin content was determined in the flesh of white *B. vulgaris* Snow Ball (497 mg/kg fw). In addition, the total saponin content in peel and flesh in yellow beet (26054 mg/kg fw) was much higher than the total content in peel and flesh in red beet Tytus (8364 mg/kg fw) and white beet Snow Ball (1204 mg/kg fw). This is the first report on the profile of saponins in white and yellow beets.

### INTRODUCTION

Beetroot (*Beta vulgaris* L.) belongs to Chenopodiaceae family and has several varieties ranging from the white to yellow and red [Bárta *et al.*, 2020]. *B. vulgaris* is a vegetable consumed worldwide due to its health benefits. It can be eaten as raw, boiled, steamed and roasted [Kavalcová *et al.*, 2015]. Many studies confirm that the consumption of this vegetable helps protect against several diseases due to the presence of compounds implicated in numerous health benefits [Chhikara *et al.*, 2019; Clifford *et al.*, 2015]. These bioactive compounds include betalains [Pietrkowski *et al.*, 2010; Wybraniec *et al.*, 2011], carotenoids, polyphenols (including flavonoids) [Chhikara *et al.*, 2019], inorganic nitrate [dos Santos Baião *et al.*, 2021; Lidder & Webb, 2013] and saponins [Mikołajczyk-Bator *et al.*, 2016; Sparg *et al.*, 2004].

Saponins are natural glycosides that are known for their physicochemical (biosurfactant) properties and biological activities; therefore, they are commercially significant compounds with applications in the food, cosmetic and pharmaceutical industries [Güçlü-Üstündağ & Mazza, 2007; Rai

*et al.*, 2021]. A recent study has indicated multiple and complex activities of triterpene saponins, including anti-inflammatory, anti-bacterial, anti-allergic, hepatoprotective and anti-tumor ones; therefore, foods rich in saponins may reduce the risk of development of selected diseases [Fang *et al.*, 2020].

Due to the considerable application potential of saponins, in recent years there has been a significant increase in research on these compounds in terms of their uses as eco-friendly, biodegradable biosurfactants [Muhammad & Khan, 2018; Schmitt *et al.*, 2014] and pharmaceuticals [Mbaveng *et al.*, 2018; Xu *et al.*, 2018]. Beetroot, as a readily available and inexpensive vegetable, appears to be a good source of saponins [Mroczek *et al.*, 2012; Spórna-Kucab & Wybraniec, 2020]. Triterpene saponins present in *Beta vulgaris* L. are complex molecules consisting of the aglycone – hederagenin, akebonoic acid, oleanolic acid or gypsogenin coupled to sugar chain units. The number/type of sugars and different possibilities of sugar chain composition cause great natural diversity of saponin structures in *Beta vulgaris* L. [Mikołajczyk-Bator *et al.*, 2016]. The glycosidic bond is present between the aglycone and one (monodesmoside) or two (didesmoside) sugar

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chains at C-28 (via an ester) or C-3 (via an ether). The glycone can consist of hexoses, pentoses, 6-deoxyhexoses and uronic acids [Mikołajczyk-Bator *et al.*, 2016].

Saponins are present in the leaves and roots of *Beta vulgaris* L. [Mroczek *et al.*, 2019] and were found in the roots of red beet cultivars [Mikołajczyk-Bator *et al.*, 2016; Mroczek *et al.*, 2019] as well as in the sugar beets. Beetroot varieties differ in terms of their profile and content. Moreover, the differences in the content of saponins are also noticed in roots and leaves of the same variety [Edelmann *et al.*, 2020].

White and yellow beet saponins have never been studied so far, which might be due to the low availability of these varieties compared to red beet. Thus, for a comprehensive characterization of the composition of triterpene saponins, their accurate profiles were studied in selected red (Ceryl, Chrobry, Forono, Tytus) as well as yellow (Boldor) and white (Snow Ball) varieties harvested in Poland.

## MATERIALS AND METHODS

### Sample materials

Red, yellow and white *B. vulgaris* cultivars – Ceryl, Chrobry, Forono, Tytus, Boldor and Snow Ball, were collected in Poland. The red cultivars (Ceryl, Chrobry, Forono, Tytus) were harvested from Spójnia company (Nochowo, Poland) and the yellow cultivar from Bejo company (Ożarów Mazowiecki, Poland) in September 2019. The seeds of white cultivar were harvested from Torseed company (Toruń, Polska) and grown in September 2021. The fresh roots were washed under running water and peeled out to obtain the peel and flesh for further quantitative and qualitative analyses of saponins. Individual plant parts, the peel and flesh of studied *B. vulgaris* cultivars, were weighed before extraction and stored in a freezer.

### Solvents and reference compounds

Respective standards from a previous study on *B. vulgaris* (Red Sphere cultivar) were used for the saponin identification [Spórna-Kucab & Wybraniec, 2020]. Ethanol was purchased from Avantor Performance Materials Poland S.A. (Gliwice, Poland). LC-MS-grade acetonitrile and formic acid (purity  $\geq 98\%$ ) were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Oleanolic acid standard was purchased from Sigma-Aldrich. 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 21°C was deionized in a Milli-Q purification system (Merck, Darmstadt, Germany).

### Sample extraction

A 100-g portion of each peel or flesh was extracted with 300 mL of 80% (v/v) ethanol by ultrasound-assisted maceration for 30 min. The procedure was repeated three times, and each time extracts were combined, partially concentrated at 25°C under reduced pressure and freeze-dried. Finally, the freeze-dried extracts were weighed and used for further studies on the quantitative and qualitative profile of saponins by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) system.

### LC-MS – instrumentation and conditions

Qualitative and quantitative analyses were performed by high-performance liquid chromatography and electrospray ionization mass spectrometry (LC-ESI-MS/MS) using an LCMS-8030 system (Shimadzu, Kyoto, Japan). The LC system consisted of a SIL-20ACXR autosampler, a degasser, and a Nexera LC-20ADXR binary pump equipped with a gradient controller. LC separation was performed using a 100 $\times$ 4.6 mm, 5.0  $\mu$ m Kinetex C<sub>18</sub> chromatography column (Phenomenex, Torrance, CA, USA) protected by a 4 $\times$ 2 mm guard column of the same material (Phenomenex) and thermostated at 40°C. LabSolutions software version 5.91 SP1 was used for data acquisition in LC-ESI-MS/MS.

In the LC system, the mobile phase was composed of acetonitrile (A) and 2% (v/v) aqueous formic acid (B). The flow rate was kept constant at 0.5 mL/min. Oleanolic acid and extracts were analyzed in different gradient elutions. For extracts, the gradient elution was programmed as follows: from 5% to 60% A, 0–62 min; from 60% to 80% A, 62–65 min, 80% to 5% A, 65–66 min. For oleanolic acid, the gradient elution was as follows: from 20% to 99% A, 0–7 min; 99% A, 7–15 min.

Triple quadrupole mass spectrometer with an electrospray ion source coupled to the LC system described above was used for MS/MS experiments. The following instrumental parameters were applied in ESI-MS/MS analysis of saponins: curved desolvation line (CDL) and heat block temperature of 230°C, nebulizing gas flow rate of 1.5 L/min, electrospray voltage of 4.5 kV and the capillary temperature at 250°C. The relative collision energies for MS/MS analyses were set at -35 V, and argon was used as the collision gas. Data were recorded in a negative ion mode using a scan mode with  $m/z$  ranging from 100 to 2000 Da and the selected ion monitoring (SIM).

### Quantitative and qualitative analysis of saponins

Oleanolic acid is an aglycone of the most saponins identified in the *B. vulgaris* roots, thus, it was utilized to plot the calibration curve. A stock solution of the external standard – oleanolic acid (20.0  $\mu$ g/mL), was prepared in water and stored at 8°C until further use. Calibration solutions were prepared by 1:1 (v/v) stepwise dilution of the stock solution in order to get five calibration points over a concentration range from 1.25 to 20.0  $\mu$ g/mL. Then, 10  $\mu$ L of the standard was injected three times to the LC-ESI-MS/MS system. The calibration curve was prepared by plotting the peak area ratios of the standard from MS chromatograms against their concentrations showing a linear response with the coefficient of determination ( $R^2$ ) of 0.9996.

In the analysis of saponins, freeze-dried extracts (100 mg) were diluted in demineralized water (1 mL) and centrifuged at 2504 $\times$ g for 5 min, then 20  $\mu$ L of each sample was injected three times to the LC-ESI-MS/MS system. The content of individual saponins as well as their total content in the studied extract was estimated from the external standard calibration curve plotted for oleanolic acid. The peak areas from LC-ESI-MS chromatograms were used for quantification. The use of aglycone in the analysis of glycosides bears the risk of receiving a less accurate result than in the case of using single, separated standards of saponins. However, at present, such standards are not easily available and obtaining them

from the plant is time-consuming and challenging. Therefore, using the calibration curve based on oleanolic acid seems to be a good solution in saponin analysis.

Three independent LC-ESI-MS/MS analyses were run for each sample to evaluate instrumental precision. The MS chromatograms allowed identifying compounds below the LOQ. Compounds with signal-to-noise ratio lower than 10:1 were below LOQ.

Content of saponins was expressed in mg/kg fresh weight (fw) of peel or flesh.

### Statistical analysis

Data were reported as the mean  $\pm$  standard deviation (SD) of three measurements. One-way analysis of variance (ANOVA) was used for the statistical analysis of the data with the help of Statistica, version 7.1 (StatSoft, TIBCO Software Inc. Palo Alto, CA, USA). Differences between means were determined using Fisher's test and were found significant at  $p < 0.05$ .

## RESULTS AND DISCUSSION

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

The complete structural characterization of saponins in plant materials is complicated and time-consuming. The LC-ESI-MS/MS is a viable method used to characterize natural compounds based on their fragmentation [Spórna-Kucab *et al.*, 2019, 2020; Wybraniec *et al.*, 2010].

The analysis of extracts of peel and flesh of four red (Ceryl, Chrobry, Forono, Tytus), one yellow (Boldor) and one white (Snow Ball) *B. vulgaris* cultivars by LC-ESI-MS/MS revealed the presence of 24 triterpene saponins consisting of oleanolic acid, hederagenin, akebonoic acid and gypsogenin as the aglycones (Figure 1). The compounds identified as well as their retention times,  $[M-H]^-$  and MS/MS data are summarized in Table 1.

Ten compounds (saponins 3, 5, 7–9, 17, 18, 21, 22, 24) were identified by comparison with the reference compounds isolated in a previous research [Spórna-Kucab & Wybraniec, 2020]. In contrast, the remaining 14 compounds were tentatively identified by the interpretation of their fragmentation patterns obtained from mass spectra (MS/MS experiments) and by comparison with the previous experiments reported in the literature [Mikołajczyk-Bator *et al.*, 2016; Mroczek *et al.*, 2012, 2019].

### Saponins with oleanolic acid as the aglycone

Previous research on saponins show that *B. vulgaris* contains significant amounts of oleanolic acid derivatives, which we also confirmed in our research. Here, seventeen saponins as oleanolic acid derivatives were identified in the peel and flesh samples of all analyzed beetroot cultivars (Table 1, Figure 2, Figure 3, Figure 4, Figure 5).

Oleanolic acid as well as saponins containing oleanolic acid are of great interest to the food, cosmetic and pharmaceutical industries because they exhibit various biological properties [Dubois *et al.*, 1990; Hikino *et al.*, 1985; Lemmich *et al.*, 1995; Parus, 2013; Yoshikawa *et al.*, 1994]. Oleanolic acid itself exhibits anti-inflammatory, antibacterial and antiseptic

[Ismaili *et al.*, 2001] as well as hepatoprotective [Hikino *et al.*, 1985] and hypoglycemic effects [Yoshikawa *et al.*, 1994].

The following sugar moieties were found in the identified *B. vulgaris* saponins: uronic acid (UrA), deoxyhexose (dHex), pentose (Pen), hexose (Hex), substituted sugar residues of the acetal (Act) and dioxolane (Diox) type (Table 1). Different sugar moieties as well as a different number of sugar units were noticed. The previous research results show that the activity of saponins containing oleanolic acid depends on the structure of the sugar chain [De Tommasi *et al.*, 1991; Yoshikawa *et al.*, 1994] and can exhibit wider spectrum of activities than oleanolic acid, including *e.g.* immunostimulating [Dubois *et al.*, 1990], cytotoxic, anti-carcinogenic, anti-mutagenic, antiviral and protozoicidal. Moreover, saponins are well known for their hemolytic properties, which are not always desired; however, this strongly depends on the type of sugar, the number of sugar groups and the spatial arrangement of sugar chains [Lemmich *et al.*, 1995]. Moreover, the tumor-specificity of the cytotoxic action seems to be influenced by the structure of the sugar portion of the saponins [Kuroda *et al.*, 2001].

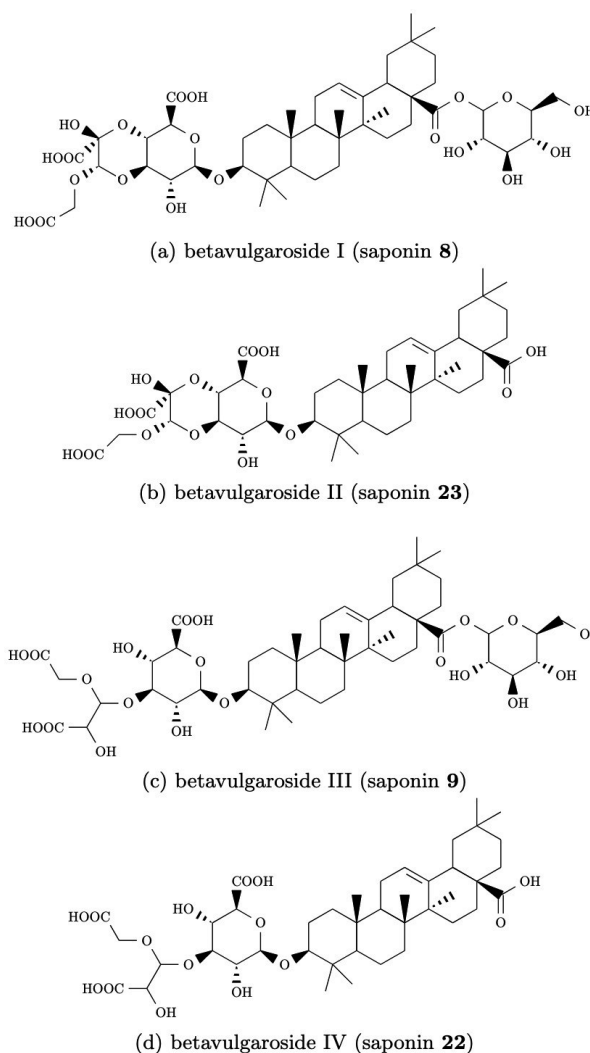


FIGURE 1. Chemical structures of the main saponins detected in *Beta vulgaris* L.: (a) betavulgaroside I, (b) betavulgaroside II, (c) betavulgaroside III, and (d) betavulgaroside IV.

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

No.	Saponin	Trivial name	t <sub>R</sub> (min)	m/z [M-H] <sup>-</sup>	m/z from MS <sup>2</sup> of [M-H] <sup>-</sup>
1	Act-Hex-Hex-UrA-oleanolic acid	betavulgaroside V	36.3	1117	997; 955; 793; 631; 455
2	Act-Hex-UrA-akebonoic acid	betavulgaroside VIII	38.2	939	777; 615; 439
3	Act-Hex-Hex-UrA-oleanolic acid	betavulgaroside V	38.4	1117	997; 955; 793; 631; 455
4	Hex-Pen-Hex-UrA-oleanolic acid		38.6	1087	967; 925; 763; 631; 455
5	Act-Hex-Pen-UrA-oleanolic acid	betavulgaroside IX	39.4	1087	925; 763; 631; 455
6	Act-Hex-Hex-UrA-oleanolic acid	betavulgaroside V	40.1	1117	997; 955; 793; 631; 455
7	Hex-Pen-UrA-oleanolic acid		41.0	925	763; 631; 455
8	Diox-Hex-UrA-oleanolic acid	betavulgaroside I	41.5	953	909; 793; 631; 455
9	Act-Hex-UrA-oleanolic acid	betavulgaroside III	41.8	955	835; 793; 673; 631; 455
10	Act-dHex-UrA-oleanolic acid		45.7	939	777; 631; 455
11	Hex-Pen-UrA-akebonoic acid	betavulgaroside X	46.3	909	747; 615; 439
12	Act-dHex-UrA-oleanolic acid		47.0	939	777; 631; 455
13	Pen-UrA-hederagenin		47.2	779	647; 471
14	C <sub>5</sub> H <sub>4</sub> O <sub>5</sub> -Hex-UrA-akebonoic acid		47.8	921	439
15	Act-UrA-hederagenin	betavulgaroside VII	48.0	809	647; 471
16	Hex-UrA-gypsogenin		48.4	807	645; 469
17	Hex-Pen-UrA-oleanolic acid		49.7	925	763; 631; 455
18	Act-Hex-UrA-oleanolic acid	betavulgaroside III	49.8	955	835; 793; 673; 631; 455
19	Act-UrA-akebonoic acid		51.0	777	615; 439
20	C <sub>4</sub> H <sub>4</sub> O <sub>5</sub> -Hex-UrA-oleanolic acid		51.4	925	763; 631; 455
21	Pen-UrA-oleanolic acid		54.3	763	631; 455
22	Act-UrA-oleanolic acid	betavulgaroside IV	55.6	793	673; 631; 455
23	Diox-UrA-oleanolic acid	betavulgaroside II	56.0	791	631; 455
24	UrA-oleanolic acid		56.4	631	455

Act – acetal substituent, Diox – dioxolane substituent, Hex – hexose, dHex – deoxyhexose, Pen – pentose, UrA – uronic acid, t<sub>R</sub> – retention time.

Oleanolic acid in the unbound form has not been identified, but its derivative with uronic acid was detected. The main and simplest saponin with the pseudomolecular ion [M-H]<sup>-</sup> at m/z 631 was tentatively identified as saponin **24** based on its fragmentation pattern and by comparison to the reference compound previously isolated from *B. vulgaris* cultivar Red Sphere [Spórna-Kucab & Wybraniec, 2020]. Compound **24** fragmented to m/z 455, which corresponded to oleanolic acid. The presence of a daughter ion for the studied compounds at m/z 455 indicated the loss of the uronic acid (631–455=176) for saponin **24** [Mroczek *et al.*, 2012, 2019; Spórna-Kucab & Wybraniec, 2020]. Previous research indicates that uronic acid attached to the oleanolic acid may significantly enhance its activity against retinivirus, which is responsible for most colds [De Tommasi *et al.*, 1991]. This saponin was also identified previously in the *B. vulgaris* cultivar Red Sphere [Mroczek *et al.*, 2012, 2019; Spórna-Kucab & Wybraniec, 2020].

Saponin profiles in *B. vulgaris* revealed the presence of three monosubstituted with pentose (saponin **21**), acetal (saponin **22**) or dioxolane (saponin **23**) derivatives of saponin **24**. The presence of daughter ions for compounds at m/z 631 indicated the loss of pentose (763–631=132) for saponin **21**, acetal-type substituent (793–631=162) for saponin **22** and dioxolane-type substituent (791–631=160) for saponin **23**, respectively. The structures of saponin **21** and **22** were confirmed with the reference compound from *B. vulgaris* cultivar Red Sphere [Spórna-Kucab & Wybraniec, 2020]. Compound **21** has been previously identified in leaves of *B. vulgaris* cultivars Red Sphere, Forono, Egyptian and Round Dark [Mroczek *et al.*, 2019]. The structure and fragmentation pattern of saponin **22**, commonly known as betavulgaroside IV, was previously described [Mikołajczyk-Bator *et al.*, 2016; Yoshikawa *et al.*, 1996]. Saponin **23** was detected with [M-H]<sup>-</sup> at m/z 791 and the fragmentation daughter ions at m/z 631 and 455. Previous research, revealed two saponins with m/z 791 [Mikołajczyk-Bator *et al.*, 2016]. These



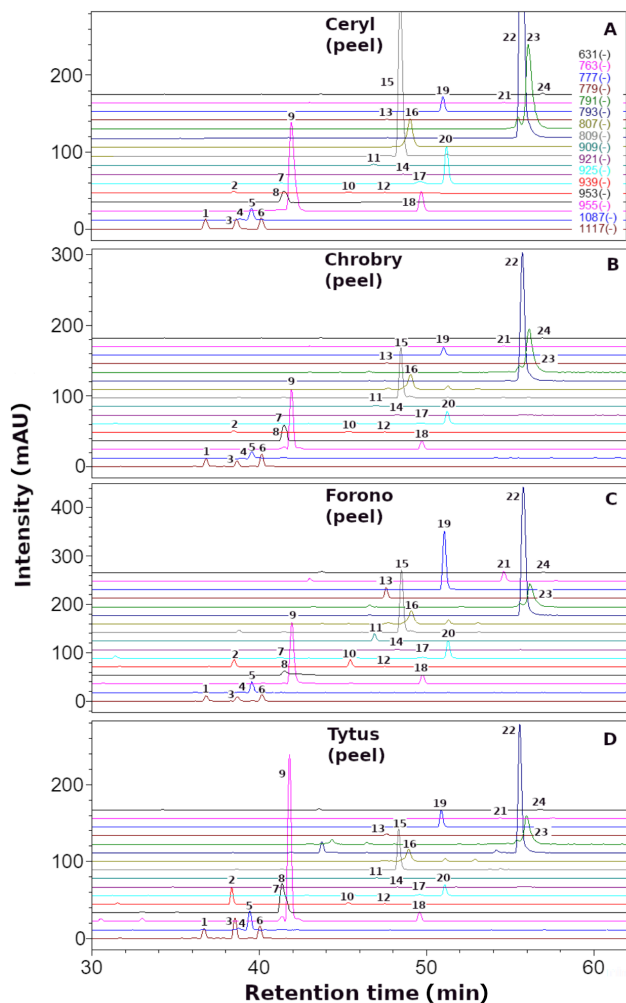


FIGURE 2. Single-ion monitoring chromatograms obtained by LC-ESI-MS in a negative ion mode for saponins of red *Beta vulgaris* L. peels: (a) Ceryl, (b) Chrobry, (c) Forono, and (d) Tytus.

saponins differ in the presence of the aglycones: oleanolic acid ( $m/z$  455) or akebonoic acid ( $m/z$  439). Herein, the MS/MS spectrum of **23** exhibited an aglycone at  $m/z$  455; therefore, this saponin has been identified as betavulgaroside II. Previous research showed that hexose attachment to oleanolic acid and glucuronic acid enhanced the antiviral activity of saponins [De Tommasi *et al.*, 1991]; however, to the best of our knowledge, there is no research on the effect of a single substituent of another sugar on the properties of saponins.

The monosubstituted with hexose saponin **24** was not detected in any peel and flesh sample but simultaneous presence of hexose and dioxalane (saponin **8**), hexose and acetal-type (saponin **9**, **18**) or hexose and  $C_4H_4O_5$  (saponin **20**) substituents was noticed.

Saponins **8** ( $[M-H]^-$  at  $m/z$  953), **9** and **18** ( $[M-H]^-$  at  $m/z$  955) were confirmed with the authentic standards isolated and described in previous experiments [Spórna-Kucab & Wybraniec, 2020]. These saponins were thoroughly described using NMR by Yoshikawa *et al.* [1996], which named them as betavulgaroside I (saponin **8**) and III (saponin **9**, **18**) (Table 1). Betavulgaroside I and III were also isolated from

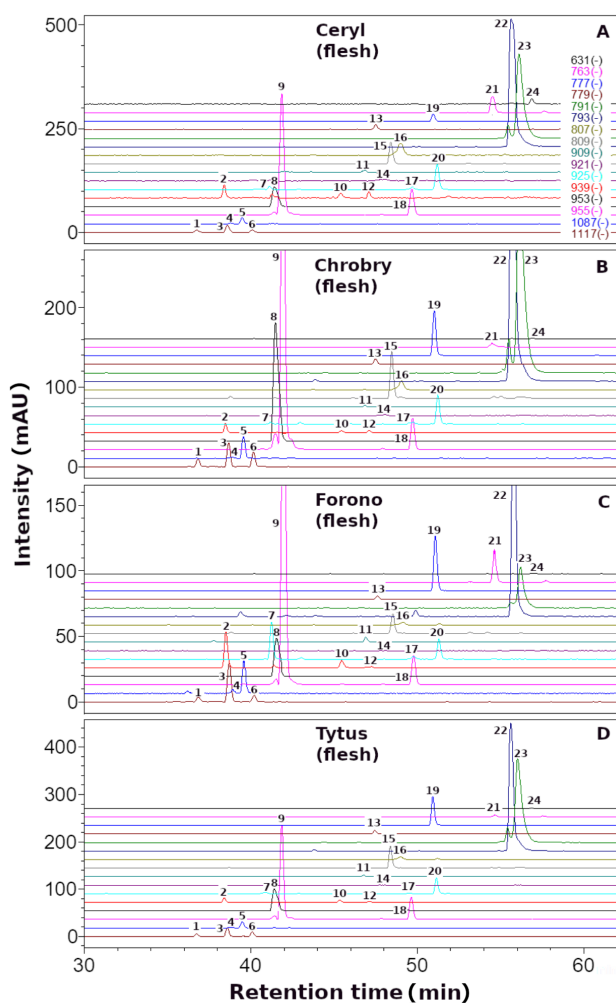


FIGURE 3. Single-ion monitoring chromatograms obtained by LC-ESI-MS in a negative ion mode for saponins of red *Beta vulgaris* L. flesh: (a) Ceryl, (b) Chrobry, (c) Forono, and (d) Tytus.

*Achyranthes fauriei* and named achyranthoside B and C, respectively [Ida *et al.*, 1994]. Achyranthoside B may find application in the treatment of cancer, as research shows that it is toxic to human colorectal cancer cells and murine melanoma cells [Ida *et al.*, 1994].

The fragmentation pattern of saponin **20** ( $[M-H]^-$  at  $m/z$  925) was already described in the studied *B. vulgaris* cultivar Nochowski [Mikołajczyk-Bator *et al.*, 2016]; therefore, its tentative identification was feasible.

Hexose-hexose and hexose-pentose substituents in saponin **24** ( $[M-H]^-$  at  $m/z$  631) were not identified. But hexose-hexose-acetal and hexose-pentose-hexose substituents were detected in saponins **1**, **3** and **6** ( $[M-H]^-$  at  $m/z$  1117) as well as saponin **4** ( $[M-H]^-$  at  $m/z$  1087). It is worth noting that tetraglycoside saponins have stronger bactericidal properties than triglycoside saponins [Francis *et al.*, 2002; Konishi *et al.*, 1998]. Moreover, antibacterial properties generally depend on the attached sugar; therefore, these compounds may be very interesting for further studies of their antimicrobial activities. Saponin **6** was named betavulgaroside V and was also isolated from *Achyranthes fauriei* and named achyranthoside D

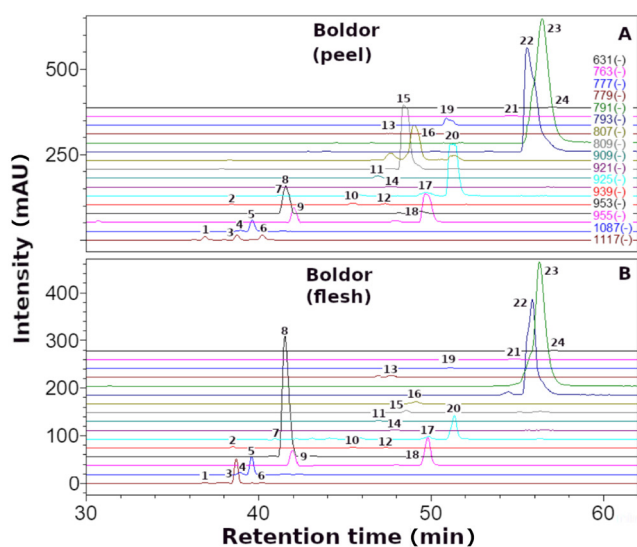


FIGURE 4. Single-ion monitoring chromatograms obtained by LC-ESI-MS in a negative ion mode for saponins of yellow *Beta vulgaris* L.: (a) peel, and (b) flesh.

[Kuwada *et al.*, 2020]. Saponin **3** was isolated in previous research and used here as a reference compound [Spórna-Kucab & Wybraniec, 2020]. For saponins detected with  $[M-H]^-$  at  $m/z$  1117, the previous study [Mikołajczyk-Bator *et al.*, 2016] of *B. vulgaris* cultivars indicated the presence of four positional isomers of two hexose, one uronic acid moiety, and an acetal-type substituent attached to the oleanolic acid by an ester bond at the C-28 position or another bond at the C-3 position. Saponin **4** was tentatively identified by comparison with previously reported data [Mikołajczyk-Bator *et al.*, 2016]. Previous study found that saponin **4** had a positional isomer with identical aglycone ions detected at  $m/z$  455 and identical moieties (*i.e.*, uronic acid, two hexoses and pentose) connected at different positions. Hence, the more polar isomer of **4** was not identified, presumably because of its low content.

The saponin **24** might also be bisubstituted with pentose-hexose (saponin **7**, **17**) and trisubstituted with pentose-hexose-acetal (saponin **5**). The signal detected with  $[M-H]^-$  at  $m/z$  1087 (saponin **5**) gave a base peak in the MS chromatogram. Saponin **5** showed molecular negative ions at  $m/z$  925, 763, 631, and 455, suggesting the presence of substituted sugar residues of acetal ( $1087-925=162$  Da), hexose ( $925-763=162$  Da), pentose ( $763-631=132$ ) and uronic acid ( $631-455=176$ ), respectively. The two saponins detected with  $[M-H]^-$  at  $m/z$  925 (saponin **7**, **17**), which were already described in *B. vulgaris* cultivar Nochowski [Mikołajczyk-Bator *et al.*, 2016], were found in this analysis. Saponins **5**, **7**, **17** were identified by comparison with the reference compounds isolated previously from *B. vulgaris* cultivar Red Sphere [Spórna-Kucab & Wybraniec, 2020]. The chemical structure of compound **5**, named betavulgaroside IX, was thoroughly described by H-NMR and C-NMR [Yoshikawa *et al.*, 1998].

The saponin **24** bisubstituted with deoxyhexose and acetal substituent was detected (saponins **10**, **12**) and these compounds showed a pseudomolecular  $[M-H]^-$  ion at  $m/z$  939.

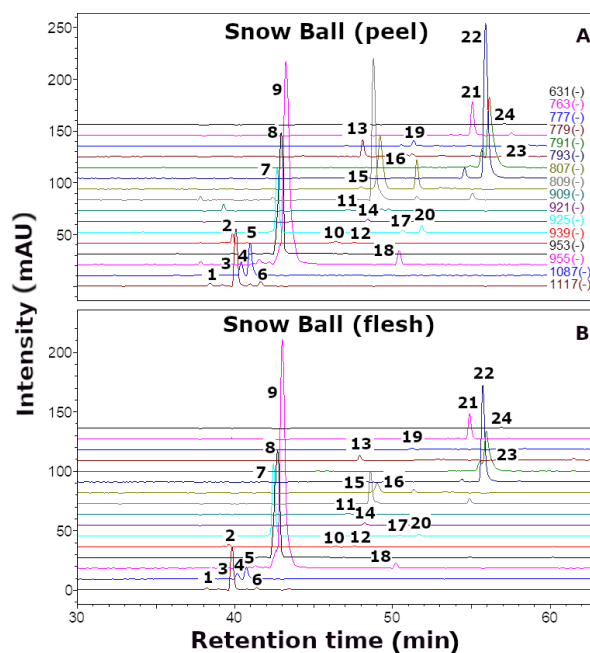


FIGURE 5. Single-ion monitoring chromatograms obtained by LC-ESI-MS in a negative ion mode for saponins of white *Beta vulgaris* L.: (a) peel, and (b) flesh.

Similar retention times of saponins **10** and **12** may indicate that these compounds might be isomers. Previous research [Mikołajczyk-Bator *et al.*, 2016] indicates three saponins with oleanolic acid in *B. vulgaris*, with two of them having a similar structure and polarity. Therefore, these compounds were tentatively identified based on the interpretation of their fragmentation patterns.

#### Saponins with akebonoic acid as the aglycone

Four saponins, which comprised akebonoic acid as an aglycone, were identified and profiled using LC-ESI-MS/MS in the peel and flesh samples of all analyzed beetroot cultivars (Table 1, Figure 2, Figure 3, Figure 4, Figure 5).

The simplest saponin with a pseudomolecular ion  $[M-H]^-$  detected at  $m/z$  615 corresponded to akebonoic acid ( $m/z$  439), whereas uronic acid ( $m/z$  176) was not detected. Herein, the MS/MS spectrum revealed akebonoic acid as the aglycone for saponins with  $[M-H]^-$  at  $m/z$  777 (saponin **19**), 909 (saponin **11**), 921 (saponin **14**) and 939 (saponin **2**). These saponins were previously identified in *B. vulgaris* cultivar Nochowski [Mikołajczyk-Bator *et al.*, 2016]. Additionally, saponin with a pseudomolecular ion detected at  $m/z$  777 was identified in Swiss chard (*B. vulgaris*) [Mroczek *et al.*, 2021].

Saponin **19** had a negative pseudomolecular ion at  $m/z$  777 and daughter ions at  $m/z$  615 and 439. The MS spectrum of compound **19** revealed an aglycone ion at  $m/z$  439, which corresponded to akebonoic acid. Saponins with akebonoic acid were previously identified and profiled by LC-ESI-MS/MS in *B. vulgaris* cultivar Nochowski [Mikołajczyk-Bator *et al.*, 2016]. Findings of research suggest two positional isomers of saponins with  $[M-H]^-$  at  $m/z$  777 differing in polarity. The fragmentation pattern as well as the polarity of saponin **19** indicate the presence

of an acetal-type substituent attached to the akebonoic acid (777–615=162) and uronic acid moiety (615–439=176).

Saponin **11** had a negative pseudomolecular ion detected at  $m/z$  909, yielding fragmentation ions at  $m/z$  747, 615 and 439. The  $m/z$  fragments corresponded to the loss of hexose (–162 Da) and pentose moieties (–132 Da) and further uronic acid (–176 Da), respectively. The ion detected at  $m/z$  439 suggested akebonoic acid to be the aglycone, which allowed identifying compound **11** as betavulgaroside X. This compound was previously detected in the *B. vulgaris* cultivar Nochowski [Mikołajczyk-Bator et al., 2016]. Moreover, the structure of compound **11** was determined by Yoshikawa et al. [1996].

Saponin **14** [M–H]<sup>–</sup> ion was detected at  $m/z$  921, which provided the product ion in the MS/MS spectra at  $m/z$  439 characteristic for akebonoic acid. The review of previous findings [Mikołajczyk-Bator et al., 2016] indicates only one saponin in the *B. vulgaris* cultivars with [M–H]<sup>–</sup> at  $m/z$  921; therefore, saponin **14** was tentatively identified by comparison with previously reported data.

Saponin **2** yielded a product ion in the MS/MS spectra at  $m/z$  439 characteristic for akebonoic acid. Analysis of *B. vulgaris* cultivar Nochowski indicates the presence of three akebonoic acids as the aglycones [Mikołajczyk-Bator et al., 2016]. The dominant saponin in *B. vulgaris* including akebonoic acid glycosides is a well-known betavulgaroside VIII [Yoshikawa et al., 1998]. The remaining saponins with akebonoic acid were identified in trace amounts in the previous research [Mikołajczyk-Bator et al., 2016]; therefore, we did not detect these compounds in the studied material.

To the best of our knowledge, there are no studies on the bioactivity of purified saponins with akebonoic acid as the aglycone. The saponins might be very useful for the pharmaceutical industry because akebonoic acid is considered a potential candidate for the treatment of type-2 diabetes due to its antidiabetic activity [Dirir et al., 2021]. The previous study showed that fractions containing saponins with akebonoic acid and gypsogenin obtained from *Chenopodium bonus-henricus* L. exhibited hepatoprotective activity [Kokanova-Nedialkova et al., 2020].

#### Saponins with hederagenin and gypsogenin as the aglycones

In the present study, hederagenin and gypsogenin as the aglycones were detected in three saponins identified with [M–H]<sup>–</sup> at  $m/z$  779 (saponin **13**), 807 (saponin **16**) and 809 (saponin **15**) (Table 1, Figure 2, Figure 3, Figure 4, Figure 5). Compound **13** detected with [M–H]<sup>–</sup> at  $m/z$  779 was identified based on its fragmentation pattern (MS/MS experiments) and by comparison with the literature data [Mikołajczyk-Bator et al., 2016]. Literature findings indicate only one saponin detected with [M–H]<sup>–</sup> at  $m/z$  779. Moreover, the fragmentation of saponin **13** to ions at  $m/z$  647 and hederagenin as an aglycone at  $m/z$  471 confirms the presence of pentose (779–647=132) including uronic acid (647–471=176) in the structure.

Saponin **15** presented a pseudomolecular ion [M–H]<sup>–</sup> at  $m/z$  809 releasing fragment ions at  $m/z$  647 (loss of acetal-type substituent) as well as  $m/z$  471 (loss of uronic acid) and was tentatively identified as betavulgaroside VII. Saponin **15** was earlier identified in *B. vulgaris* [Mikołajczyk-Bator et al., 2016;

Mroczek et al., 2012]. Previous findings [Mikołajczyk-Bator et al., 2016; Mroczek et al., 2012] indicate two saponin isomers detected with [M–H]<sup>–</sup> at  $m/z$  809, significantly differing in their polarity, consequently, the confirmation of saponin **15** was more convenient.

Correspondingly, previous research [Mikołajczyk-Bator et al., 2016] suggested only one saponin with a molecular ion [M–H]<sup>–</sup> detected at  $m/z$  807. A high content of saponin **16** in yellow *B. vulgaris* cultivar enabled its fragmentation, which indicated the loss of hexose (162 Da) and uronic acid (176 Da) from gypsogenin as an aglycone ([M–H]<sup>–</sup> at  $m/z$  469), respectively.

The beneficial effect of hederagenin derivatives has been repeatedly confirmed [Fang et al., 2020; Kuljanabhadgavad & Wink, 2009]. They have been proven to exhibit the following activities: antimicrobial, antioxidant, molluscidal, fungicidal [Kuljanabhadgavad & Wink, 2009], anti-inflammatory, anti-arthritis, anticomplementary and cytotoxic [Fang et al., 2020]. Cytotoxic activity was also confirmed for gypsogenin derivatives [El Hazzam et al., 2020]; however, they have not been thoroughly tested for their effects. Therefore, their activity can be very interesting; hence it is important to look for their new sources. Hederagenin glycosides were detected in a previous study of saponins from the *B. vulgaris* cultivars Red Sphere, Rocket, Wodan and Nochowski [Mikołajczyk-Bator et al., 2016; Mroczek et al., 2012].

#### Saponin quantitative analysis

The quantification of the individual saponins of beetroot peel and flesh was carried out using oleanolic acid as a standard. The oleanolic acid was the aglycone of the most identified saponins. The qualitative profiles of the peel and flesh saponins of all analyzed beetroot cultivars were identical, but the content of individual compounds was highly varied across the samples (Table 2, Table 3). The total saponin content ranged from 707 mg/kg fw (Snow Ball) to 6834 mg/kg fw (Forono) for peel samples and from 497 mg/kg fw (Snow Ball) to 6864 mg/kg fw (Boldor) for flesh samples. The quantitative profiles of saponins divided into four groups according to their aglycone – oleanolic acids, akebonoic acid, gypsogenin or hederagenin – are discussed below.

#### Saponins with oleanolic acid as the aglycone

Among 17 derivatives of oleanolic acid, compounds **8**, **9**, **22** and **23** (betavulgaroside I–IV) were the main saponins determined in the peel and flesh of *B. vulgaris* cultivars (Table 2, Table 3). The content of saponin **22** was the highest in most of beetroot peel (452–7361 mg/kg fw) and flesh (423–2090 mg/kg fw) samples with the exception of the peel of Tytus cultivar, flesh of Boldor cultivar and flesh and peel of Snow Ball cultivar wherein saponins **9** (675 mg/kg fw), **8** (2603 mg/kg fw) and **9** (164–173 mg/kg fw), respectively, were dominant. In a previous study, a high content of compound **9** (9543 mg/kg dw) was found in beetroot flesh of red Red Sphere cultivar [Mroczek et al., 2019]. The content of saponin **23** was high in most of the analyzed cultivars (36.1–5516 mg/kg fw), except for cultivars Chrobry (peel) (6.7 mg/kg fw) and Forono (4.5–5.2 mg/kg fw).

TABLE 2. Content of individual saponins and total saponins in fresh peel of *Beta vulgaris* L. cultivars (mg/kg fresh weight) analyzed by LC-DAD-ESI-MS/MS.

No.	Saponin	Red cultivars				Yellow cultivar	White cultivar
		Ceryl	Chrobry	Forono	Tytus	Boldor	Snow Ball
1	Act-Hex-Hex-UrA-oleanolic acid	0.021±0.003 <sup>f</sup>	19.4±0.60 <sup>d</sup>	82.7±1.2 <sup>b</sup>	37.9±1.1 <sup>c</sup>	97.3±2.0 <sup>a</sup>	1.2±0.05 <sup>c</sup>
2	Act-Hex-UrA-akebonoic acid	2.9±0.15 <sup>e</sup>	3.4±0.14 <sup>e</sup>	101±2.5 <sup>a</sup>	53.2±1.8 <sup>b</sup>	1.6±0.09 <sup>f</sup>	3.2±0.16 <sup>d</sup>
3	Act-Hex-Hex-UrA-oleanolic acid	32.5±0.78 <sup>d</sup>	15.3±0.30 <sup>f</sup>	65.8±2.1 <sup>c</sup>	77.8±2.1 <sup>a</sup>	67.8±1.2 <sup>b</sup>	29.3±0.73 <sup>e</sup>
4	Hex-Pen-Hex-UrA-oleanolic acid	0.61±0.030 <sup>e</sup>	0.023±0.003 <sup>f</sup>	1.3±0.08 <sup>d</sup>	2.7±0.12 <sup>c</sup>	16.7±0.6 <sup>a</sup>	6.8±0.26 <sup>b</sup>
5	Act-Hex-Pen-UrA-oleanolic acid	41.4±0.65 <sup>d</sup>	17.8±0.63 <sup>c</sup>	140±3.8 <sup>b</sup>	74.3±1.4 <sup>c</sup>	369±5.1 <sup>a</sup>	17.4±0.35 <sup>e</sup>
6	Act-Hex-Hex-UrA-oleanolic acid	38.2±0.69 <sup>c</sup>	33.6±0.70 <sup>d</sup>	1.2±0.06 <sup>f</sup>	48.5±1.7 <sup>b</sup>	96.1±2.4 <sup>a</sup>	1.9±0.09 <sup>e</sup>
7	Hex-Pen-UrA-oleanolic acid	0.25±0.012 <sup>d</sup>	0.012±0.002 <sup>e</sup>	0.37±0.02 <sup>c</sup>	0.33±0.02 <sup>c</sup>	1.2±0.06 <sup>b</sup>	30.5±0.79 <sup>a</sup>
8	Diox-Hex-UrA-oleanolic acid	11.8±0.15 <sup>f</sup>	54.8±0.80 <sup>c</sup>	55.2±1.0 <sup>d</sup>	154±3.8 <sup>b</sup>	1200±16 <sup>a</sup>	152±3.0 <sup>c</sup>
9	Act-Hex-UrA-oleanolic acid	2.2±0.11 <sup>f</sup>	174±2.3 <sup>c</sup>	1069±22 <sup>a</sup>	675±14 <sup>b</sup>	118±2.2 <sup>e</sup>	173±3.5 <sup>d</sup>
10	Act-dHex-UrA-oleanolic acid	0.51±0.022 <sup>f</sup>	0.85±0.04 <sup>e</sup>	98.1±2.0 <sup>a</sup>	3.3±0.14 <sup>d</sup>	30.4±0.6 <sup>b</sup>	22.6±0.67 <sup>c</sup>
11	Hex-Pen-UrA-akebonoic acid	0.072±0.009 <sup>c</sup>	0.050±0.007 <sup>c</sup>	0.20±0.01 <sup>d</sup>	2.8±0.16 <sup>b</sup>	105±2.4 <sup>a</sup>	<LOQ
12	Act-dHex-UrA-oleanolic acid	0.81±0.040 <sup>e</sup>	0.35±0.022 <sup>d</sup>	1.40±0.06 <sup>b</sup>	0.33±0.02 <sup>c</sup>	15.3±0.4 <sup>a</sup>	0.023±0.004 <sup>f</sup>
13	Pen-UrA-hederagenin	42.0±0.90 <sup>b</sup>	0.14±0.008 <sup>c</sup>	150±4.1 <sup>a</sup>	1.8±0.10 <sup>d</sup>	32.6±0.60 <sup>c</sup>	0.032±0.004 <sup>f</sup>
14	C <sub>5</sub> H <sub>4</sub> O <sub>5</sub> -Hex-UrA-akebonoic acid	0.032±0.004 <sup>e</sup>	0.032±0.004 <sup>c</sup>	0.053±0.006 <sup>a</sup>	0.044±0.006 <sup>b</sup>	0.12±0.008 <sup>d</sup>	<LOQ
15	Act-UrA-hederagenin	639±8.5 <sup>c</sup>	133±4.2 <sup>d</sup>	989±20 <sup>b</sup>	152±1.8 <sup>c</sup>	2283±44 <sup>a</sup>	71.0±1.4 <sup>f</sup>
16	Hex-UrA-gypsogenin	152±5.5 <sup>b</sup>	35.7±1.3 <sup>e</sup>	16.2±0.21 <sup>f</sup>	65.2±1.5 <sup>c</sup>	856±14 <sup>fa</sup>	37.4±0.74 <sup>d</sup>
17	Hex-Pen-UrA-oleanolic acid	6.7±0.21 <sup>d</sup>	0.86±0.04 <sup>e</sup>	21.1±0.90 <sup>b</sup>	8.3±0.20 <sup>c</sup>	137±2.0 <sup>a</sup>	0.023±0.004 <sup>f</sup>
18	Act-Hex-UrA-oleanolic acid	73.5±1.2 <sup>c</sup>	25.6±0.50 <sup>e</sup>	150±2.2 <sup>b</sup>	35.6±0.90 <sup>d</sup>	415±9.1 <sup>a</sup>	6.2±0.31 <sup>f</sup>
19	Act-UrA-akebonoic acid	46.8±1.1 <sup>d</sup>	20.4±0.38 <sup>e</sup>	881±11 <sup>a</sup>	63.6±0.90 <sup>c</sup>	274±6.1 <sup>b</sup>	2.1±0.11 <sup>f</sup>
20	C <sub>4</sub> H <sub>4</sub> O <sub>5</sub> -Hex-UrA-oleanolic acid	255±5.4 <sup>c</sup>	70.6±1.3 <sup>e</sup>	528±12 <sup>b</sup>	82.3±1.6 <sup>d</sup>	1735±20 <sup>a</sup>	2.4±0.12 <sup>f</sup>
21	Pen-UrA-oleanolic acid	45.5±0.48 <sup>c</sup>	0.071±0.008 <sup>f</sup>	62.3±1.4 <sup>b</sup>	2.3±0.10 <sup>c</sup>	68.0±1.9 <sup>a</sup>	15.3±0.46 <sup>d</sup>
22	Act-UrA-oleanolic acid	1194±15 <sup>c</sup>	452±5.0 <sup>c</sup>	2410±51 <sup>b</sup>	547±34 <sup>d</sup>	7361±117 <sup>a</sup>	88.2±1.7 <sup>f</sup>
23	Diox-UrA-oleanolic acid	497±13 <sup>b</sup>	6.7±0.30 <sup>e</sup>	5.2±0.16 <sup>f</sup>	173±4.0 <sup>c</sup>	5516±160 <sup>a</sup>	44.9±0.90 <sup>d</sup>
24	UrA-oleanolic acid	6.1±0.30 <sup>b</sup>	0.073±0.009 <sup>e</sup>	5.2±0.22 <sup>c</sup>	1.9±0.10 <sup>d</sup>	16.3±0.44 <sup>a</sup>	<LOQ
	Total	3089±72 <sup>c</sup>	1065±12 <sup>c</sup>	6834±98 <sup>b</sup>	2263±29 <sup>d</sup>	20812±228 <sup>a</sup>	707±18.4 <sup>f</sup>

Data are expressed as mean ± standard deviation ( $n=3$ ). Values in each row having the same letter are not significantly different ( $p>0.05$ ). Results are expressed as oleanolic acid equivalents. LOQ – limit of quantification. The assigned saponin numbers correspond to those listed in Table 1.

Among the remaining compounds, a higher content of saponin **20** was found in selected *B. vulgaris* samples. The cultivar with the highest content of this saponin was Boldor (peel) (1735 mg/kg fw). Saponin **20** was previously determined in a high content in beetroot of an Egyptian cultivar (1052 mg/kg dw) [Mroczek *et al.*, 2019].

Saponins **4**, **12**, **17**, **24** were found in *B. vulgaris* cultivars in low amounts (0.02–137.56 mg/kg fw). Moreover, the content of saponins **12**, **17**, **24** in Snow Ball cultivar (flesh) was below the limit of quantification (LOQ). Interestingly, a previous report [Mroczek *et al.*, 2019] indicates that compounds **4** (200–3933 mg/kg dw) and **17** (170–1123 mg/kg dw) were present mainly in leaves of *B. vulgaris* cultivars [Mroczek *et al.*, 2019], whereas compound **24** was detected in a low content (46.9–482 mg/kg dw) in leaves and roots.

According to Mroczek *et al.* [2019], saponin **1** mainly occurred in leaves of *B. vulgaris* cultivars (37.6–780 mg/kg dw). Here, a low content of saponin **1** (0.021–97.3 mg/kg fw) was found in the peel and flesh of *B. vulgaris* cultivars, which may confirm a high content of this compound in the leaves.

The content of saponins **5**, **6** and **18** in the peel (96.1–415 mg/kg fw) and saponin **3** in the flesh (318 mg/kg) of yellow *B. vulgaris* was higher than in white (0.83–29.3 mg/kg fw) and red (0.21–150 mg/kg fw) cultivars. Therefore, yellow *B. vulgaris* seems to be a rich source of these compounds.

The content of saponins **7**, **10** and **21** in *B. vulgaris* cultivars was low. The highest content of saponin **7**, **10** and **21** was determined in red cultivars: Forono (saponin **7** and **10**) and Ceryl (saponin **21**). Saponins **7** (563–2954 mg/kg dw) and **21** (172–623 mg/kg dw) seem



TABLE 3. Content of individual saponins and total saponins in fresh flesh of *Beta vulgaris* L. cultivars (mg/kg fresh weight) analyzed by LC-DAD-ESI-MS/MS.

No.	Saponin	Red cultivars				Yellow cultivar	White cultivar
		Ceryl	Chrobry	Forono	Tytus	Boldor	Snow Ball
1	Act-Hex-Hex-UrA-oleanolic acid	6.8±0.28 <sup>c</sup>	33.8±0.50 <sup>a</sup>	4.9±0.16 <sup>d</sup>	24.4±0.50 <sup>b</sup>	2.3±0.09 <sup>e</sup>	0.52±0.02 <sup>f</sup>
2	Act-Hex-UrA-akebonoic acid	5.9±0.24 <sup>c</sup>	31.4±0.90 <sup>b</sup>	0.23±0.011 <sup>f</sup>	42.8±0.83 <sup>a</sup>	5.7±0.13 <sup>d</sup>	0.92±0.04 <sup>c</sup>
3	Act-Hex-Hex-UrA-oleanolic acid	35.6±0.55 <sup>e</sup>	99.2±2.2 <sup>b</sup>	58.8±1.4 <sup>d</sup>	93.1±1.0 <sup>c</sup>	318±9.0 <sup>a</sup>	19.5±0.39 <sup>f</sup>
4	Hex-Pen-Hex-UrA-oleanolic acid	3.6±0.16 <sup>c</sup>	4.5±0.22 <sup>b</sup>	3.6±0.20 <sup>c</sup>	1.5±0.06 <sup>e</sup>	21.3±0.44 <sup>a</sup>	2.9±0.14 <sup>d</sup>
5	Act-Hex-Pen-UrA-oleanolic acid	28.8±0.53 <sup>e</sup>	91.0±4.4 <sup>b</sup>	45.3±1.4 <sup>d</sup>	67.7±1.5 <sup>c</sup>	234±3.1 <sup>a</sup>	6.1±0.27 <sup>f</sup>
6	Act-Hex-Hex-UrA-oleanolic acid	9.8±0.16 <sup>c</sup>	63.1±2.7 <sup>a</sup>	0.29±0.016 <sup>f</sup>	50.2±1.4 <sup>b</sup>	2.2±0.08 <sup>d</sup>	0.83±0.04 <sup>e</sup>
7	Hex-Pen-UrA-oleanolic acid	15.4±0.28 <sup>d</sup>	0.14±0.008 <sup>e</sup>	50.4±2.4 <sup>a</sup>	0.041±0.005 <sup>f</sup>	0.21±0.01 <sup>c</sup>	32.2±0.64 <sup>b</sup>
8	Diox-Hex-UrA-oleanolic acid	166±5.1 <sup>d</sup>	784±22 <sup>b</sup>	52.8±2.6 <sup>f</sup>	279±4.0 <sup>c</sup>	2603±43 <sup>a</sup>	133±2.6 <sup>e</sup>
9	Act-Hex-UrA-oleanolic acid	799±10 <sup>c</sup>	1905±35 <sup>a</sup>	2.7±0.14 <sup>f</sup>	1120±22 <sup>b</sup>	36.3±0.42 <sup>e</sup>	164±2.9 <sup>d</sup>
10	Act-dHex-UrA-oleanolic acid	0.18±0.009 <sup>f</sup>	6.2±0.20 <sup>d</sup>	11.6±0.20 <sup>c</sup>	23.0±0.30 <sup>a</sup>	0.55±0.03 <sup>e</sup>	18.1±0.58 <sup>b</sup>
11	Hex-Pen-UrA-akebonoic acid	1.2±0.070 <sup>d</sup>	5.4±0.20 <sup>c</sup>	6.0±0.16 <sup>b</sup>	14.5±0.40 <sup>a</sup>	0.61±0.03 <sup>e</sup>	0.031±0.005 <sup>f</sup>
12	Act-dHex-UrA-oleanolic acid	2.9±0.14 <sup>c</sup>	7.5±0.30 <sup>a</sup>	0.47±0.03 <sup>e</sup>	3.0±0.16 <sup>b</sup>	1.9±0.08 <sup>d</sup>	<LOQ
13	Pen-UrA-hederagenin	2.7±0.12 <sup>c</sup>	0.24±0.014 <sup>e</sup>	4.1±0.12 <sup>b</sup>	4.2±0.14 <sup>a</sup>	0.43±0.02 <sup>d</sup>	<LOQ
14	C <sub>5</sub> H <sub>4</sub> O <sub>5</sub> -Hex-UrA-akebonoic acid	0.032±0.004 <sup>d</sup>	0.053±0.007 <sup>c</sup>	0.035±0.004 <sup>d</sup>	0.071±0.009 <sup>b</sup>	0.083±0.009 <sup>a</sup>	<LOQ
15	Act-UrA-hederagenin	120±1.4 <sup>c</sup>	202±5.6 <sup>b</sup>	28.6±0.60 <sup>d</sup>	234±4.2 <sup>a</sup>	2.8±0.09 <sup>f</sup>	12.5±0.26 <sup>e</sup>
16	Hex-UrA-gypsogenin	0.36±0.020 <sup>f</sup>	5.9±0.20 <sup>c</sup>	0.37±0.02 <sup>f</sup>	10.3±0.20 <sup>b</sup>	1.8±0.06 <sup>e</sup>	3.1±0.10 <sup>d</sup>
17	Hex-Pen-UrA-oleanolic acid	4.1±0.20 <sup>d</sup>	15.4±0.34 <sup>b</sup>	2.5±0.12 <sup>c</sup>	8.3±0.20 <sup>c</sup>	19.1±0.32 <sup>a</sup>	<LOQ
18	Act-Hex-UrA-oleanolic acid	0.21±0.011 <sup>f</sup>	141±5.6 <sup>a</sup>	45.7±1.3 <sup>b</sup>	0.42±0.02 <sup>c</sup>	2.9±0.08 <sup>c</sup>	2.4±0.12 <sup>d</sup>
19	Act-UrA-akebonoic acid	38.0±1.4 <sup>d</sup>	193±4.5 <sup>c</sup>	80.3±1.7 <sup>d</sup>	309±4.2 <sup>a</sup>	1.1±0.06 <sup>e</sup>	0.11±0.007 <sup>f</sup>
20	C <sub>4</sub> H <sub>4</sub> O <sub>5</sub> -Hex-UrA-oleanolic acid	0.52±0.030 <sup>e</sup>	245±5.0 <sup>c</sup>	54.1±2.0 <sup>b</sup>	322±4.2 <sup>b</sup>	873±16 <sup>a</sup>	0.59±0.04 <sup>c</sup>
21	Pen-UrA-oleanolic acid	105±2.8 <sup>a</sup>	0.17±0.009 <sup>f</sup>	41.7±1.0 <sup>b</sup>	0.49±0.03 <sup>c</sup>	24.4±0.34 <sup>c</sup>	16.4±0.98 <sup>d</sup>
22	Act-UrA-oleanolic acid	1260±12 <sup>c</sup>	1998±55 <sup>b</sup>	423±4.5 <sup>c</sup>	2090±22 <sup>a</sup>	532±9.1 <sup>d</sup>	47.7±1.4 <sup>f</sup>
23	Diox-UrA-oleanolic acid	889±22 <sup>c</sup>	1032±18 <sup>b</sup>	4.5±0.15 <sup>f</sup>	1403±24 <sup>a</sup>	558±8.1 <sup>d</sup>	36.1±0.72 <sup>e</sup>
24	UrA-oleanolic acid	2.3±0.12 <sup>a</sup>	0.12±0.008 <sup>e</sup>	0.032±0.005 <sup>d</sup>	0.39±0.02 <sup>c</sup>	0.45±0.03 <sup>b</sup>	<LOQ
	Total	3497±52 <sup>d</sup>	6864±82 <sup>a</sup>	922±14 <sup>c</sup>	6101±85 <sup>b</sup>	5242±63 <sup>c</sup>	497±11 <sup>f</sup>

Data are expressed as mean ± standard deviation ( $n=3$ ). Values in each row having the same letter are not significantly different ( $p>0.05$ ). Results are expressed as oleanolic acid equivalents. LOQ – limit of quantification. The assigned saponin numbers correspond to those listed in Table 1.

to be present mainly in the leaves of *B. vulgaris* cultivars; therefore their content in beet roots may be at a quite low level [Mroczek et al., 2019].

#### Saponins with akebonoic acid as the aglycone

Saponin **19** was the main akebonoic acid derivative determined in the peel and flesh of *B. vulgaris* cultivars (0.11–881 mg/kg fw) (Table 2, Table 3). Its content was the highest in the peel of Forono cultivar, whereas the lowest in the flesh of Snow Ball cultivar. The contents of the remaining saponins **2**, **11** and **14** with akebonoic acid were much lower. The content of saponin **14** was very low in all studied samples (0.032–0.12 mg/kg fw), especially in the peel and flesh of Snow Ball cultivar, where the determined value was below the limit of quantification. In turn, higher contents of saponins

**2** and **11** were determined in the peel of Forono (101 mg/kg fw) and Boldor (105 mg/kg fw) cultivars.

#### Saponins with hederagenin and gypsogenin as the aglycones

Three saponins, which consisted of hederagenin (saponins **13** and **15**) or gypsogenin (saponin **16**) as an aglycone, were identified in the studied cultivars. Among these compounds, saponin **15** was dominant (Table 2, Table 3) and its highest content was determined in the peel of the yellow cultivar Boldor (2283 mg/kg fw). Interestingly, its content in flesh of yellow cultivar was merely 2.8 mg/kg fw. The peels of the studied cultivars turned out to be the richest sources of saponin **16**, the highest content of which was detected in the peels of yellow cultivar Boldor (peel) (856 mg/kg fw). The highest content of saponin **13** was determined in Forono

(peel) (150 mg/kg fw) but still it was lower than contents of saponins **15** and **16**.

## CONCLUSIONS

In summary, a simple procedure for saponin quantification with oleanolic acid, hederagenin, akebonoic acid and gypso-genin glycosides was developed. Herein, 24 saponins were detected in six *B. vulgaris* cultivars, of which saponins of the white and yellow cultivars were identified for the first time.

Our results report that the qualitative profile of saponins was identical for the peel and flesh of red, white and yellow cultivars, while the quantitative profile depended on the cultivar and part of the root. Among the identified compounds, betavulgaroside I, II, III and IV were dominant in the studied cultivars.

*B. vulgaris* seems to be a good source of these compounds for future research because their contents in peels and flesh were high, which is uncommon. The highest content of saponins was found in the peels of the yellow *B. vulgaris* cultivar (Boldor). In contrast, the lowest content was determined in the flesh of the white cultivar (Snow Ball).

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## CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

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## REFERENCES

- Bárta, J., Bártová, V., Šindelková, T., Jarošová, M., Linhartová, Z., Mráz, J., Bedrníček, J., Smetana, P., Samková, E., Laknerová, I. (2020). Effect of boiling on colour, contents of betalains and total phenolics and on antioxidant activity of colourful powder derived from six different beetroot (*Beta vulgaris* L.). *Polish Journal of Food and Nutrition Sciences*, 70(4), 377–385. <https://doi.org/10.31883/pjfn/128613>
- Chhikara, N., Kushwaha, K., Sharma, P., Gat, Y., Panghal, A. (2019). Bioactive compounds of beetroot and utilization in food processing industry: a critical review. *Food Chemistry*, 272, 192–200. <https://doi.org/10.1016/j.foodchem.2018.08.022>
- Clifford, T., Howatson, G., West, D.J., Stevenson, E.J. (2015). The potential benefits of red beetroot supplementation in health and disease. *Nutrients*, 7(4), 2801–2822. <https://doi.org/10.3390/nu7042801>
- De Tommasi, N., Conti, C., Stein, M.L., Pizza, C. (1991). Structure and *in vitro* antiviral activity of triterpenoid saponins from *Calendula arvensis*. *Planta Medica*, 57(3), 250–253. <https://doi.org/10.1055/s-2006-960084>
- Dirir, A.M., Daou, M., Yousef, A.F., Yousef, L.F. (2021). A review of alpha-glucosidase inhibitors from plants as potential candidates for the treatment of type-2 diabetes. *Phytochemistry Reviews*, 2021, 1–31. <https://doi.org/10.1007/s11101-021-09773-1>
- dos Santos Baião, D., da Silva, D.V.T., Paschoalin, V.M.F. (2021). A narrative review on dietary strategies to provide nitric oxide as a non-drug cardiovascular disease therapy: beetroot formulations – A smart nutritional interaction. *Foods*, 10(4), art. no. 859. <https://doi.org/10.3390/foods10040859>
- Dubois, M.-A., Benze, S., Wagner, H. (1990). New biologically active triterpene-saponins from *Randia dumetorum*. *Planta Medica*, 56(5), 451–455. <https://doi.org/10.1055/s-2006-961009>
- Edelmann, M., Dawid, C., Ralla, T., Stark, T. D., Salminen, H., Weiss, J., Hofmann, T. (2020). Fast and sensitive LC-MS/MS method for the quantitation of saponins in various sugar beet materials. *Journal of Agricultural and Food Chemistry*, 68(50), 15027–15035. <https://doi.org/10.1021/acs.jafc.0c05836>
- El Hazzam, K., Hafsa, J., Sobeh, M., Mhada, M., Taourirte, M., El Kacimi, K., Yasri, A. (2020). An insight into saponins from *Quinoa* (*Chenopodium quinoa* Willd): a review. *Molecules*, 25(5), art. no. 1059. <https://doi.org/10.3390/molecules25051059>
- Fang, Z., Li, J., Yang, R., Fang, L., Zhang, Y. (2020). A review: the triterpenoid saponins and biological activities of *Lonicera* Linn. *Molecules*, 25(17), art. no. 3773. <https://doi.org/10.3390/molecules25173773>
- Francis, G., Kerem, Z., Makkar, H.P.S., Becker, K. (2002). The biological action of saponins in animal systems: a review. *British Journal of Nutrition*, 88(6), 587–605. <https://doi.org/10.1079/BJN2002725>
- Güçlü-Üstündağ, Ö., Mazza, G. (2007). Saponins: properties, applications and processing. *Critical Reviews in Food Science and Nutrition*, 47(3), 231–258. <https://doi.org/10.1080/10408390600698197>
- Hikino, H., Kiso, Y., Kinouchi, J., Sanada, S., Shoji, J. (1985). Antihepatotoxic actions of ginsenosides from *Panax ginseng* roots. *Planta Medica*, 51(1), 62–64. <https://doi.org/10.1055/s-2007-969395>
- Ida, Y., Satoh, Y., Katoh, M., Katsumata (nee Ohtsuka), M., Nagasao, M., Yamaguchi, K., Kamei, H., Shoji, J. (1994). Achyranthosides A and B, novel cytotoxic saponins from *Achyranthes fauriei* root. *Tetrahedron Letters*, 35(37), 6887–6890. [https://doi.org/10.1016/0040-4039\(94\)85032-1](https://doi.org/10.1016/0040-4039(94)85032-1)
- Ismaili, H., Tortora, S., Sosa, S., Fkih-Tetouani, S., Ildirissi, A., Della Loggia, R., Tubaro, A., Aquino, R. (2001). Topical anti-inflammatory activity of *Thymus willdenowii*. *Journal of Pharmacy and Pharmacology*, 53(12), 1645–1652. <https://doi.org/10.1211/0022357011778250>
- Kavalcová, P., Bystrická, J., Tomáš, J., Karovičová, J., Kovarovič, J., Lenková, M. (2015). The content of total polyphenols and antioxidant activity in red beetroot. *Potravinárstvo – Slovak Journal of Food Sciences*, 9(1), 77–83. <https://doi.org/10.5219/441>

17. Kokanova-Nedialkova, Z., Nedialkov, P., Kondeva-Burdina, M. (2020). Ultra-high-performance liquid chromatography – high-resolution mass spectrometry profiling and hepatoprotective activity of purified saponin and flavonoid fractions from the aerial parts of wild spinach (*Chenopodium bonus-henricus* L.). *Zeitschrift Für Naturforschung C*, 76(7–8), 261–271.  
<https://doi.org/10.1515/znc-2020-0144>
18. Konishi, M., Hano, Y., Takayama, M., Nomura, T., Hamzah, A.S., Ahmad, R.B., Jasmani, H. (1998). Triterpenoid saponins from *Hedyotis nudicaulis*. *Phytochemistry*, 48(3), 525–528.  
[https://doi.org/10.1016/S0031-9422\(98\)00038-7](https://doi.org/10.1016/S0031-9422(98)00038-7)
19. Kuljanabhadgavad, T., Wink, M. (2009). Biological activities and chemistry of saponins from *Chenopodium quinoa* Willd. *Phytochemistry Reviews*, 8(2), 473–490.  
<https://doi.org/10.1007/s11101-009-9121-0>
20. Kuroda, M., Mimaki, Y., Sashida, Y., Kitahara, M., Yamazaki, M., Yui, S. (2001). Securiosides A and B, novel acylated triterpene bisdesmosides with selective cytotoxic activity against M-CSF-stimulated macrophages. *Bioorganic & Medicinal Chemistry Letters*, 11(3), 371–374.  
[https://doi.org/10.1016/S0960-894X\(00\)00674-0](https://doi.org/10.1016/S0960-894X(00)00674-0)
21. Kuwada, K., Kawase, S., Nakata, K., Shinya, N., Narukawa, Y., Fuchino, H., Kawahara, N., Kiuchi, F. (2020). LC–MS analysis of saponins of *Achyranthes* root in the Japanese market. *Journal of Natural Medicines*, 74(1), 135–141.  
<https://doi.org/10.1007/s11418-019-01355-y>
22. Lemmich, E., Cornett, C., Furu, P., Jørstian, C.L., Knudsen, A.D., Olsen, C.E., Salih, A., Thiilborg, S.T. (1995). Molluscicidal saponins from *Catunaregam nilotica*. *Phytochemistry*, 39(1), 63–68.  
[https://doi.org/10.1016/0031-9422\(94\)00866-R](https://doi.org/10.1016/0031-9422(94)00866-R)
23. Lidder, S., Webb, A. (2013). Vascular effects of dietary nitrate (as found in green leafy vegetables and beetroot) via the nitrate–nitrite–nitric oxide pathway. *British Journal of Clinical Pharmacology*, 75(3SI), 677–696.  
<https://doi.org/10.1111/j.1365-2125.2012.04420.x>
24. Mbaveng, A.T., Ndontsa, B.L., Kuete, V., Nguékeu, Y.M.M., Çelik, İ., Mbouangouere, R., Tane, P., Efferth, T. (2018). A naturally occurring triterpene saponin ardisiacrispin B displayed cytotoxic effects in multi-factorial drug resistant cancer cells via ferroptotic and apoptotic cell. *Phytomedicine*, 43, 78–85.  
<https://doi.org/10.1016/j.phymed.2018.03.035>
25. Mikołajczyk-Bator, K., Błaszczak, A., Czyżniejewski, M., Kachlicki, P. (2016). Characterisation and identification of triterpene saponins in the roots of red beets (*Beta vulgaris* L.) using two HPLC–MS systems. *Food Chemistry*, 192, 979–990.  
<https://doi.org/10.1016/j.foodchem.2015.07.111>
26. 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(50), 12397–12402.  
<https://doi.org/10.1021/jf303952x>
27. Mroczek, A., Kapusta, I., 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>
28. Mroczek, A., Klimczak, U., Kowalczyk, M. (2021). Determination of saponins in leaves of four swiss chard (*Beta vulgaris* L.) cultivars by UHPLC–CAD/QTOF–MS/MS. *Polish Journal of Food and Nutrition Sciences*, 71(2), 147–159.  
<https://doi.org/10.31883/pjfn/134623>
29. Muhammad, M.T., Khan, M.N. (2018). Eco-friendly, biodegradable natural surfactant (*Acacia Concinna*): an alternative to the synthetic surfactants. *Journal of Cleaner Production*, 188, 678–685.  
<https://doi.org/10.1016/j.jclepro.2018.04.016>
30. Parus, A. (2013). Właściwości farmakologiczne saponin. *Postępy Fitoterapii*, 3, 200–20204 (in Polish).
31. Pietrzkowski, Z., Nemzer, B., Spórna, A., Stalica, P., Tresher, W., Keller, R., Jimenez, R., Michałowski, T., Wybraniec, S. (2010). Influence of betalain-rich extract on reduction of discomfort associated with osteoarthritis. *New Medicine*, 1, 12–17.
32. Rai, S., Acharya-Siwakoti, E., Kafle, A., Devkota, H. P., Bhattarai, A. (2021). Plant-derived saponins: a review of their surfactant properties and applications. *Sci*, 3(4), art. no. 44.  
<https://doi.org/10.3390/sci3040044>
33. Schmitt, C., Grassl, B., Lespes, G., Desbrières, J., Pellerin, V., Reynaud, S., Gigault, J., Hackley, V.A. (2014). Saponins: A renewable and biodegradable surfactant from its microwave-assisted extraction to the synthesis of monodisperse lattices. *Biomacromolecules*, 15(3), 856–862.  
<https://doi.org/10.1021/bm401708m>
34. Sparg, S.G., Light, M.E., Van Staden, J. (2004). Biological activities and distribution of plant saponins. *Journal of Ethnopharmacology*, 94(2–3), 219–243.  
<https://doi.org/10.1016/j.jep.2004.05.016>
35. Spórna-Kucab, A., Kumorkiewicz, A., Szymur, N., Szneler, E., Wybraniec, S. (2019). Separation of betacyanins from flowers of *Amaranthus cruentus* L. in a polar solvent system by high-speed counter-current chromatography. *Journal of Separation Science*, 42(9), 1676–1685.  
<https://doi.org/10.1002/jssc.201801172>
36. Spórna-Kucab, A., Wróbel, N., Kumorkiewicz-Jamro, A., Wybraniec, S. (2020). Separation of betacyanins from *Iresine herbstii* Hook. ex Lindl. leaves by high-speed countercurrent chromatography in a polar solvent system. *Journal of Chromatography A*, 1626, art. no. 461370.  
<https://doi.org/10.1016/j.chroma.2020.461370>
37. Spórna-Kucab, A., Wybraniec, S. (2020). High-speed counter-current 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/pjfn/116416>
38. Wybraniec, S., Stalica, P., Spórna, A., Mizrahi, Y. (2010). Profiles of betacyanins in epidermal layers of grafted and light-stressed cacti studied by LC–DAD–ESI–MS/MS. *Journal of Agricultural and Food Chemistry*, 58(9), 5347–5354.  
<https://doi.org/10.1021/jf100065w>
39. Wybraniec, S., Stalica, P., Spórna, A., Nemzer, B., Pietrzkowski, Z., Michałowski, T. (2011). Antioxidant activity of betanidin: electrochemical study in aqueous media. *Journal of Agricultural and Food Chemistry*, 59(22), 12163–12170.  
<https://doi.org/10.1021/jf2024769>
40. Xu, J., Wang, S., Feng, T., Chen, Y., Yang, G. (2018). Hypoglycemic and hypolipidemic effects of total saponins from *Stauntonia chinensis* in diabetic db/db mice. *Journal of Cellular and Molecular Medicine*, 22(12), 6026–6038.  
<https://doi.org/10.1111/jcmm.13876>

41. Yoshikawa, M., Matsuda, H., Harada, E., Murakami, T., Wariishi, N., Yamahara, J., Murakami, N. (1994). Elatoside E, a new hypoglycemic principle from the root cortex of *Aralia elata* Seem.: Structure-related hypoglycemic activity of oleanolic acid glycosides. *Chemical and Pharmaceutical Bulletin*, 42(6), 1354–1356. <https://doi.org/10.1248/cpb.42.1354>
42. Yoshikawa, M., Murakami, T., Kadoya, M., Matsuda, H., Muraoka, O., Yamahara, J., Murakami, N. (1996). Medicinal 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(6), 1212–1217. <https://doi.org/10.1248/cpb.44.1212>
43. Yoshikawa, M., Murakami, T., Kadoya, M., Yamahara, J., Matsuda, H. (1998). Medicinal 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(11), 1758–1763. <https://doi.org/10.1248/cpb.46.1758>