Polish Journal of Food and Nutrition Sciences

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 1, pp. 67–74
DOI: 10.31883/pjfns/116416
http://journal.pan.olsztyn.pl

Original article
Red Beetroot Thematic Issue

High-Speed Counter-Current Chromatography in Separation and Identification of Saponins from *Beta vulgaris* L. Cultivar Red Sphere

Aneta Spórna-Kucab, Sławomir Wybraniec*

Department of Analytical Chemistry, Institute C-1, Faculty of Chemical Engineering and Technology, Cracow University of Technology, ul. Warszawska 24, 31–155 Cracow, Poland

Key words: saponins, *Beta vulgaris* L., high-speed counter-current chromatography

Saponins, natural plant compounds exhibiting health benefits, were extracted from *B. vulgaris* L. cultivar (cv.) Red Sphere and separated by high-speed counter-current chromatography (HSCCC) in a new solvent system composed of: TBME-BuOH-ACN-H_2O (1:2:1:5, *v/v/v/v*). The system was used in the head-to-tail mode. The flow rate of the mobile phase was 3 mL/min and the column rotation speed was 860 rpm. The retention of the stationary phase was 47%. The LC-MS/MS analyses were performed for the identification of separated saponins in the crude extract as well as HSCCC fractions,.

Significantly different elution orders of the analytes were observed in the HSCCC and HPLC systems, indicating the complementarity of both the techniques in the fractionation of saponins. Moreover, during the HSCCC separation, acetal-type saponins were eluted faster than pentose/hexose-type saponins and then dioxolane-type saponins. The separation of saponins in the HSCCC solvent system enabled the fractionation and preconcentration of 13 saponins for further fragmentation experiments in the LC-MS/MS system. Nine saponins were detected for the first time ever in *B. vulgaris* L. cv. Red Sphere.

INTRODUCTION

The widely described health benefits of red beet root (*Beta vulgaris* L.) have led to increased scientific and consumer interest in this vegetable [Chhikara *et al*., 2019; Clifford *et al*., 2015; Nemzer *et al*., 2011]. Red beet is rich in several bioactive compounds which can be useful in the treatment of many diseases, such as hypertension, atherosclerosis, type 2 diabetes, and dementia [Clifford *et al*., 2015; Nemzer *et al*., 2011].

Beet root is a rich source of highly bioactive saponins which are associated with haemolytic [Voutquenne *et al*., 2003]; molluscicidal [Huang *et al*., 2003]; anti-inflammatory [da Silva *et al*., 2002]; antifungal, anti-yeast activity, and antibacterial [de Lucca *et al*., 2002]; as well as with antiparasitic activity [Traore *et al*., 2000]. They exhibit also cytotoxic, antitumoric [González *et al*., 2003], and antiviral effects [Gosse *et al*., 2002].

Saponins are divided into two groups: steroidal saponins and triterpenoid saponins. Some authors distinguish a third group called steroidal amines or steroidal alkaloids [Bruneton, 1995]. Triterpenoid saponins are a common group of saponins consisting of a triterpenoid aglycone containing 30 carbon atoms and comprising a pentacyclic structure [Sparg *et al*., 2004]. Triterpene saponins are glycosides containing one or two sugar chains connected *via* an ester to C-28 or ether to C-3. According to the literature, hexoses, pentoses, 6-deoxyhexoses, and uronic acids are primary sugar units in triterpene saponins. Moreover, the uronic acid moiety is bonded only to C-3 [Mikołajczyk-Bator *et al*., 2016; Yoshikawa *et al*., 1996, 1998]. So far, oleanolic as well as hederagenin, akebonoic, and gypsogenin aglycones were identified in red beet roots [Mikołajczyk-Bator *et al*., 2016].

The profile of triterpenoid saponins in the roots of red beet cv. Red Sphere (*Beta vulgaris* L.) was previously studied using reversed liquid chromatography and mass spectrometry by Mroczek *et al.* [2012, 2019] and 13 saponins have been described wherein 11 compounds contained oleanolic acids aglycone. Another research on saponins in red beet root cv. Nochowski was that by Mikołajczyk-Bator *et al.* [2016], wherein 44 saponins were characterized, including 22 compounds that were described for the first time ever. Moreover, 27 saponins, which contained oleanolic acids aglycone, were identified using the LC-MS/MS/MS technique.

The structural characterisation of triterpenoid saponins in the roots of red beet is very difficult and time-consuming due to the complexity of the matrix [Mikołajczyk-Bator *et al*., 2016; Mroczek *et al*., 2012, 2019], therefore, fractionation of the crude samples enables faster and more accurate identification of significantly preconcentrated saponins [Thakur *et al*., 2014]. High-speed counter-current chromatography is a very important technique in the separation of natural plant compounds [Jerz *et al*., 2008, 2010; Spórna-Kucab

* Corresponding Author: E-mail: anetasporna@chemia.pk.edu.pl (A. Spórna-Kucab)

© Copyright by Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences
© 2020 Author(s). This is an open access article licensed under the Creative Commons Attribution-NonCommercial-NoDerivs License (http://creativecommons.org/licenses/by-nc-nd/3.0/).

et al., 2019; Wybraniec *et al*., 2009, 2010] which enables subsequent identification of new structures of target compounds [Choi *et al*., 2015; Figueiredo *et al*., 2017]. Moreover, HSCCC is perceived as a rapid and convenient technique for the isolation of saponins [Thakur *et al*., 2014]. Additionally, it is worth noting that during HSCCC separations, no loss of target compounds arising from their irreversible adsorption onto the solid matrix is observed, often taking place in the conventional liquid chromatography, because mobile and stationary phases are liquids [Spórna-Kucab *et al*., 2013a, 2015; 2016; 2018a,b].

In this study, a crude saponin mixture from *Beta vulgaris* L. cv. Red Sphere was separated for the first time by semi-preparative HSCCC in a new solvent system. The HSCCC technique enabled the identification of new saponins in *Beta vulgaris* L. cv. Red Sphere.

MATERIALS AND METHODS

Plant material and reagents

Red beet roots (*Beta vulgaris* L.) of cv. Red Sphere were purchased from a local market in Cracow in June 2019. The roots were grounded and forthwith extracted.

For the HSCCC experiments and for analytes extraction, HPLC-grade butanol (BuOH), *tert*-butylmethylether (TBME), acetonitrile (ACN), and ethanol (EtOH) were obtained from Avantor Performance Materials Poland S.A. (Gliwice, Poland). LC-MS grade acetonitrile and formic acid (purity ≥98%) were obtained from Sigma-Aldrich (St. Louis, United States).

Crude pigment extracts

Fresh and grounded in a blender (thermomix, Vorwerk, Wuppertal, Germany) roots from *Beta vulgaris* L. cv. Red Sphere (1.5 kg) were extracted by maceration three times for 1 h, using 1 L of 80% ethanol each time. The extract obtained was filtered and pre-concentrated by a rotary evaporator (Heidolph, Schwabach, Germany) at 25°C to 100 mL under reduced pressure. Then, the extract was loaded into an RP-C_{18} cartridge pre-conditioned with ethanol and water. The cartridge was washed with water and then with 10%, 20%, 50%, and 100% ethanol. The eluates were pre-concentrated by a rotary evaporator at 25°C and freeze-dried. The eluates obtained were monitored by LC-DAD-ESI-MS/MS. Finally, 196.6 mg of saponins were eluted with 50% ethanol and 115.4 mg of saponins with 100% ethanol.

HSCCC separation

The separation of saponins (Table 1) was accomplished on a semi-preparative AECS QuikPrep HSCCC *J*-type hydrodynamic chromatograph (London, United Kingdom) with 121 mL capacity and 2.0 mm i.d..

A new biphasic system consisting of TBME-BuOH-ACN-H_2O (1:2:1:5, *v/v/v/v*) was prepared for the HSCCC run. The solvent system was prepared in a separating funnel. Required volumes of solvents were mixed in a separating funnel. Then, the two phases for the HSCCC separation were divided shortly before use and degassed by ultrasonication for 10 min. The upper organic phase was used as the stationary phase and the lower aqueous phase as the mobile phase in the 'head-to-tail' mode. Per definition, this mode of separation is named reversed-phase comparable to C_{18}-HPLC also using the aqueous phase as the eluting solvent phase [Spórna-Kucab *et al*., 2019; Wybraniec *et al*., 2010].

The HSCCC column was entirely filled with the upper phase (stationary phase). The rotation of the HSCCC instrument was started at 860 rpm. Then, the lower aqueous phase (mobile phase) was pumped at a flow rate of 3.0 mL/min (K-501 pump, Knauer, Berlin, Germany).

After column equilibration, the sample solution was injected by an injection valve. This solution was prepared by dissolving 300 mg of the extract in 4 mL of the lower phase. The effluent from the column was continuously monitored with a UV-Vis detector at 210 nm (Knauer). Twelve fractions were collected in a fraction collector (Foxy Jr., Knauer) at 2-min intervals and then analysed by LC-DAD-ESI-MS/MS (Figures 1–3). The HSCCC fractions were weighed after their pre-concentration on a rotary evaporator at 25°C and freeze-drying.

LC-DAD-ESI-MS/MS analysis

The eluates from the RP-C_{18} cartridge as well as a crude extract (Figure 1) and the HSCCC fractions (Figures 2 and 3) were analysed by LC-DAD-ESI-MS/MS using an LCMS-8030 mass spectrometric system coupled to an LC-20ADXR pump with a gradient elution mode at 40ºC in the acetonitrile (A) and 2% aqueous formic acid (B) system: 5% A in B at 0 min, a gradient to 60% A in B at 12 min, then 80% A in B at 15 min. The injection volume was 5 μL and the flow rate was 0.5 mL/min. LC-DAD-ESI-MS/MS analyses were conducted on a 100 mm × 4.6 mm I.D., 5.0 μm Kinetex C_{18} chromatographic column from Phenomenex (Torrance, United States).

The LC-MS/MS system was controlled with LabSolutions software (Shimadzu, Japan), which was operated in a negative mode, at electrospray voltage of 4.5 kV, capillary temp. of 250°C, and N_2 used as the sheath gas. Scan range was from *m/z* 100 to 2000. Argon was used to improve trapping efficiency and as the collision gas for CID experiments. The collision energy for MS analyses was set at 50 V.

RESULTS AND DISCUSSION

MS/MS analysis of saponins

The first study by Mroczek *et al.* [2012, 2019] on *B. vulgaris* L. cv. Red Sphere reported thirteen saponins with pentose and hexose substituents. Another research on saponins in *B. vulgaris* L. cv. Nochowski conducted by Mikołajczyk-Bator *et al.* [2016] revealed 27 acetal-, dioxolane- as well as pentose/hexose-type saponins.

In this study, the MS/MS analysis of the crude extract of *Beta vulgaris* L. (Figure 1) revealed the presence of thirteen acetal-, dioxolane- as well as pentose/hexose-type saponins. All of the identified saponins and their MS/MS data are listed in Table 1.

The saponins detected in our research are derivatives of oleanolic acid. The MS/MS data show the presence of five acetal- and two dioxolane-type substituents, which have been never described in *B. vulgaris* L. cv. Red Sphere. These types

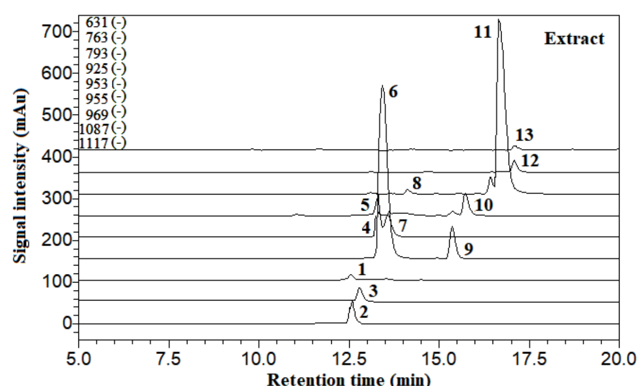

FIGURE 1. ESI-MS chromatogram of saponins from *B. vulgaris* L. cultivar Red Sphere extract.

of compounds were found by Mikołajczyk-Bator [2016] in *B. vulgaris* L. cv. Nochowski.

Fragmentation of saponins with oleanolic acid as the aglycone leads to yielding the daughter ion $[M-H]^-$ at *m/z* 631 which corresponds to hexuronic acid-oleanolic acid. The simplest saponin with the pseudomolecular ion $[M-H]^-$ at *m/z* 631 (peak **13** with t_R = 17.1 min) was identified in the studied *B. vulgaris* L. extract. As expected, this compound (**13,** HexUA-oleanolic acid) fragmented to *m/z* 455 which corresponded to oleanolic acid. HexUA-oleanolic acid was previously identified in the *B. vulgaris* L. cv. Red Sphere [Mroczek *et al*., 2012, 2019].

The peak **12** (t_R = 17.1 min) corresponded to the compound which exhibited the pseudomolecular ion $[M-H]^-$ at *m/z* 763. The fragmentation of this compound (**12)** yielded daughter ions at *m/z* 631 and 455 because of the losses of pentose (132 Da) and hexuronic acid (176 Da), and therefore compound **12** was identified as Pen-HexUA-oleanolic acid. Compound **12** was previously identified in the *B. vulgaris* L. cultivars Red Sphere and Nochowski [Mikołajczyk-Bator *et al*., 2016; Mroczek *et al*., 2012, 2019].

Peaks **8** (t_R = 14.1 min) and **11** (t_R = 16.7 min) corresponded to compounds with the identical pseudomolecular ions $[M-H]^-$ at *m/z* 793 and aglycone ions at *m/z* 455 which correspond to oleanolic acid. The fragmentation of the pseudomolecular ion $[M-H]^-$ at *m/z* 793 for compound **8** yielded daughter ions at *m/z* 631 and 455 because of the losses of 162 Da and 176 Da, confirming the presence of hexose and hexuronic acid. Finally, compound **8** was identified as Hex-HexUA-oleanolic acid. However, the fragmentation of compound **11** with the same pseudomolecular ion $[M-H]^-$ at *m/z* 793 resulted in the formation of daughter ions at *m/z* 673 and 631, indicating the loss of 120 Da and 162 Da characteristic for the acetal-type substituent; and this compound can be assigned to Act-HexUA-oleanolic acid. The research of saponins profile in the *B. vulgaris* L. cv. Red Sphere did not reveal the presence of compound **11** [Mroczek *et al*., 2012, 2019] but this compound was previously observed in the *B. vulgaris* L. cv. Nochowski [Mikołajczyk-Bator *et al*., 2016]. The structure of compound **11** was determined by Yoshikawa *et al*. [1996] who named this saponin betavulgaroside IV.

The identical pseudomolecular ions $[M-H]^-$ at *m/z* 925 were ascribed to peaks **5** (t_R = 13.3 min) and **10** (t_R = 15.8 min). These saponins consisted of the same aglycone ion at *m/z* 455, corresponding to oleanolic acid. Moreover, the daughter ions at *m/z* 631 and 793 (losses of pentose (132 Da) and hexose (162 Da), respectively) were observed for both saponins. The compounds **5** and **10** (Pen-Hex-HexUA-oleanolic acids) were previously identified in *B. vulgaris* L. cv. Red Sphere [Mroczek *et al*., 2012, 2019].

Peaks **4** (t_R = 13.3 min) and **7** (t_R = 13.6 min) corresponded to compounds which revealed pseudomolecular ion $[M-H]^-$

TABLE 1. Saponins tentatively identified by negative ion ESI-MS/MS in *B. vulgaris* L. cultivar Red Sphere.

No.	Saponin structure	t_R [min]	*m/z* $[M-H]^-$	*m/z* from MS/MS of $[M-H]^-$
1	HexUA-Hex-HexUA-oleanolic acid*	12.5	969	unknown
2	Act-Hex-Hex-HexUA-oleanolic acid	12.5	1117	997;955;835;793;631;455
3	Act-Hex-Pen-HexUA-oleanolic acid	12.8	1087	967;925;763;631;455
4	Diox-Hex-HexUA-oleanolic acid	13.3	953	909;793;631;455
5	Pen-Hex-HexUA-oleanolic acid	13.3	925	793;631;455
6	Act-Hex-HexUA-oleanolic acid	13.5	955	835;793;673;631;455
7	Diox-Hex-HexUA-oleanolic acid	13.6	953	909;793;631;455
8	Hex-HexUA-oleanolic acid	14.1	793	631;455
9	Act-Hex-HexUA-oleanolic acid	15.4	955	835;793;673;631;455
10	Pen-Hex-HexUA-oleanolic acid	15.8	925	793;631;455
11	Act-HexUA-oleanolic acid	16.7	793	673;631;455
12	Pen-HexUA-oleanolic acid	17.1	763	631;455
13	HexUA-oleanolic acid	17.1	631	455

*proposed structure Hex – hexose; Pen – pentose; HexUA – hexuronic acid; Act – acetal substituent; Diox – dioxolane substituent.

TABLE 2. Saponin distribution in the recovered HSCCC fractions obtained from *B. vulgaris* L. cultivar Red Sphere extract.

No.	*m/z* [M-H]$^-$	Relative content of pigments (%)*												Total HPLC peak area (×10^{-5})
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	
1	969								60.7	26.4	12.9			0.9
2	1117				80.3	19.7								37.8
3	1087			86.7	13.3									33.8
4	953						71.7	28.3						30.1
5	925							30.0	41.0	29.0				20.2
6	955			0.8	78.0	21.2								294.8
7	953						54.6	45.4						36.6
8	793										14.3	71.4	14.3	7.0
9	955	10.0	63.3	26.7										53.1
10	925				59.4	24.2	16.4							44.4
11	793	8.0	68.0	11.0	13.0									104.6
12	763		12.0	13.2	18.0	48.1	8.7							19.4
13	631					12.6	12.9	74.5						3.5
Fraction mass (mg)		30.3	94.2	55.2	31.2	30.9	6.0	6.6	3.0	1.5	0.9	0.9	0.6	

*analysed by LC-MS.

at *m/z* 953 and fragment ions at *m/z* 909, 793, 631, and 455. These compounds were characterised for the first time in *B. vulgaris* L. cv. Red Sphere. The fragmentation of the [M-H]$^-$ primarily yielded daughter ions at *m/z* 909 and 793 because of the loss of CO_2 and a dioxolane-type substituent, respectively. The MS/MS data exhibited additionally daughter ions at *m/z* 631 and 455, being characteristic for hexuronic acid-oleanolic acid and oleanolic acid. These two compounds were identified as Diox-Hex-HexUA-oleanolic acids. It is worth noting that saponins with dioxolane-type substituents were detected in *B. vulgaris* L. cv. Red Sphere for the first time ever, but this type of the structure was determined by Yoshikawa *et al*. [1996] who named it betavulgaroside I. The compounds **6** and **9** with t_R 13.5 and 15.4 min, respectively, exhibited identical [M-H]$^-$ ions at *m/z* 955, which corresponded to acetal-type substituent saponins because of the loss of 120 Da (daughter ion at *m/z* 835) and 162 Da (daughter ion at *m/z* 793) during fragmentation. The daughter ion at *m/z* 835 was not noticed during the fragmentation of compounds **6** and **9** identified by Mroczek *et al*. [2019] in *B. vulgaris* L. cv. Red Sphere. These acetal-saponins (compounds **6** and **9**) were also identified by Mikołajczyk-Bator *et al*. [2016] in *B. vulgaris* L. cv. Nochowski. Based on the MS/MS fragmentation of compound **6** and **9** as well as previous MS/MS data [Mikołajczyk-Bator *et al*., 2016], it can be deduced that these molecules consisted of aglycone ion at *m/z* 455 – oleanolic acid as well as acetal-type substituent, hexose and hexuronic acid. Taking into account the above elucidations, compounds **6** and **9** were designated as Act-Hex-HexUA-oleanolic acid.

Peak **1** (t_R = 12.5 min) corresponding to the compound revealing the pseudomolecular ion [M-H]$^-$ at *m/z* 969 was tentatively identified as hexuronic acid-hexose-hexuronic acid derivative of oleanolic acid (HexUA-Hex-HexUA-oleanolic acids). The concentration of compound **1** was not sufficient to identify daughter ions. Mikołajczyk-Bator *et al.* [2016] identified presumably identical saponin in *B. vulgaris* L. cv. Nochowski. The elution of compound **1** in further HSCCC fractions indicates that it is not an acetal-type saponin, because such saponins are eluted in early fractions.

The compound **3** (t_R = 12.8 min) was characterised by the pseudomolecular ion [M-H]$^-$ at *m/z* 1087 and fragment ions at *m/z* 967, 925, 763, 631, and 455. The fragmentation of compound **3** resulted in the formation of primary daughter ions at *m/z* 967 and 925 because of the loss of 120 Da and 162 Da (cleavage and loss of the acetyl-type substituent, respectively). The presence of the daughter ions at *m/z* 763 and 631 indicated the loss of the hexose and pen-

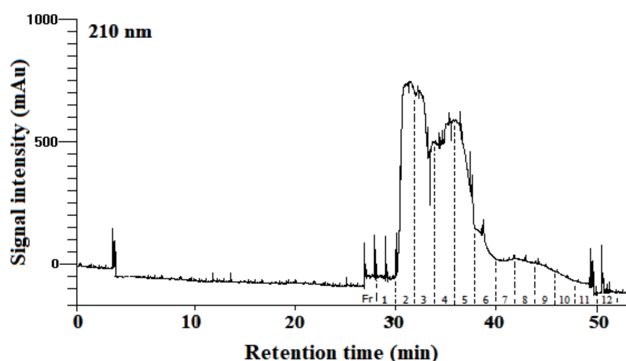

FIGURE 2. HSCCC chromatogram of *B. vulgaris* L. cultivar Red Sphere extract (300 mg) separated into 12 fractions in a solvent system: TBME-BuOH-ACN-H2O (1:2:1:5, *v/v/v/v*) at a flow rate of 3.0 mL/min in the head-to-tail mode; velocity 860 rpm; optical detection at a wavelength of 210 nm.

tose from the structure of compound **3**. Finally, this compound was identified as Act-Hex-Pen-HexUA-oleanolic acid. Compound **3**, named as betavulgaroside IX, was identified for the first time by Yoshikawa *et al*. [1996]. Betavulgaroside IX was only identified in Nochowski cv. by Mikołajczyk-Bator *et al*. [2016].

The MS spectra of the compound t_R = 12.5 min the pseudomolecular ion [M-H]$^-$ at *m/z* 1117 which fragmented into ions at *m/z* 997, 955, 835, 793, 631, and 455, indicated the presence of saponin with acetal-type substituent. The fragmentation of this compound (**2**) resulted in the formation of primary daughter ions at *m/z* 997 and 955 (losses of 120 and 162 Da) corresponding to the cleavage and loss of the acetal-type substituent, respectively. The presence of the daughter ions at *m/z* 793 and 631 indicated the loss of the two hexoses from the structure of compound **2**. The daughter ions at *m/z* 631 and 455 confirmed the presence of hexuronic acid--oleanolic acid and oleanolic acid, respectively. Taking into account the above elucidations, compound **2** was identified as Act-Hex-Hex-HexUA-oleanolic acid. The compound **2** has been never identified in *B. vulgaris* L. cv. Red Sphere but was identified in Nochowski cv. by Mikołajczyk-Bator *et al*. [2016]. Moreover, compound **2** was thoroughly described using NMR by Yoshikawa *et al*. [1996] who named it betavulgaroside V.

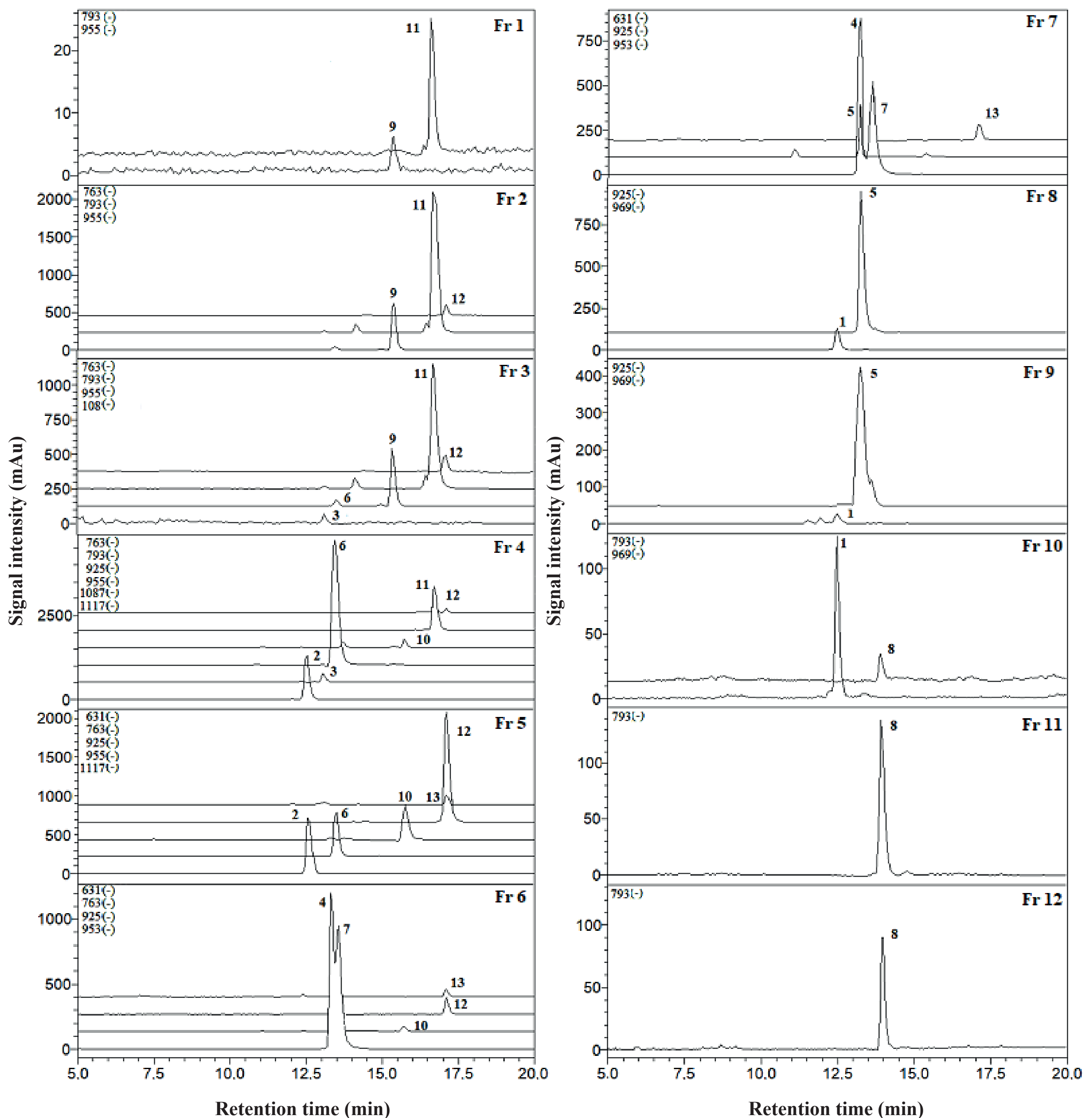

FIGURE 3. ESI-MS chromatograms of saponins analysed in the fractions (1–12) separated from the extract of *B. vulgaris* L. cultivar Red Sphere by HSCCC.

HSCCC separation of saponins

The crude extract (300 mg) was fractionated by the HSCCC technique and 12 fractions were collected and analysed by LC-DAD-ESI-MS/MS analysis (Table 2, Figures 1–3).

The HSCCC separation monitored at 210 nm (Figure 2) was realized in a new solvent system TBME-BuOH-ACN-H_2O (1:2:1:5, *v/v/v/v*) in a head-to-tail mode which corresponds to the reversed-phase mode in HPLC. Saponins were previously separated by HSCCC technique in a solvent system TBME-BuOH-ACN-H_2O (1:3:1:5, *v/v/v/v*) showing retention of the stationary phase amounting to 35% [Thakur *et al*., 2014], therefore, a new solvent system with higher retention was prepared. After the separation in the new solvent system, the retaining amount of stationary phase in the coil-system was calculated to be 47%. Elution order and separation effectiveness of saponins in the HSCCC and the C_{18} reversed--phase HPLC were compared in order to demonstrate the complementarity of these techniques.

The most polar HexUA-Hex-HexUA-oleanolic acid (**1**) with the pseudomolecular ion [M-H]- at m/z 969 was eluted in fractions 8–10 and it was completely separated from Act-Hex--Hex-HexUA-oleanolic acid (**2)** with the pseudomolecular ion at *m/z* 1117. Separation of compounds **1** and **2** was not possible in the HPLC system applied due to their identical retention times, therefore, HSCCC allowed for their complete separation.

Likewise, compounds Act-Hex-Hex-HexUA-oleanolic acid (**2**) and Act-Hex-Pen-HexUA-oleanolic acid (**3**) differed by their elution profiles in the HPLC and HSCCC technique. Compound **2** ($[M-H]^-$ at *m/z* 1117) and compound **3** ($[M-H]^-$ at *m/z* 1087) differed by one sugar moiety. Compound **2** with hexose in the structure as eluted later in the HSCCC than compound **3** with pentose. Different elution profiles of compounds **2** and **3** in the HPLC and the HSCCC mad their complete separation feasible.

Similar structures for Diox-Hex-HexUA-oleanolic acid (**4**) ([M-H]- at m/z 953) and Pen-Hex-HexUA-oleanolic acid (**5**) ($[M-H]^-$ at *m/z* 925) presumably contributed to their coelution in the HPLC system. Compound **4** consisted of dioxolane-type substituent, whereas compound **5** consisted of a pentose. The elution order of compounds **4** and **5** was identical in the HPLC and the HSCCC but the HSCCC technique enabled their quite effective separation.

A complete separation of Pen-Hex-HexUA-oleanolic acid (**5**) ($[M-H]^-$ at *m/z* 925) and Act-Hex-HexUA-oleanolic acid (**6**) ($[M-H]^-$ at *m/z* 955) was observed in spite of their similar chromatographic properties indicated in the C_{18}-HPLC system. The difference in the structure consists in the presence of the acetal-type substituent in compound **6** instead of pentose in compound **5**. The presence of the acetal-type substituent presumably contributed to the faster elution of compound **6** in comparison to compound **5** in the HSCCC system. Similarly, the separation of the compounds **5** and **6** by HSCCC and HPLC also proves the complementarity of the two separation systems.

The principal compound present in the extract, Act-Hex--HexUA-oleanolic acid (**6**) ($[M-H]^-$ at *m/z* 955), differed from Diox-Hex-HexUA-oleanolic acid (**4**) and Diox-Hex-HexUA-oleanolic acid (**7**) ($[M-H]^-$ at *m/z* 953) by the presence of acetal-type substituent in compound **6** instead of dioxolane-type substituent in compound **4** and **7**. The differences in the structures translated into their properties during separation by the HSCCC technique. Acetal-type saponin (Act-Hex-HexUA-oleanolic acid, compound **6**) was eluted faster than Diox-Hex-HexUA-oleanolic acid (compounds **4** and **7**).

Separation of Hex-HexUA-oleanolic acid (**8**) ($[M-H]^-$ at *m/z* 793) which was present mainly in the fractions 11 and 12 was very effective. This saponin only partially coeluted with HexUA-Hex-HexUA-oleanolic acid in fraction 10 in the HSCCC.

Act-Hex-HexUA-oleanolic acid (**9**) ($[M-H]^-$ at *m/z* 955) and Pen-Hex-HexUA-oleanolic acid (**10**) ($[M-H]^-$ at *m/z* 925), similarly to compounds **5** and **6**, were completely separated by the HSCCC according to differences in their structures (acetal-type substituent and pentose, respectively). Similarities can also be seen in the elution order.

Furthermore, comparison of the elution order of Act-HexUA-oleanolic acid (**11**) ($[M-H]^-$ at *m/z* 793) and Pen-HexUA-oleanolic acid (**12**) ($[M-H]^-$ at *m/z* 763) confirmed that the acetal-type saponins are eluted faster in the HSCCC system than the pentose-type saponins. Because compounds **11** and **12** are closely related, their complete separation by HSCCC was impossible. The separation of these saponins could be feasible in highly polar solvent systems with salts [Spórna-Kucab *et al*., 2013a].

HexUA-oleanolic acid (**13**) ($[M-H]^-$ at *m/z* 631), eluted as the last saponin in the HPLC, was not completely separated by the HSCCC technique but its different elution profiles in the HPLC and the HSCCC systems afford the possibility for its recovery using both techniques.

CONCLUSION

In this study, separation of saponins from *B. vulgaris* L., cultivar Red Sphere, has been realized for the first time using high-speed counter-current chromatography in a new solvent system consisting of *tert*-butyl-methyl ether-butanol-acetonitrile-water. The previously described [Thakur *et al*., 2014] solvent system for the separation of saponins by the HSCCC had much lower retention of the stationary phase, therefore, the new solvent system had been prepared. Separation and concentration of the compounds during the HSCCC process enabled tentative identification of 13 saponins by MS/MS technique. Nine saponins were detected for the first time in *B. vulgaris* L. cv. Red Sphere. Additionally, saponin with the pseudomolecular ion $[M-H]^-$ at *m/z* 969 has been tentatively identified for the first time and its possible structure has been proposed.

Analysis of the saponin elution order showed some tendencies. Acetal-type saponins were eluted faster than pentose/hexose-type saponins as well as dioxolane-type saponins. Moreover, the saponins differed in the elution order in the HPLC and HSCCC systems, therefore, their elution in HSCCC is rather dependent on the steric conditions than on polarity. The combination of the HPLC and HSCCC results in complementary elution orders and makes them a very versatile tool for the isolation of saponins which may open up the possibility of utilizing these compounds commercially.

RESEARCH FUNDING

This research was financed by the Polish National Science Centre for years 2019–2020 (Project No. 2019/03/X/ST4/00968).

CONFLICT OF INTEREST

Authors declare no conflict of interest.

REFERENCES

1. Bruneton, J. (1995). *Pharmacognosy. Phytochemistry, Medicinal Plants*. Lavoisier Publishing, Paris, France, pp. 538–544.
2. 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.
3. Choi, S.J., Choi, J., Jeon, H., Bae, S., Ko, J., Kim, J., Yoon, K.D. (2015). Application of high-performance countercurrent chromatography for the isolation of steroidal saponins from *Liriope plathyphylla*. *Journal of Separation Science, 38*(1), 18–24.
4. Clifford, T., Howatson, G., West, D., Stevenson, J. (2015). Review: The potential benefits of red beetroot supplementation in health and disease. *Nutrients, 7*(4), 2801–2822.
5. da Silva, B.P., De Sousa, A.C., Silva, G.M., Mendes, T.P., Parente, J.P. (2002). A new bioactive steroidal saponin from *Agave attenuata*. *Zeitschrift fur Naturforschung C – Journal of Biosciences, 57*(5–6), 423–428.
6. de Lucca, A., Bland, J.M., Vigo, C.B., Cushion, M., Selitrennikoff, C.P., Peter, J., Walsh, T.J. (2002). CAY-1, a fungicidal saponin from *Capsicum sp*. fruit. *Medical Mycology, 40*(2), 131–137.
7. Figueiredo, F., Celano, R., Silva, D., Costa, F., Hewitson, P., Ignatova, S., Piccinelli, A., Leitão, S., Leitão, G. (2017). Countercurrent chromatography separation of saponins by skeleton type from *Ampelozizyphus amazonicus* for off-line ultra-high-performance liquid chromatography/high resolution accurate mass spectrometry analysis and characterisation. *Journal of Chromatography A, 1481*, 92–100.
8. González, A.G., Hernández, J.C., León, F., Padrón, J.I., Estévez, F., Quintana, J., Bermejo, J. (2003). Steroidal saponins from the bark of *Dracaena draco* and their cytotoxic activities. *Journal of Natural Products*, *66*(6), 793–798.
9. Gosse, B., Gnabre, J., Bates, R.B., Dicus, C.W., Nakkiew, P., Huang, R.C.C. (2002). Antiviral saponins from *Tieghemella heckelii*. *Journal of Natural Products, 65*(12), 1942–1944.
10. Huang, H.C., Liao, S.C., Chang, F.R., Kuo, Y.H., Wu, Y.C. (2003). Molluscicidal saponins from *Sapindus mukorossi*, inhibitory agents of golden apple snails, *Pomacea canaliculata*. *Journal of Agricultural and Food Chemistry, 51*(17), 4916–4919.
11. Jerz, G., Wybraniec, S., Gebers, N., Winterhalter, P. (2010). Target-guided separation of *Bougainvillea glabra* betacyanins by direct coupling of preparative ion-pair high-speed countercurrent chromatography and electrospray ionization mass-spectrometry. *Journal of Chromatography A, 1217*(27), 4544–4554.
12. Jerz, G., Skotzki, T., Fiege, K., Winterhalter, P., Wybraniec, S. (2008). Separation of betalains from berries of *Phytolacca americana* by ion pair high-speed counter-current chromatography. *Journal of Chromatography A, 1190*(1–2), 63–73.
13. Mikołajczyk-Bator, K., Błaszczyk, 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.
14. 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.
15. 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.
16. Nemzer, B., Pietrzkowski, Z., Spórna, A., Stalica, P., Thresher, W., Michałowski, T., Wybraniec, S. (2011). Betalainic and nutritional profiles of pigment-enriched red beet root (*Beta vulgaris* L.) dried extracts. *Food Chemistry, 127*(1), 42–53.
17. Sparg, S.G., Light, M.E., Staden, J. (2004). Review: Biological activities and distribution of plant saponins. *Journal of Ethnopharmacology, 94*(2–3), 219–243.
18. Spórna-Kucab, A., Kumorkiewicz, A., Szmyr, 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.
19. Spórna-Kucab, A., Milo, A., Kumorkiewicz, A., Wybraniec, S. (2018a). Studies on polar high-speed counter-current chromatographic systems in separation of amaranthine-type betacyanins from *Celosia species*. *Journal of Chromatography B, 1073*, 96–103.
20. Spórna-Kucab, A., Bernaś, K., Grzegorczyk, A., Malm, A., Skalicka-Woźnika, K., Wybraniec, S., (2018b). Liquid chromatographic techniques in betacyanin isomers separation from *Gomphrena globosa* L. flowers for the determination of their antimicrobial activities. *Journal of Pharmaceutical and Biomedical Analysis, 161*, 83–93.
21. Spórna-Kucab, A., Hołda, E., Wybraniec, S. (2016). High-speed counter-current chromatography in separation of betacyanins from flowers of red *Gomphrena globosa* L. cultivars. *Journal of Chromatography B, 1033–1034*, 421–427.
22. Spórna-Kucab, A., Ignatova, S., Garrard, I., Wybraniec, S. (2013a). Versatile solvent systems for the separation of betalains from processed *Beta vulgaris* L. juice using counter-current chromatography. *Journal of Chromatography B, 941*, 54–61.
23. Spórna-Kucab, A., Garrard, I., Ignatova, S., Wybraniec, S. (2015). New solvent systems for gradient-counter-current chromatography in separation of betanin and its derivatives from processed *Beta vulgaris* L. juice. *Journal of Chromatography A, 1380*, 29–37.
24. Thakur, M., Jerz, G., Tuwalska, D., Gilabert-Oriol, R., Wybraniec, S., Winterhalter, P. (2014). High-speed countercurrent chromatographic recovery and off-line electrospray ionization mass spectrometry profiling of bisdesmodic saponins from *Saponaria officinalis* possessing synergistic toxicity enhancing properties on targeted antitumor toxins. *Journal of Chromatography B, 955–956*, 1–9.
25. Traore, F., Faure, R., Ollivier, E., Gasquet, M., Azas, N., Debrauwer, L., Keita, A., Timon-David, P., Balansard, G. (2000). Structure and antiprotozoal activity of triterpenoid saponins from *Glinus oppositifolius*. *Planta Medica, 66*(4), 368–371.

26. Voutquenne, L., Guinot, P., Thoison, O., Sevenet, T., Lavaud, C. (2003). Oleanolic glycosides from *Pometia ridleyi*. *Phytochemistry, 64*(3), 781–789.
27. Wybraniec, S., Jerz, G., Gebers, N., Winterhalter, P. (2010). Ion--pair high speed countercurrent chromatography fractionation of a high molecular weight variation of acyl-oligosaccharide linked betacyanins from purple bracts of *Bougainvillea glabra*. *Journal of Chromatography B, 878*(5–6), 538–550.
28. Wybraniec, S., Stalica, P., Jerz, G., Klose, B., Gebers, N., Winterhalter, P., Spórna, A., Szaleniec, M., Mizrahi, Y. (2009). Separation of polar betalain pigments from cacti fruits of *Hylocereus polyrhizus* by ion-pair high-speed countercurrent chromatography. *Journal of Chromatography A, 1216*(41), 6890–6899.
29. Yoshikawa, M., Murakami, T., Kadoya, M., Yamahara, J., Matsuda, H. (1998). Medical Foodstuffs. XV. Sugar Beet. (2): Structures of Betavulgarosides V, VI, VII, VIII, IX and X from the roots and leaves of sugar beet (*Beta vulgaris* L., Chenopodiaceae). *Chemical and Pharmaceutical Bulletin, 46*, 1758–1763.
30. Yoshikawa, M., Murakami, T., Kadoya, M., Yamahara, J., Matsuda, H. (1996). Medical Foodstuffs. III. Sugar Beet. (1): Hypoglycemic oleanolic acid oligoglycosides, Betavulgarosides I, II, III, and IV, from the root of *Beta vulgaris* L. (Chenopodiaceae). *Chemical and Pharmaceutical Bulletin, 44*, 1212–1217.

Submitted: 10 September 2019. Revised: 22 November and 9 December 2019. Accepted: 10 January 2020. Published on-line: 5 February 2020.