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Isolation and Characterization of Anthocyanins in Four Varieties of *Vigna subterranea* (*Fabaceae*)

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Abstract

By chromatographic methods (HPLC, TLC) coupled with spectral methods (LC-MS, TLC-MS) and chemical revelation tests, anthocyanins from four *Vigna subterranea* varieties (M4, D3, KVS350, KVS97) were isolated and identified as malvidin 3-O-β-D-glucopyranoside, paeonidin-3-O-β-D-glucopyranoside, petunidin-3-O-β-D-glucopyranoside, cyanidin 3-O-β-D-glucopyranoside, delpinidin-3-O-β-D-glucopyranoside.

Keywords

Fabaceae, *Vigna subterranea*, Malvidin Paeonidin, Petunidin, Cyanidin, Delphinidin

1. Introduction

Vigna subterranea called voandzou is a plant of the fabaceae family. This plant is native to West Africa and introduced in several sub-regions of Africa. The seeds are widely consumed in many regions [1]. It tolerates acidic and slightly poor soils quite well. Legumes of the genus Vigna are among the most consumed foods in Africa south of the Sahara [2]. Known by the common names "souma" in Moore (Burkina Faso) and "oule-ndâ" in Ngambaye (Chad), voandzou is the second most economically important legume after cowpea (*Vigna unguiculata* (L.)) in sub-Saharan countries. It is a plant that adapts well to harsh climatic conditions [3]. The seeds are very nutritious and chemical analyses have shown

that they contain 32.72% of total essential amino acids and 66.10% of total non-essential amino acids [4] [5]. Moreover, the seeds and leaves are used in the treatment of certain diseases such as abscesses and infected wounds [2]. The juice of the green leaves is applied to the eyes to treat epilepsy. The roots are used as an aphrodisiac and the crushed seeds mixed with water are used to treat cataracts by applying to the eyes [2]. In Nigeria, the plant is administered in the treatment of venereal diseases [2]. In Chad, voandzou flour is used in the treatment of blood pressure. For food and nutritional security in this context of climate change, seed legumes such as voandzou are very important. However, despite its many advantages, voandzou has remained an underutilized crop and one of the most neglected crops in scientific research [6].

Previous work conducted on the seed oil of *Vigna subterranea*, reveals the presence of the main fatty acids such as palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid and behenic acid [7]. In the seed coat are tannins and anthocyanin compounds [8].

The present work on the four varieties (M4, D3, KVS350, KVS97) of *V. subterranea* isolated five anthocyanin compounds and identified as malvidin 3-O-β-D-glucopyranoside, paeonidin-3-O-β-D-glucopyranoside, petunidin-3-O-β-D-glucopyranoside, cyanidin-3-O-β-D-glucopyranoside, delpinidin-3-O-β-D-glucopyranoside.

2. Materials and Methods

2.1. Plant Material

The study focuses on four (4) varieties of *V. subterranea* including two varieties (KVS350 and KVS 97) from Burkina Faso and two (D3 and M4) from Chad harvested in late October 2012. These different varieties are provided respectively by Institute of Environment and Agricultural Research (IEAR) of Ouagadougou in Burkina Faso and Chadian Institute of Agronomic Research for Development (CIARD) of Deli in Chad.

2.2. Extraction, Separation and Purification of Anthocyanins

The different varieties of *Vigna subteranea* seeds were cold macerated for 72 hours in 1% acidified methanol. After filtration, the different filtrates were concentrated.

After filtration and concentration, 100 mL of distilled water was added to each concentrated extract. The solutions of the obtained extracts were passed over amberlite (**Figure 1**). After deposition and fixation of the extract on the amberlite XAD-7 resin, the column is washed with 200 mL of 1% acidified water to remove the water soluble substances. Thus, anthocyanins and other phenolic compounds remain fixed on the resin and are recovered by elution with 1% acidified methanol (**Figure 2**). Note that amberlite XAD-7 is prepared in ethanol or methanol. The resulting mixture is poured into the column and allowed to stand. Before the aqueous extract is deposited, the column is washed with water to remove the alcohol [9].

(a) (b)

Figure 1. (a) Fixation of *V. subterranea* seed extract on amberlite XAD. (b) Elution of *V. subterranea* seed extract with acidified methanol.

Figure 2. Pre-purified extracts of four varieties of *V. subterranea* seeds.

2.3. Identification and Elucidation of Purified Compounds

The identification and elucidation of the purified compounds are performed by TLC, TLC-MS and HPLC-MS.

Thin layer chromatography (TLC)

- TLC in normal phase

It is done on the silica plate (silica gel 60 F254) with as mobile phase: ethyl acetate-formic acid-acetic acid-water (AEFAW: 100:11:26, vol).

- HPTLC in reverse phase

It is done on the high performance silica plate in reverse phase with the aceto-nitrile—water—TFA system (20:80:2, vol.) as mobile phase.

TLC-MS and HPLC-MS

❖ Chromatographic conditions

- TLC-MS: silica glass plate HPTLC reverse phase; mobile phase acetonitrile—water—TFA (20:80: 2, vol.).
- HPLC-MS: C18 column (150 mm, 4 mm, 3 μm) with the elution gradient given in Table 1.

❖ Mass detection conditions.

The mass detection conditions are listed in Table 2.

Table 1. HPLC analysis conditions.

Time (min)	% A ($HCOOH/H_2O$, 1: 60, mL)	% B ($HCOOH/H_2O/CH_3OH$, 0.5: 24.5: 75, mL)
0	70	30
20	40	60
30	40	60
35	70	30

Volume injected: 5 µL; flow rate: 0.5 mL/min.

Table 2. Mass spectra acquisition conditions.

Parameter Acquisition					
Source Type	ESI	Ion Polarity	Positive	Nebulizer Set	1.0 Bar
Focus	Not Active	Capillary Set	4500 V	Dry Radiator Set	190˚C
Scan Start	50 m/z	Set End Offset Plate	−500 V	Dry Gas Set	6.0 L/min
End of Scan	1000 m/z	RF Collision Cell Set	250.0 Vpp	Determined Set Value	Source

3. Results and Discussion

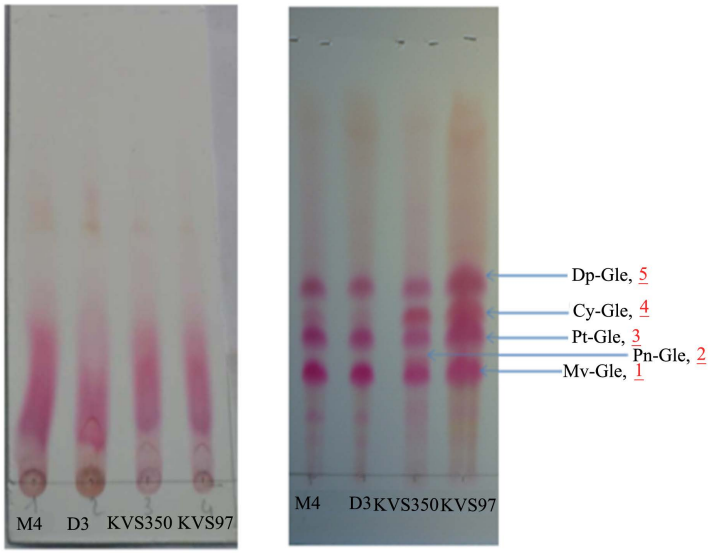
Qualitative analysis in HPTLC after TLC, of the crude extracts on silica in reverse phase using the acetonitrile-water-TFA system as mobile phase gave for each of the varieties M4, D3, KVS350, KVS97 five spots (**Figure 3(b)**). These spots correspond respectively to compounds **1**, **2**, **3**, **4** and **5** for each variety whose Rf are recorded in **Table 3**. Analysis of these spots shows that the four varieties are qualitatively identical. However, the Chad varieties (M4 and D3) appear identical and different from the varieties KVS350 and KVS97 from Burkina Faso, which are also identical to each other. Indeed, compounds **2** and **4** are less remarkable in varieties M4 and D3 (Chad).

The red color of the spots confirms the anthocyanin revelation tests. Indeed, red colorations in acidic medium, blue in basic medium indicate the presence of oxyanthocyanins: position 3 is substituted [8].

The HPLC profile of the four varieties is qualitatively identical: five peaks with retention times of 15.5 min, 14.5 min, 13 min, 12 min and 10.5 min, respectively (**Table 3**, **Figure 4**).

The relative intensities of these chromatograms indicate that these compounds are not present in the same proportions.

Quantitative analysis of these chromatograms shows that for the Chad varieties M4 and D3, we have the following intensities: $A_1 > A_3 > A_5 > A_4 > A_2$. This shows that the compounds are not present in the M4 and D3 extracts in the same proportions.



(a) (b)

Figure 3. Thin layer chromatograms (TLC) of *V. subterranea* extracts. (a) Normal phase TLC; (b) Reverse phase HPTLC.

Table 3. Spectral and chromatographic data of anthocyanins from *V. subterranea* seeds.

Compounds	Rf in CCM (×100)	HPLC	LC-MS	TLC-MS
	Acetonitrile—water—TFA	$t_{R\ (min)}$	m/z	m/z
1	24	15.5	$[M]^+$ 493; [M-162] 331	$[M]^+$ 493; [M-162] 331
2	28	14.5	$[M]^+$ 463; [M-162] 301	
3	33	13	$[M]^+$ 479; [M-162] 317	$[M]^+$ 479; [M-162] 317
4	38	12	$[M]^+$ 449; [M-162] 287	$[M]^+$ 449; [M-162] 287
5	43	10.5	$[M]^+$ 465; [M-162] 303	$[M]^+$ 465; [M-162] 303

The intensities of these chromatograms also show that the anthocyanic compounds of the extracts of varieties KVS350 and KVS97 are not in the same proportions. Thus, we have $A_1 > A_4 > A_3 > A_5 > A_2$.

Signals **2** and **4** of the extracts of varieties M4 and D3 are low while the same signals are relatively high for varieties KVS350 and KVS97 (Figure 4). This confirms the results observed in TLC.

For each variety, the mass spectra of the different signals yield five molecular ions with m/z: 493 (tR = 15.5 min), 463 (tR = 14.5 min), 479 (tR = 13 min), 449 (tR = 12 min), and 465 (tR = 10.5 min) (Table 3 and Figure 5 and Figure 6). These molecular ions correspond to the compounds with the crude formulas: $C_{22}H_{25}O_{12}$, $C_{21}H_{20}O_{12}$, $C_{22}H_{23}O_{12}$, $C_{21}H_{21}O_{11}$ and $C_{21}H_{21}O_{12}$. Furthermore, analysis of fragments at m/z: 331 for (tR = 15.5 min), m/z: 301 for (tR = 14.5 min), m/z:

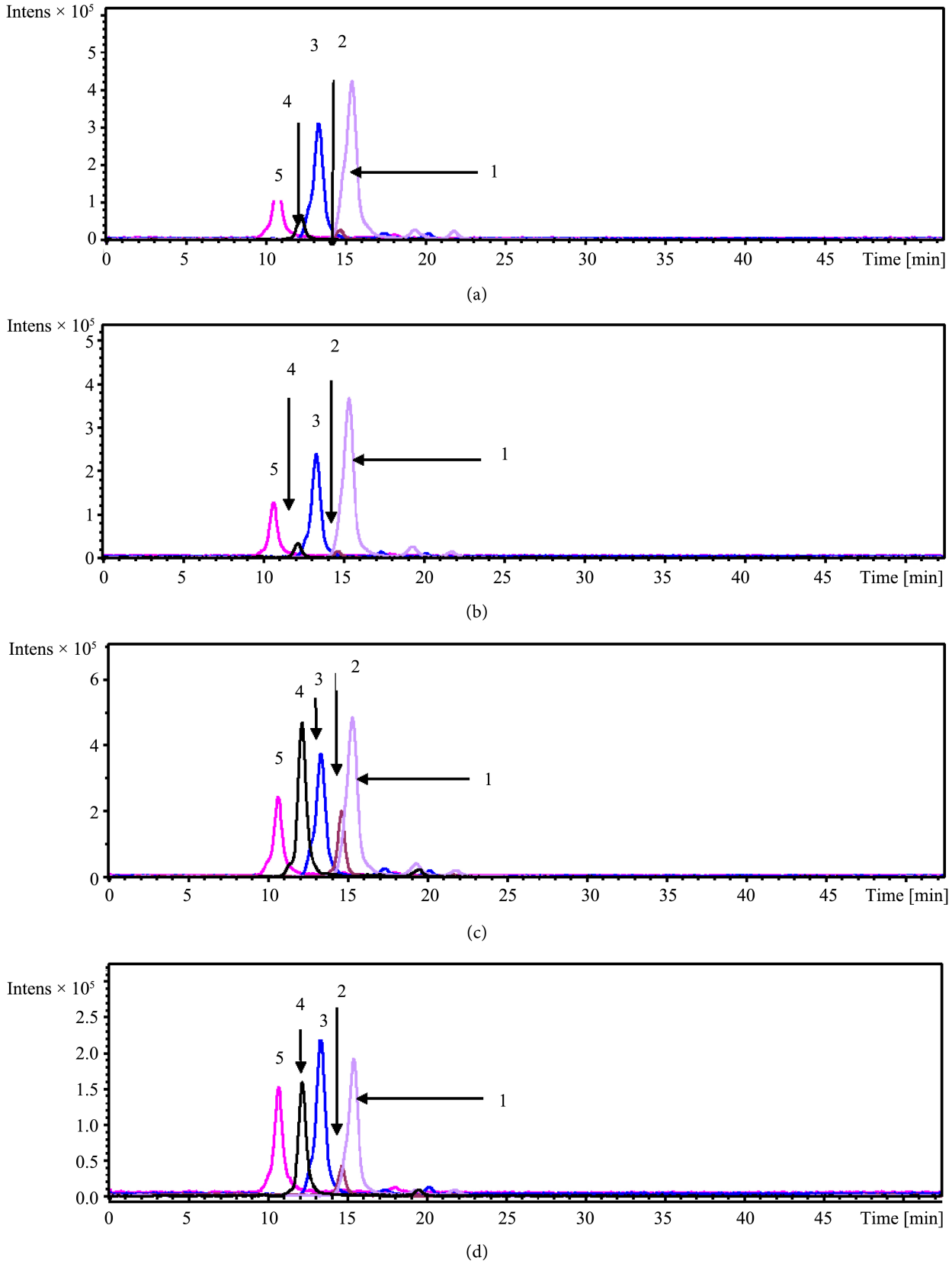
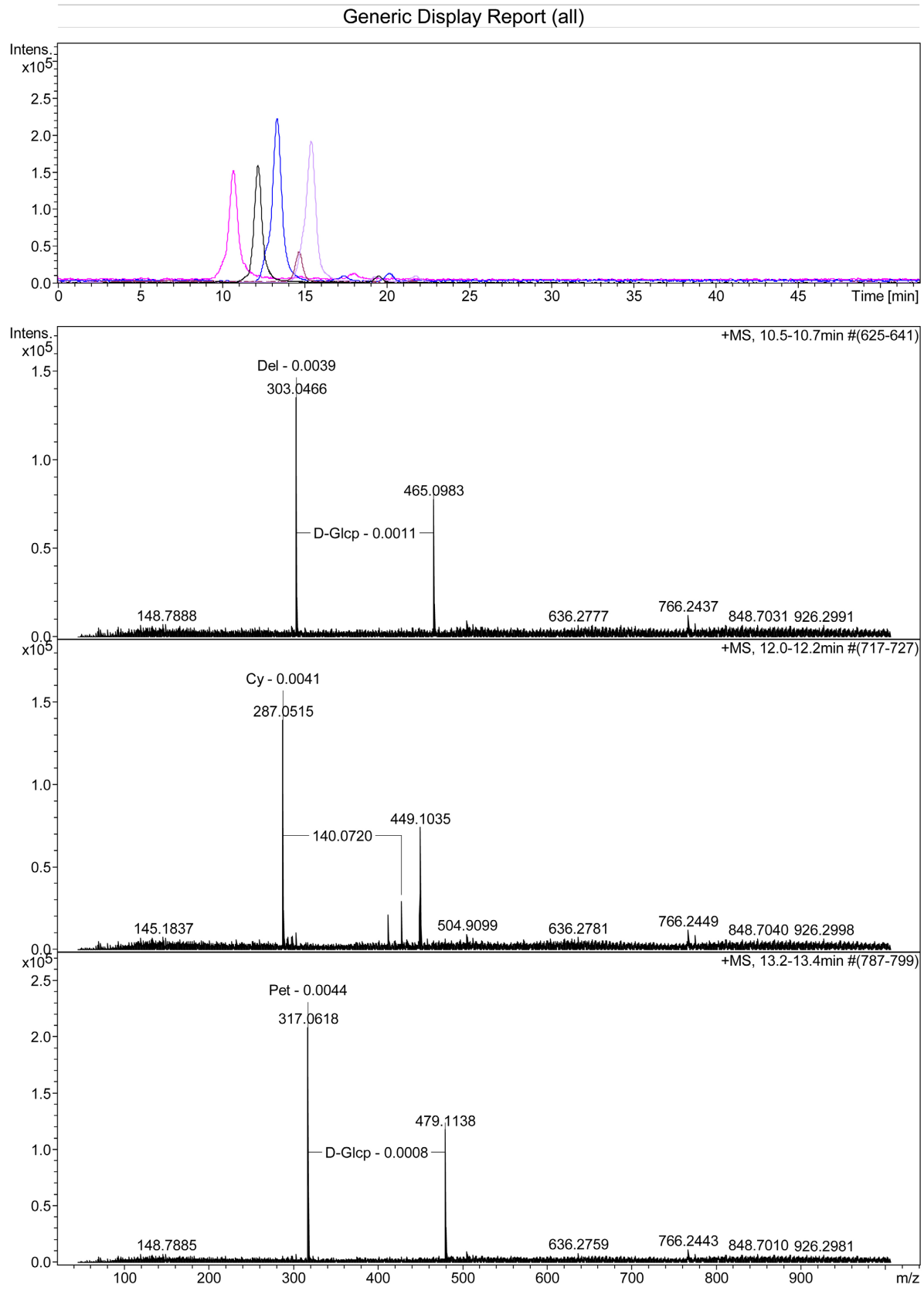


Figure 4. HPLC chromatograms of methanol extracts of four varieties of *V. subterranea*. (a): M4; (b): D3; (c): KVS350; (d): KVS97.



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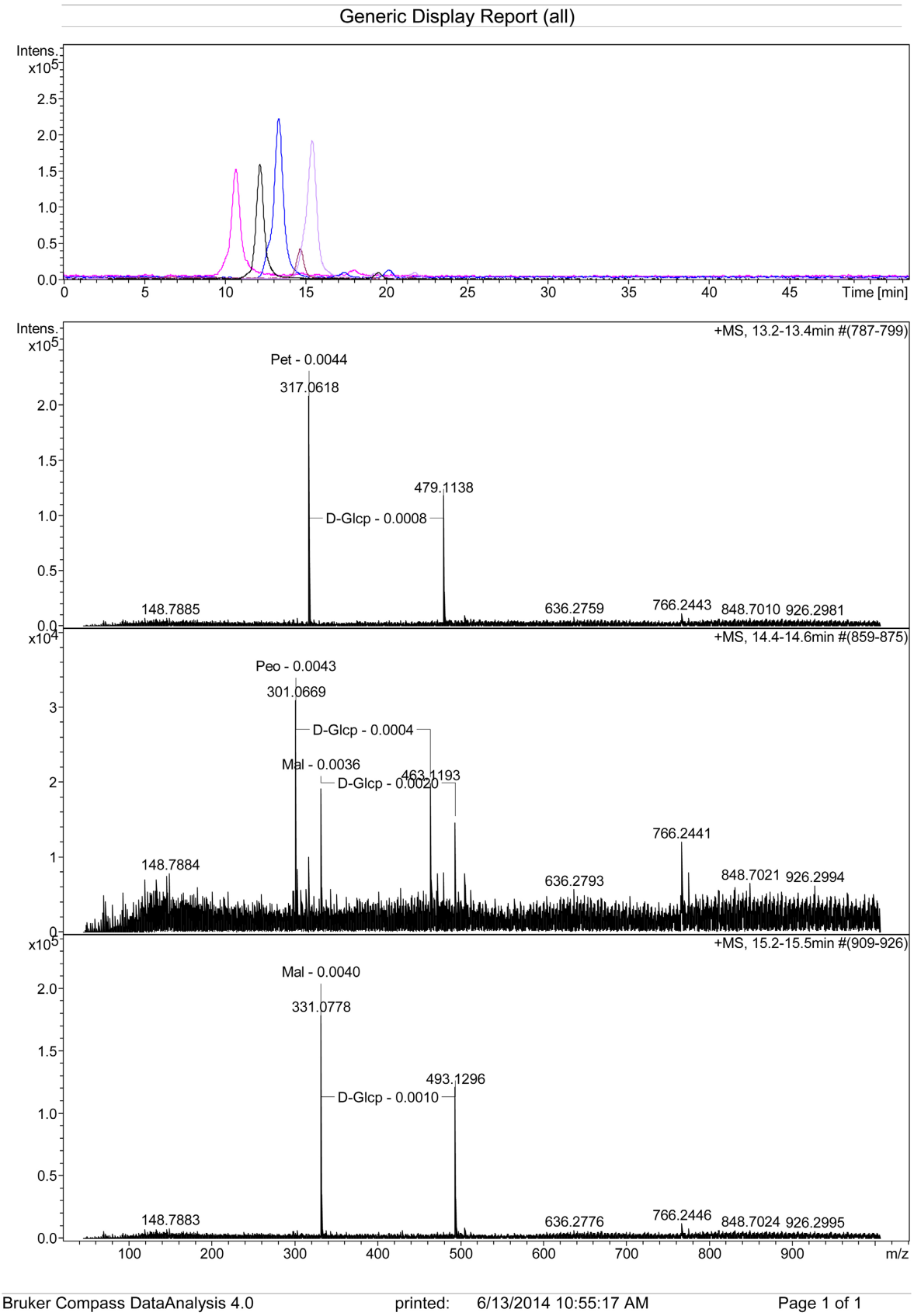
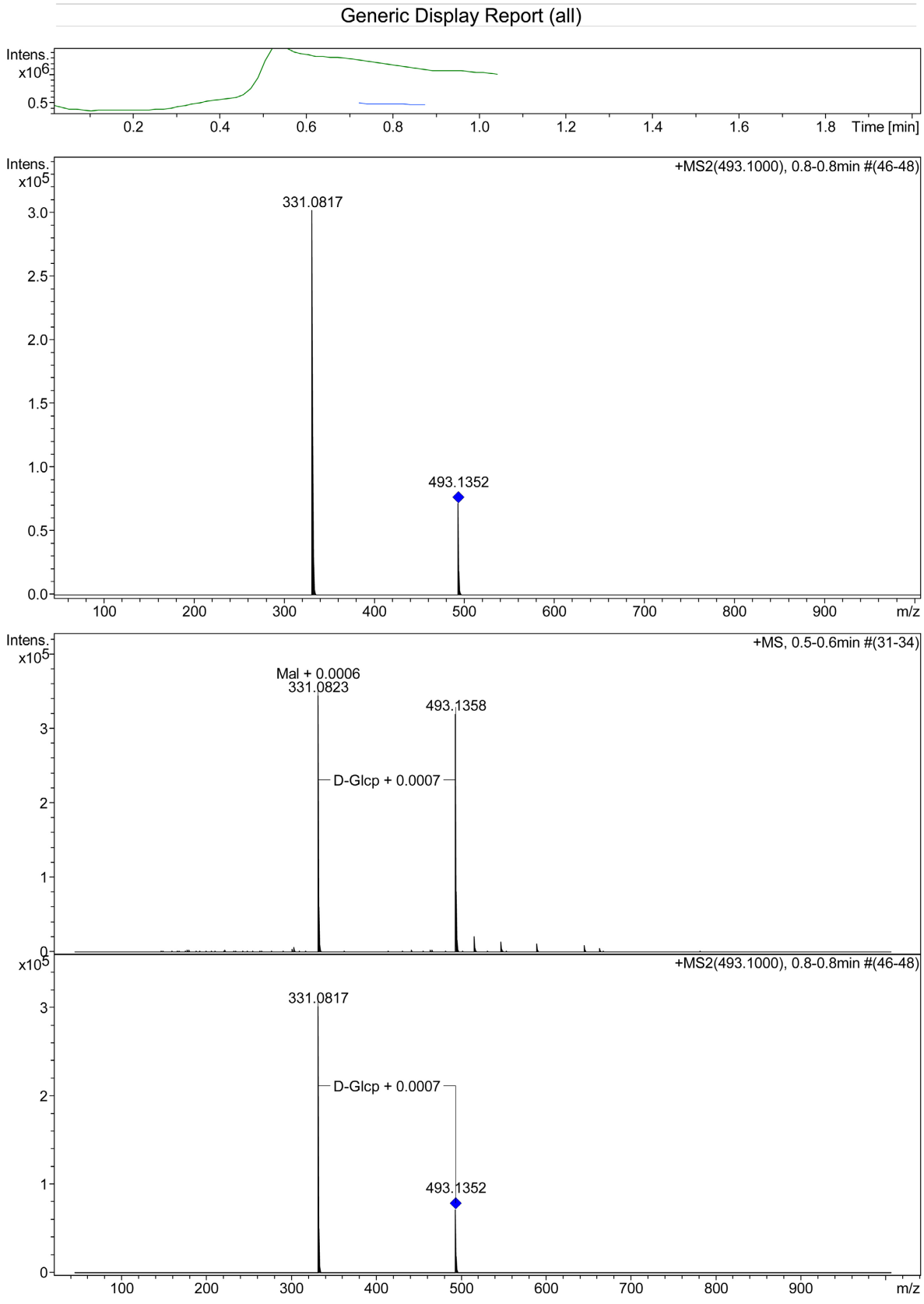


Figure 5. LC-MS spectra of M4 extract.



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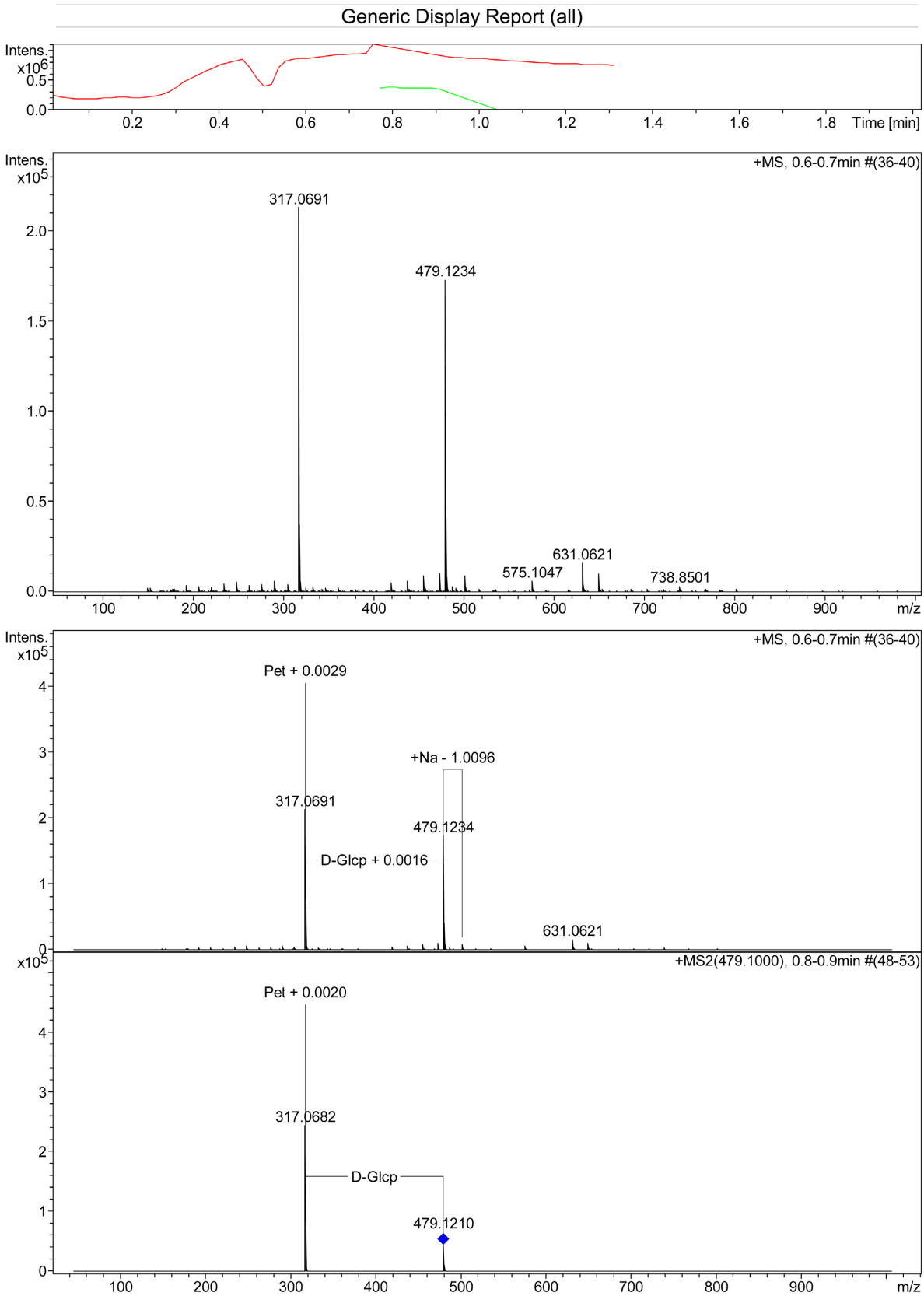

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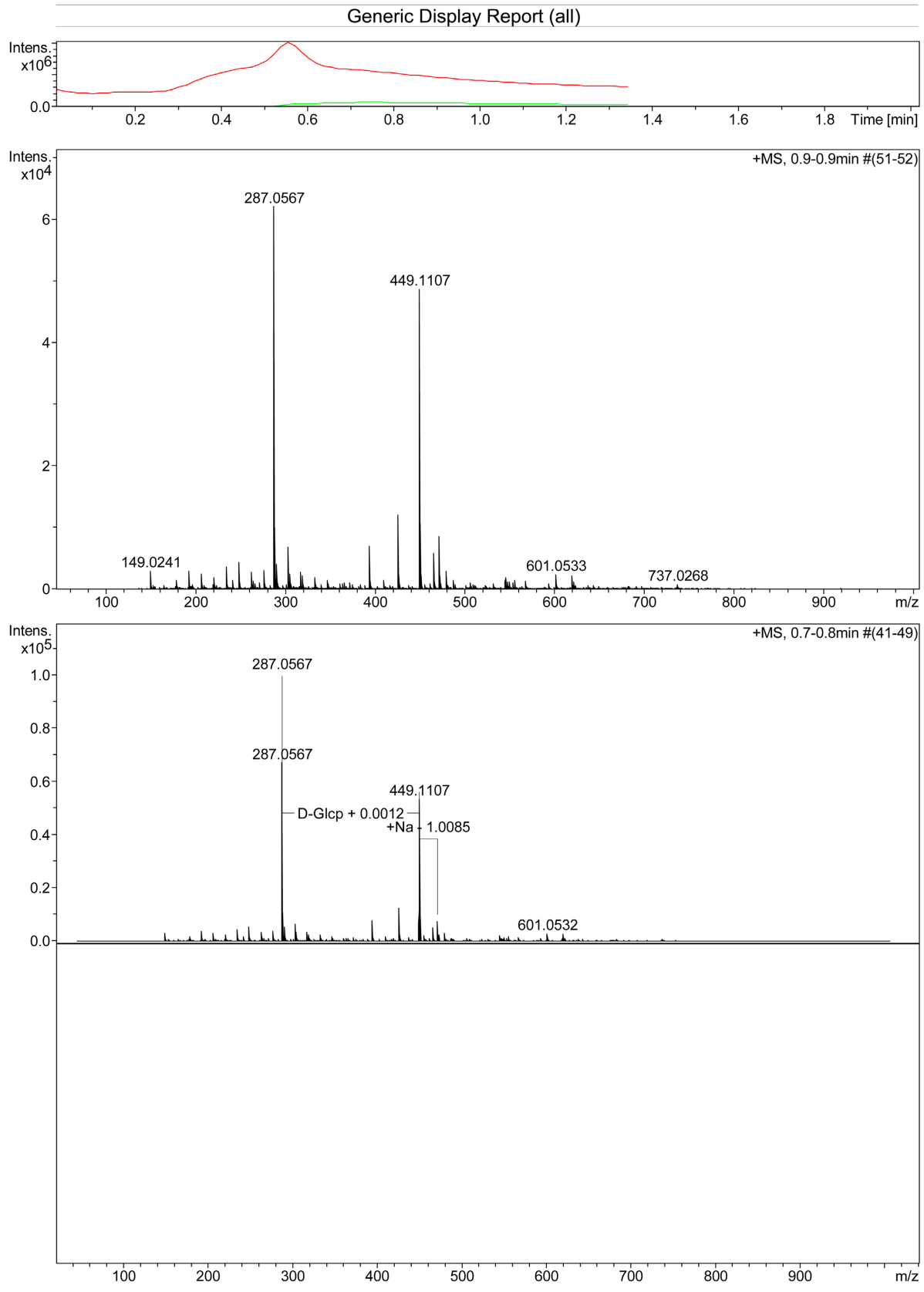
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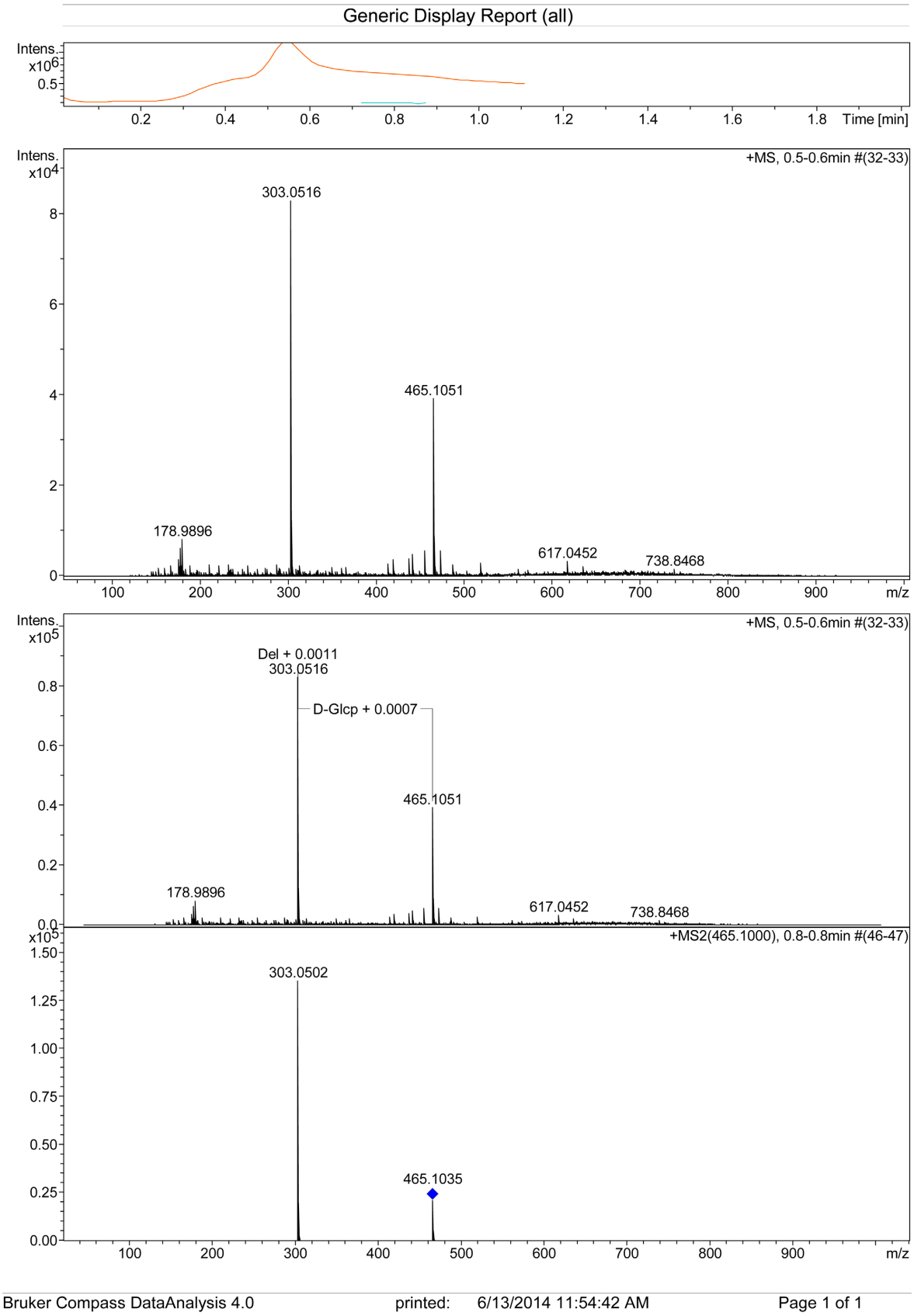


Figure 6. HPTLC-MS spectra (reverse phase) of the extract D3-spot 1 to 4.

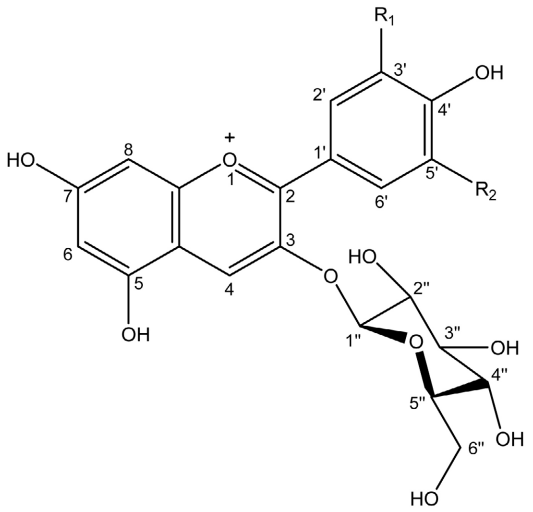
317 for (tR = 13 min), m/z: 287 for (tR = 12 min) and m/z: 303 for (tR = 10.5 min) respectively for each variety, corresponds to the loss of [M-162]+ from a residue of the glycosyl. This shows that these different compounds are monoglycosiles. Compounds with retention times of 15.5 min, 14.5 min, 13 min, 12 min, 10.5 min were identified as Mv-Glc, Pn-Glc, Pt-Glc, Cy-Glc and Dp-Glc.

In TLC-MS, only the four majority spots (**Figure 3(b)**) were analyzed for each variety. The analysis of the mass spectra (TLC-MS) of spot 1, spot 3, spot 4 and spot 5 of the extracts of M4, D3, KVS350, KVS97 give respectively for each variety mentioned above molecular ions at m/z: 493 (**1**); 479 (**3**); 449 (**4**); 465 (**5**) corresponding to the molecular formulas $[C_{22}H_{25}O_{12}]^+$, $[C_{22}H_{23}O_{12}]^+$, $[C_{21}H_{21}O11]^+$, $[C_{21}H_{21}O_{12}]^+$. Secondary ions at m/z: 331 (**1**) for spot 1, at m/z: 317 (**3**) for spot 3, at m/z: 287 (**4**) for spot 4, and at m/z: 303 (**5**) for spot 5 are attributed to the loss of $[C_6H_{10}O_5]^+$, the glycosyl residue (**Table 3** and **Figure 5** and **Figure 6**). The spectra of spot 1 (**1**), spot 3 (**3**), spot 4 (**4**) and spot 5 (**5**) correspond to compounds Mv-Glc, Pt-Glc, Cy-Glc and Dp-Glc.

We deduce that the compound (**2**) not identified in TLC-MS corresponds to the compound Pn-Glc identified in LC-MS.

The compounds identified in LC-MS: Mv-Glc, Pn-Glc, Pt-Glc, Cy-Glc and Dp-Glc correspond respectively to compounds **1**, **2**, **3**, **4**, **5**.

Compounds **1**, **2**, **3**, **4**, **5** isolated from varieties M4, D3 (Chad) and KVS350, KVS97 (Burkina Faso) were identified as malvidin 3-O-*β*-D-glycopyranoside respectively, paeonidin-3-O-*β*-D-glycopyranoside, petunidin-3-O-*β*-D-glycopyranoside, cyanidin-3-O-*β*-D-glycopyranoside, and dephinidin-3-O-*β*-D-glycopyranoside (**Figure 7**).



1: $R_1 = R_2 =$ OMe: malvidin-3-*O*-*β*-D-glycopyranoside
2: $R_1 =$ OMe et $R_2 =$ H: paeonidin-3-*O*-*β*-D- glycopyranoside
3: $R_1 =$ OMe et $R_2 =$ OH: petunidin-3-*O*-*β*-D-glycopyranoside
4: $R_1 =$ OH et $R_2 =$ H: cyanidin-3-*O*-*β*-D-glycopyranoside
5: $R_1 = R_2 =$ OH: dephinidin-3-*O*-*β*-D-glycopyranoside

Figure 7. Complete structures of compounds 1, 2, 3, 4, 5 isolated from *V. subterranea* seeds.

4. Conclusion

Five anthocyanin compounds were isolated and identified in the four varieties of *Vigna subterranea* seeds, two from Chad (M4 and D3) and two from Burkina Faso (KVS350 and KVS97). These anthocyanins were identified and characterized by chromatographic methods (HPLC, TLC) coupled with mass (LC-MS, TLC-MS) and chemical revelation tests. These are: malvidin-3-O-β-D-glycopyranoside, paeonidin-3-O-β-D-glycopyranoside, petunidin-3-O-β-D-glycopyranoside, cyanidin-3-O-β-D-glycopyranoside, and dephinidin-3-O-β-D-glycopyranoside. These anthocyanin compounds are present in the extracts M4, D3, KVS350 and KVS97 in different proportions.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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