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**Identification of glucosinolates in seeds of three Brassicaceae species known to
hyperaccumulate heavy metals**

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Plants from the Brassicaceae family are known to contain secondary metabolites called glucosinolates. Our goal was to establish by LC-MS the glucosinolate profile of seeds of three Brassicaceae species known to hyperaccumulate heavy metals. We investigated *Alyssum fallacinum* auct. non Hausskn., *Iberis intermedia* Guers., and *Noccaea caerulescens* (J. Presl & C. Presl) F.K. Mey. Our results indicate that *A. fallacinum* seeds contain glucoiberin and

glucoibervirin, which had not been previously identified in this plant. Furthermore, we report for the first time the presence of glucoiberin, glucoibervirin, glucotropaeolin, and sinigrin in *I. intermedia*. We have detected for the first time glucoconringiin in *N. caerulescens*. In addition, glucosinalbin, 4-hydroxyglucobrassicin, and glucomoringin were also detected.

Keywords

glucosinolate, *Alyssum fallacinum*, *Noccaea caerulescens*, *Iberis intermedia*, LC-MS

Introduction. - The long history of mining operations has led to the accumulation of trace elements (TE) in the environment. TE are persistent in ecosystems and living organisms. As they are not biodegradable, they tend to concentrate easily in living organisms along food chains in the magnification process. The high toxicity of TE in soil, water resources, and crops affects public health. In spite of their toxicity, heavy metals can also exert a selective pressure on living organisms and thus drive evolution. Metal-tolerant plant species are able to grow on metal-contaminated soils while metal-hyperaccumulating plant species can extract, transport, and concentrate metals from soils into their above-ground parts. About 450 metal-hyperaccumulators have been discovered throughout the world. Among them, metal-hyperaccumulating plants belonging to the Brassicaceae family have been the most used to develop wide programs for the phytoremediation of contaminated sites. Recently, they have gained considerable interest because of their potential recovery in green chemistry. Metal-rich biomass allows the production of new catalysts, referred to as ecocatalysts. Ecocatalysts provide

increased yields in chemical production and increased regio- and chemoselectivity, which results in high added value [1] [2]. This new approach to using metal-rich biomass, such as *Alyssum* spp. and *Thlaspi* (*Noccaea*) spp. could spur the development of phytoextraction, a technique considered promising for long, yet without viable economic outlets [3].

Production of a group of plant secondary metabolites called glucosinolates (GLs) is a common feature to the Brassicaceae family [4]. Discussions on the role of GLs in defence and tolerance mechanisms in metal hyperaccumulators are often based on total GL content. However, the relationship between GLs and metal accumulation, and these relationships regarding defence against herbivores in hyperaccumulator species, are not clear. Toward a better understanding of these relationships, we chose to investigate the GL profile of seeds of three Brassicaceae species known to hyperaccumulate heavy metals, using LC-MS. We investigated *Alyssum fallacinum* auct. non Hausskn. (synonym *Alyssum baldaccii* Vierh. ex Nyár.), *Iberis intermedia* Guers., and *Noccaea caerulescens* (J. Presl & C. Presl) F.K. Mey. (synonym *Thlaspi caerulescens* J. Presl & C. Presl).

A. fallacinum is a known hyperaccumulator of Ni [5-7]. *I. intermedia*, commonly known as a variety of candytuft, is a known hyperaccumulator of Tl [8-10]. The GLs of *I. intermedia* and *A. fallacinum* have never been investigated. *N. caerulescens* is a known hyperaccumulator of Zn, Cd, and Ni [11-18]. This plant is also Pb-hypertolerant but not necessarily Pb-hyperaccumulating [19]. Glucoiberin (**1**), glucosinalbin (**6**), gluconapin, 4-hydroxyglucobrassicin (**7**), and 4-methoxyglucobrassicin were identified in *N. caerulescens* shoots (5 weeks old) by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry of the desulfo-GLs [13] [20]. Gluconasturtiin can also be present in shoots [13]. Furthermore, gluconapin, **6** (major), and sometimes neoglucobrassicin were found in the roots.

In this study, glucoputranjivin, glucomalcolmiin, **1**, and glucocapparin were also detected in leaf tissue [11]. In addition, sinigrin (**3**), **6**, and 4- α -rhamnosyloxybenzyl GL (**8**), also known as glucomoringin, were extracted from seeds and leaves [21].

The selection of the hyperaccumulator plants mentioned above was undertaken in the context of our ongoing phytoremediation programs in Southern Europe [3].

<insert the chemical formula about here>

Results and Discussion. - The seeds of three plants known to hyperaccumulate heavy metals (*A. fallacinum*, *I. intermedia*, and *N. caerulescens*) of the Brassicaceae family were extracted and analysed by LC-MS for intact GLs. We collected samples from one site each for the first two plant species from sites known to be contaminated with heavy metals (see Experimental Part). *N. caerulescens* seeds were collected from two different contaminated sites.

Glucosinolate Composition of Alyssum fallacinum seed. The chromatogram of the *A. fallacinum* seed extract displayed two major peaks at t_R 6.4 and 20.9 min (*Fig. 1a*). The compound at t_R 6.4 min had identical t_R , mass (422 a.m.u), and UV spectra as a commercial standard of **1**. The compound at t_R 20.9 min, had a mass of 406 a.m.u and a UV spectrum similar to that of a commercial standard of glucoerucin. Therefore, **2** was identified as glucoibervirin. The LC-MS chromatogram of *A. fallacinum* from our study contained the same major GLs **1** and **2** as those found in the seeds of *Alyssum peltarioides* Boiss. and *Alyssum sibiricum* Willd. from Turkey [22].

Glucosinolate Composition of Iberis intermedia seed. Four GL peaks were observed in the chromatogram of the *I. intermedia* seed extract (*Fig. 1b*). **1** and **2** were also identified in this extract. The peak at t_R 8.7 min with a mass of 358 a.m.u. was determined to be **3** by comparison

to a commercial standard. The peak at t_R 23.4 min with a mass of 408 a.m.u was found to be glucotropaeolin (**4**). This identification was confirmed by comparison of the UV and mass spectra and t_R of a commercial standard of **4**. *Iberis umbellata* L. seeds, purchased commercially, were previously found by GC-MS analysis of GL hydrolysis products to contain **1-3** [22]. The major GL was **1** (8.4 mmol/100 g sample) followed by **3** (3.9 mmol/100 g sample), and **2** (0.14 mmol/100 g sample) [22]. In the leaves, **3** and **4** (83 mmol/10 g dried leaves, 1 mmol/10 g dried leaves, respectively) were found and quantified [23]. Other *Iberis* spp. are also known to contain **1** and **2** [22, 24-27].

Glucosinolate Composition of Noccaea caerulescens seed. The chromatographic profiles of *N. caerulescens* were quite similar (*Fig. 2a and 2b*). Glucoconringiin was determined to be compound **5** at t_R 8.5 min with a mass of 390 a.m.u by comparing UV and mass spectra and t_R with an authenticated sample previously isolated in our group from *Bretschneidera sinensis* Hemsl. seeds [28]. Another minor compound at t_R 17.9 min, with a mass of 424 a.m.u was identified as **6** by comparing its UV, mass spectra, and t_R with those of a commercial standard. Compound **7** was eluted at t_R 20.8 min and had a mass of 463 a.m.u. The spectroscopic data were similar to those of **7** previously found in our group in *Brassica elongata* Ehrh. seeds [29]. In addition, the major peak at t_R 21.3 min with a mass of 570 a.m.u was identified as **8** by comparison of its UV, mass spectra and t_R with an authenticated sample [30]. All other unidentified peaks in all extracts possessed UV spectral characteristics of flavonoids [31].

We report for the first time the presence of **5** in *N. caerulescens*. However, **5** was previously deduced from the detection of 5,5-dimethylloxazolidine-2-thione, the myrosinase

hydrolysis product of **5** in *Thlaspi kovatsii* Heuff. (synonym *Thlaspi avalanum* Pančić) and *Thlaspi alpestre* Jacq. (synonym *Noccaea alpestris* (Jacq.) Kerguélen) seeds [22]. Contrary to previous studies in seeds [21], we did not detect **3** which was claimed the major GL in some accessions. In our case, the major GL was **8**, which was also the case in other accessions from Spain (Valle de Varrados and Pontaut), Luxembourg (Lellingen), and France (Navacelles) [21]. Our results confirm intraspecific variation in the GL profile in *N. caerulescens* seeds. In addition, **7**, which had only been detected in the shoots [20, 21], was detected in the seeds for the first time. We did not detect **1**, 4-methylsulfanylbutyl GL, glucoputranjivin, glucomalcomiin, nor glucocapparin, which were previously reported in leaf tissue [11].

A previous investigation has shown that when *N. caerulescens* was exposed to increased Zn concentrations, the concentration of **6** in shoots diminished, whereas in the roots, the concentration of **6** increased with Zn concentration [13]. Another study confirmed that GL concentration in shoots decreased when the concentration of Zn in leaves increased [16]. However, a separate research group demonstrated that GL production increased in shoot tissue with Zn treatments, especially 4-methylsulfanylbutyl GL, also known as glucoerucin [11]. Other researchers indicated that the total GL content in *Noccaea* leaves increased with the concentration of Ni or Cd in the soil [12]. However, the total GL amount in damaged leaves was higher than in undamaged leaves in the presence of high concentrations of Ni [12]. Conversely, it was shown that the GL content was higher in undamaged leaves in the presence of a high concentration of Cd [12]. Another investigation reported a higher GL content in undamaged than in damaged leaves of *N. caerulescens* grazed by thrips (*Frankliniella occidentalis*) and grown in Zn-contaminated soil while the anthocyanin concentration was high in damaged leaves [32]. Furthermore, GLs were shown to deter gastropods from eating *N. caerulescens* [15] [18].

Moreover, the damage caused by the cabbage whitefly (*Aleyrodes proletella*) did not affect the production of GLs in *N. caerulescens* grown in Zn-contaminated soil [16]. In addition, *N. caerulescens* was shown to contain organic acids, amino acids, metallothioneins, and phytochelatins which could be responsible for its heavy metal-hyperaccumulating capacity [11] [14]. Finally, galactolipids, anthocyanins, nicotianamine, and oxylipins were also found in the plant [11] [32].

Conclusions. - Our investigation of the three plants known to hyperaccumulate heavy metals *A. fallacinum*, *I. intermedia*, and *N. caerulescens* by LC-MS has shown the efficacy of the method in separating their GLs. We report for the first time the presence of **1-2** in *A. fallacinum* and **1-4** in *I. intermedia*. Finally, the GL profile including **5-8** in *N. caerulescens* from two sites of collection showed no differences but some discrepancies with literature data were pointed out.

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Experimental Part

General. All solvents were ACS grade and used as such. Formic acid was purchased from BDH (Toronto, ON, Canada). HPLC-grade MeOH, absolute EtOH, and triethylamine (reagent grade) were purchased from Fisher Scientific (Whitby, ON, Canada). Glucosinabin was purchased from Apin Chemicals Ltd. (Abingdon, UK). Glucoerucin and glucoiberin were

purchased from Cfm Oskar Tropitzsch (Marktredwitz, Germany). Glucotropaeolin was purchased from Chromadex (Irvine, CA, USA). Sinigrin was purchased from Sigma Aldrich (Oakville, ON, Canada). HPLC-grade H₂O was generated in the laboratory through a Nanopure Diamond Ultrapure water system by Barnstead (Dubuque, IA, USA).

Plant Material. *A. fallacinum* seeds were harvested in 2014 in Anogia-Gonies road, 1 km from Sisorha towards Gonies (estimated 35°17.9' N, 24°55.7' E (Greece)), on a serpentine soil containing high concentrations of Ni (1,350 ppm), and identified by Prof. Alan J.M. Baker (School of Botany, University of Melbourne, Australia). *I. intermedia* seeds were harvested in 2010 in Les Avinières, Saint-Laurent-Le-Minier, Gard (03°66'50''E, 43°93'13'' N, France) which is a mining site in which the soil contains Zn (up to 156,000 ppm), Pb (36,354 ppm), Cd (700 ppm), and Tl (115.1 ppm) [33] [34]. *N. caerulescens* seeds were harvested in 2012 in Les Avinières (same mining site where *I. intermedia* seeds were collected) and 2010 in Bergenbach, sges (France) on a serpentine soil containing Ni (116 mg kg⁻¹ ammonium acetate-EDTA extractable element), Zn (25 mg kg⁻¹ ammonium acetate-EDTA extractable element) and Cd (0 mg kg⁻¹ ammonium acetate-EDTA extractable element) [35] [36]. *I. intermedia* and *N. caerulescens* were identified by Prof. Claude Grison (University of Montpellier, France).

Extract Preparation. Seeds (361 mg of *A. fallacinum*, 512 mg of *I. intermedia*, 575 mg of *N. caerulescens* from Bergenbach, and 517 mg of *N. caerulescens* from Les Avinières) were frozen in liquid N₂ and ground to powder with a mortar and pestle. The powder was extracted with boiling EtOH/H₂O (7/3 v/v) (2 × 5 mL) for 5 min. The solutions were concentrated to dryness (38 mg of *A. fallacinum*, 89 mg of *I. intermedia*, 62 mg of *N. caerulescens* from Bergenbach, and 70 mg of *N. caerulescens* from Les Avinières).

HPLC-ESI-MS Analysis. The extracts were dissolved in MeOH/H₂O 7/3 (v/v) (2.5 mL for *A. fallacinum*, 6 mL for *I. intermedia*, and 5 mL for *N. caerulea* from Bergenbach and Les Avinières) and were filtered through a plug of cotton prior to analysis by a high-performance liquid chromatograph (HPLC). The analyses were performed by injecting 10 µL of extract into an Agilent Technologies HP 1100 (New Castle, DE) HPLC equipped with a quaternary pump, automatic injector, diode-array detector (wavelength range 190-600 nm), degasser, and a Hypersil ODS column (5 µm, 4.6 × 200 mm). The two mobile phase solvents, MeOH and H₂O, were prepared with 0.15% triethylamine and 0.18% formic acid added as ion-pairing reagents. Both solutions were filtered using 0.45 µm nylon membranes. The initial mobile phase was 100% HPLC-grade H₂O. At 10 min, the mobile phase was switched to a linear gradient of 100% H₂O to 100% MeOH over 60 min [37]. After each run, the initial mobile phase conditions were and the system was allowed to equilibrate. The flow rate was kept constant at 1 mL min⁻¹. The column temperature was held at room temperature. The HPLC was interfaced to an Agilent model 6120 mass spectrometer (Toronto, ON, Canada) with a Chemstation data system LC-MSD B.03.01. The ES interface was a standard ES source operating with a capillary voltage of 4 kV and temperature of 350 °C. The system was operated in the negative and positive ion ES modes. Nitrogen was used as nebulizing and drying gas at a flow of 10 L min⁻¹ (35 psig). The mass spectrometer was programmed to perform full scans between *m/z* 100 and 1,000 a.m.u.

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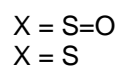
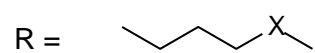
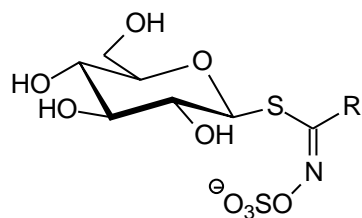
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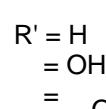
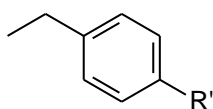
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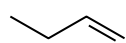
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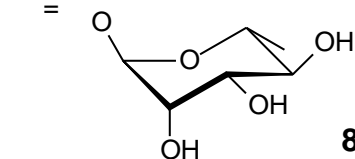
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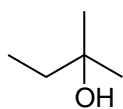
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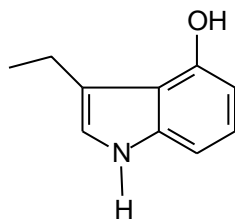
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Fig. 1.

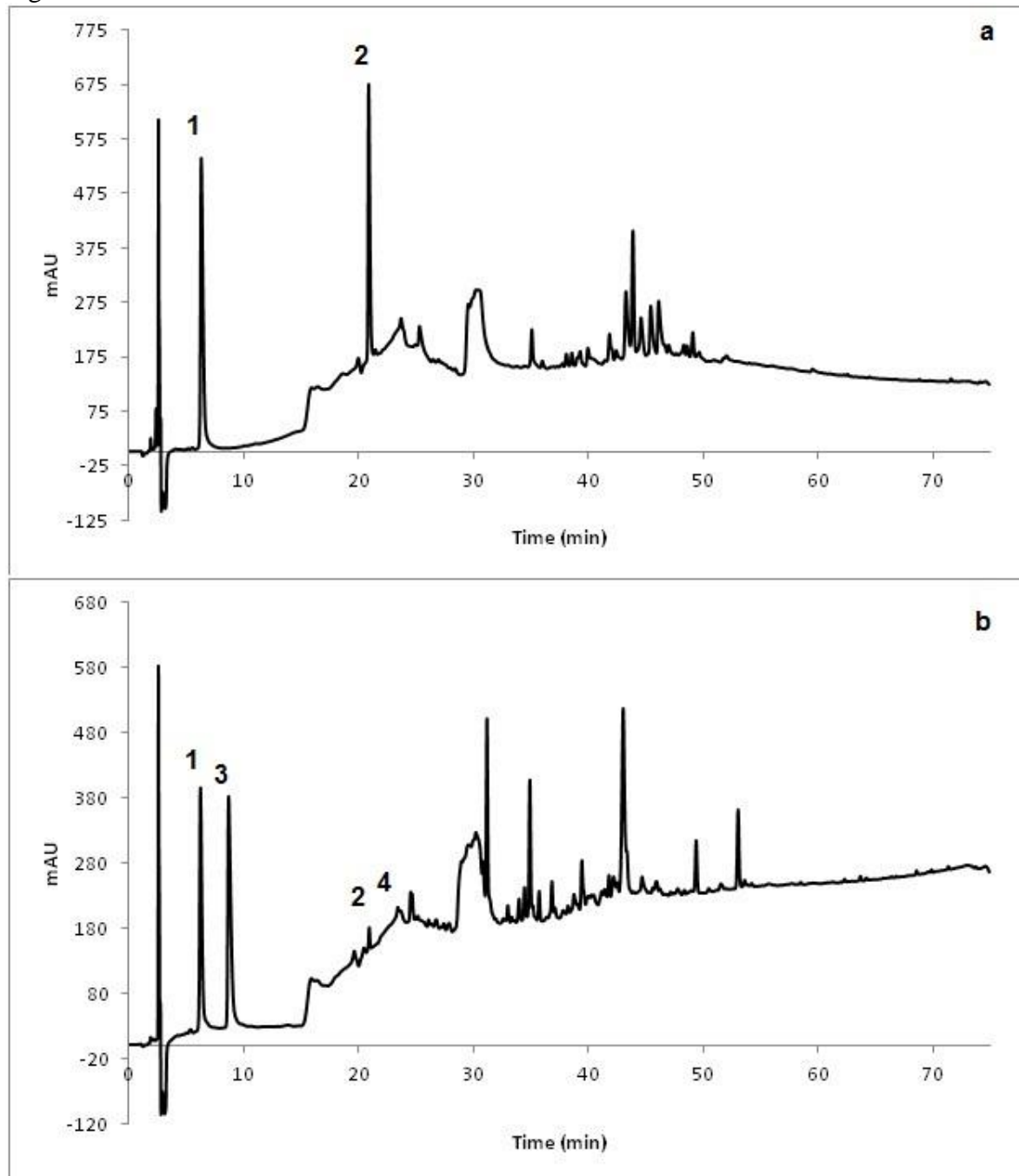


Fig. 1. HPLC chromatograms of the methanolic extract of a) *Alyssum fallacinum* seeds, b) *Iberis intermedia* seeds. Detection at 220 nm. 1: glucoiberin, 2: glucoibervirin, 3: sinigrin, 4: glucotropaeolin.

Fig. 2.

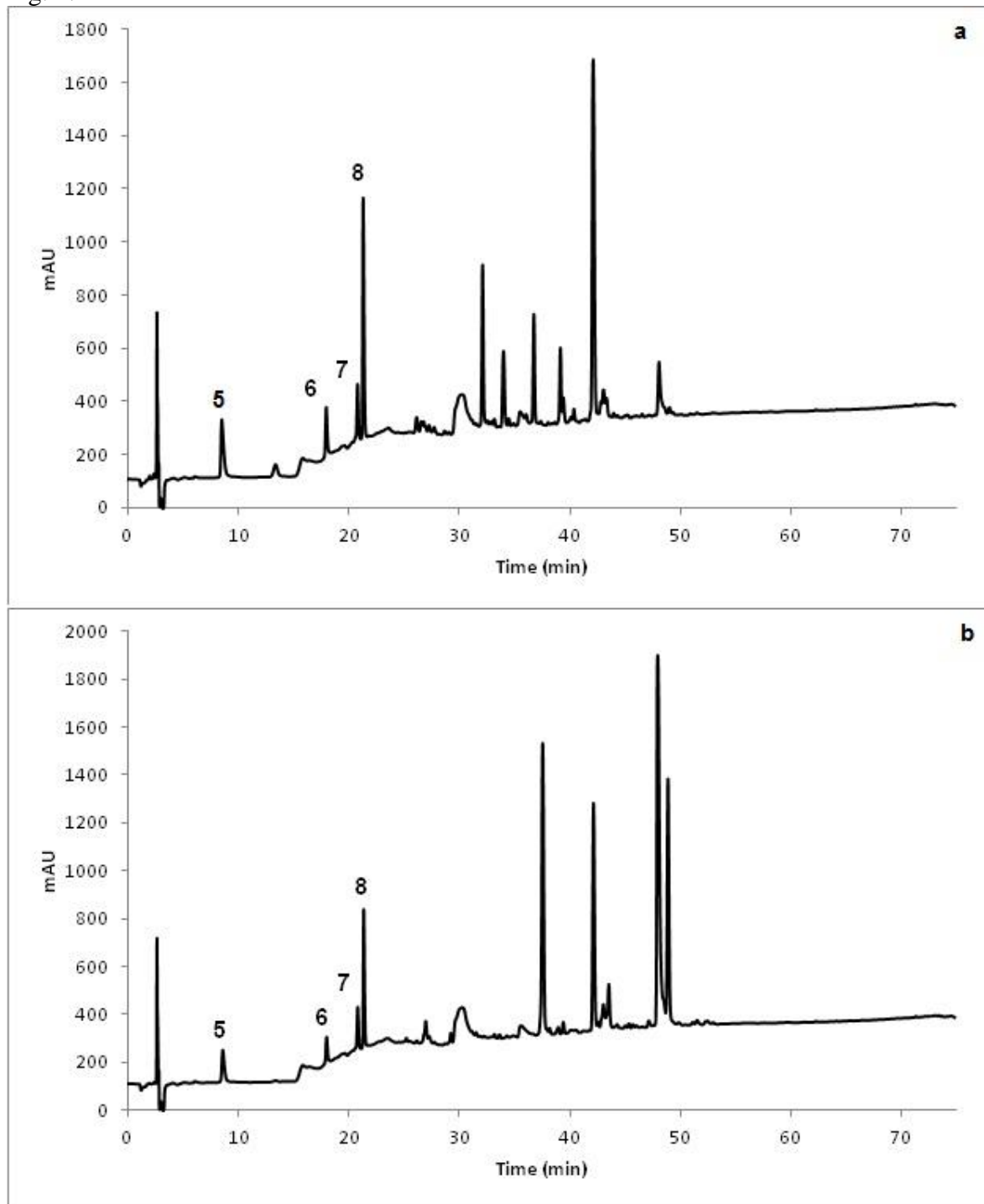


Fig. 2. HPLC chromatograms of the methanolic extract of *Nocca caerulea* seeds from a) Bergenbach and b) Avinières. Detection at 220 nm. 5: gluconringin, 6: glucosinalbin, 7: 4-hydroxyglucobrassicin, 8: glucomoringin.