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Dimitri Bréard, Thibault Barrit, Daniel Sochard, Sophie Aligon, Elisabeth Planchet, et al.. Development of a quantification method for routine analysis of glucosinolates and camalexin in brassicaceous small-sized samples by simultaneous extraction prior to liquid chromatography determination. *Journal of Chromatography B - Analytical Technologies in the Biomedical and Life Sciences*, 2022, 1205, pp.123348. 10.1016/j.jchromb.2022.123348 . hal-03708527

HAL Id: hal-03708527

<https://univ-angers.hal.science/hal-03708527>

Submitted on 3 Mar 2023

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Development of a quantification method for routine analysis of glucosinolates and camalexin in brassicaceous small-sized samples by simultaneous extraction prior to liquid chromatography determination

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ABSTRACT

Glucosinolates and camalexin are secondary metabolites that, as phytoanticipins and phytoalexins, play a crucial role in plant defence. The present work proposes an improved analytical method for routine analysis and quantification of glucosinolates and camalexin in brassicaceous small-sized samples by using the very specific desulfation process of glucosinolates analysis and the specificity of fluorescence detection for camalexin analysis. The approach is based on a simultaneous ultrasound-assisted extraction followed by a purification on an anion-exchange column. Final analyses are conducted by HPLC-UV-MS for desulfo-glucosinolates and HPLC coupled to a fluorescence detector (HPLC-FLD) for camalexin. The method is linear for glucosinolates (50-3500 μM) and camalexin (0.025-5 $\mu\text{g}\cdot\text{mL}^{-1}$) with an LOD/LOQ of 3.8/12.6 μM and 0.014/0.046 $\mu\text{g}\cdot\text{mL}^{-1}$ respectively. The method demonstrated adequate precision, accuracy and trueness on certified reference rapeseed. A practical application of our approach was conducted on different *Brassicaceae* genera (*Barbarea vulgaris*, *Brassica nigra*, *Capsella bursa-pastoris*, *Cardamine hirsuta*, *Coincya monensis*, *Sinapis arvensis*, and *Sisymbrium officinale*) and *Arabidopsis thaliana* genotypes (Columbia and Wassilewskija). Furthermore,

different plant organs (seeds and leaves) were analysed, previously inoculated or not with the pathogenic fungus *Alternaria brassicicola*.

KEYWORDS

Glucosinolates; Camalexin; Small-sized samples; Simultaneous extraction; Absolute quantification

1. Introduction

Plants produce several secondary metabolites that play a crucial role as defence compounds against pathogens and herbivores. In the *Brassicaceae* family, most species produce phytoanticipins (namely glucosinolates) as constitutive defences (Fig. 1). Some species further produce phytoalexins as inducible defences among which camalexin is the most studied compound [1] (Fig. 1). Fungal pathogens including *A. brassicicola*, a *Brassicaceae* specialist that causes black spot disease, are more or less sensitive to camalexin, glucosinolates and glucosinolates-breakdown products, present in leaves [2,3]. However, *A. brassicicola* also spreads by seeds [4,5] and little is known about the transmission of the pathogen from seed to seedling during germination and post-germinative growth as well as the role of defence compounds in this process. One reason for that is that the detection of secondary metabolites from the complex matrix of seed reserves [6] is quite difficult and that only small amounts of sample material [7] is available at the early stages of plant development. To accurately determine secondary metabolites in brassicaceous seeds and seedlings, an analytical procedure suitable for small-sized samples and adapted to maximize the recovery of glucosinolates and camalexin is therefore required. Furthermore, both glucosinolates and camalexin play important but different roles in plant protection against various pathogens. As their biosynthetic pathways are tightly regulated and interconnected [8], a method allowing identification and quantification of both types of metabolites from a single sample may assist the elucidation of their relative roles during and after germination.

Because of the great ongoing interest in glucosinolates as biologically active compounds [9–11], many protocols for extraction, identification and quantification have been published for five decades. They can be basically classified into two groups [12,13].

The first group focuses on the total content of glucosinolates and including destructive methods as well as non-destructive methods will not be discussed.

Conversely, the second group allows quantification of individual glucosinolates, like desulfo-glucosinolates, TMS-ether of desulfo-glucosinolates, isothiocyanates or intact form. The conversion of glucosinolates to isothiocyanates by myrosinase or the derivatization of desulfo-glucosinolates to TMS-ethers for GC-MS analysis is no longer conducted [12]. Current analytical determinations are now conducted on the desulfo-glucosinolates or intact forms by LC-UV, GC-FID or LC-MS, however it is uncommon to perform UV quantification on the intact form [14] because the desulfation process typically acts as an essential clean-up step. HPLC-UV and GC-FID yield comparable results for desulfo-glucosinolates and are therefore considered validated approaches [15]. However, with the advanced development of hyphenated MS based chromatographic methods, GC-FID is no longer used [16]. The detection of glucosinolates has been reported for many ionization techniques (FAB, ESI, APCI or MALDI) [17], and detector configurations such as single/triple quadrupole, IT or TOF [17]. The quantification can be accurate and precise if executed correctly. However, the lack of universal response factors in MS which is linked to several parameters (individual behaviour of analyte with settings for ionization and/or fragmentation, matrix effects, differences in linear range of detection among instruments) limits accurate quantification to few commercially available standards [12,18].

For the moment, the conversion of glucosinolates to desulfo-glucosinolates by sulfatase followed by HPLC-UV analysis [19] represents the official analytical approach outlined in European Union regulations (ISO 9167) [20] and therefore remains widely used [18]. It still provides a robust, direct and relatively low-cost approach that can be easily performed on standard laboratory equipment. Meanwhile, suggestions to improve and simplify the method have been outlined in several publications [21–23]. Moreover, desulfo-glucosinolates are chemically quite stable when kept airtight

in cool water [22] and most response factors of quantification (relative to sinigrin) are well defined and accessible by open access in publications [12]. In many cases, retention times linked to reported data are used for compound identification [24], however MS detection provides significant added analytical value by allowing a more accurate analysis [17].

Camalexin is an important component of inducible defences and contributes to the partial resistance against several pathogens [25]. Therefore, the quantification of this phytoalexin is also very important for understanding processes of plant physiology and phytopathology. The extraction protocols for camalexin are quite similar in most publications. In general 70 to 80% methanol is used as solvent at a temperature range of 20-80°C. Extraction time may last from ten minutes to one hour [26,27] and sometimes residue is re-extracted with chloroform [28–30]. Fluorometric analyses are performed using 318 nm excitation and 385 nm emission wavelength [29] but for an accurate quantification of camalexin chromatographic purification is required in order to eliminate quenching interference [26]. For ten years, LC-MS has been the predominant technique because of short analysis times and is especially effective in identifying of camalexin based on accurate mass as well as determining low concentrations [26,31].

On the other hand, the simultaneous detections of glucosinolates and camalexin has hardly been conducted from one sample. When done, it was either by a single method based only on relative quantification by UPLC-MS [32,33] or by an absolute quantification with two separate methods requiring twice as much raw material [32,34,35]. A procedure allowing the identification and quantification of glucosinolates and camalexin from a single extraction would be beneficial for the analysis of samples that are generally limited in quantity, like small seeds and seedlings. The aim of this work was therefore to provide a validated method analysis for the determination of glucosinolates and camalexin in small-sized samples (less than 250 mg of dry matter (DM) for a triplicate analysis). The proposed method comprises the simultaneous extraction of both constituents from seed and leaf samples prior to HPLC analysis and targets a broad *Brassicaceae* phylogenetic group with potentially

contrasting profiles. The approach is based on the specificity of the desulfation process of glucosinolates and the specificity of fluorescence detection for camalexin analysis.

2. Experimental

2.1. Chemicals and reagents

HPLC grade acetonitrile (MeCN) and methanol (MeOH) were purchased from HoneyWell (Charlotte, USA). Absolute ethanol (EtOH) was purchased from Carlo Erba Reagents (Val de Reuil, France). Anhydrous sodium acetate (NaOAc) and 37% hydrochloric acid (HCl) were purchased from Fisher Chemical (Illkirch, France). Sinigrin potassium salt ($\geq 99\%$) was purchased from Extrasynthese (Genay, France). Camalexin ($\geq 98\%$), DEAE-Sephadex A-25 and sulfatase type H-1 from *Helix pomatia* were purchased from Sigma-Aldrich (Darmstadt, Germany). Ultrapure water (H₂O) was obtained by a MilliQ Advantage system from Millipore (Darmstadt, Germany).

2.2. Standards and reagents solutions

Stock solution of sinigrin was prepared in H₂O at a concentration of 5050 μM . A stock solution of camalexin was prepared in 70% MeOH at a concentration of 38.8 $\mu\text{g}\cdot\text{mL}^{-1}$. The enzyme solution was prepared by dissolving and mixing 10000 units of sulfatase in 30 mL of H₂O and 30 mL of EtOH before centrifugation at 2650 *g* for 20 min at room temperature. The supernatant was taken and mixed with 90 mL of EtOH before centrifugation at 1030 *g* for 15 min at room temperature. Finally, the pellet was dissolved in a total of 25 mL of H₂O and aliquoted in 1.5 mL microtubes. All solutions were stored at -20°C before use.

The ion exchange medium was prepared by mixing 10 g of DEAE-Sephadex A-25 resin with 125 mL of H₂O and the resulting mixture was stored at 4°C.

The solution of 70% MeOH was prepared by mixing 350 mL of MeOH and 150 mL of H₂O. The 20 mM NaOAc buffer was prepared by dissolving 410 mg of NaOAc in 250 mL of H₂O and adjusting the pH at 5.5 with HCl. These solutions were prepared extemporaneously and conserved one week at 4°C.

2.3. Samples collection and preparation

Three certified rapeseed reference materials by European Commission called ERM-BC366 (seeds with low level of glucosinolates), ERM-BC190 (medium level) and ERM-BC367 (high level) were purchased from Sigma-Aldrich and stored at 4°C.

Seeds of *Arabidopsis thaliana* (L.) Heynh (= natural population), *Barbarea vulgaris* Aiton, *Brassica nigra* (L.) Koch, *Capsella bursa-pastoris* (L.) Medik., *Cardamine hirsuta* L., *Coincya monensis* (L.) Greuter and Burdet, *Sinapis arvensis* L., and *Sisymbrium officinale* (L.) Scop. were collected in 2018 in natural populations around Angers (France). Species identification was verified by Josiane Le Corff.

Seeds from these populations were sown in the spring in a substrate of 50% sand, 37.5% blond peat, and 12.5% black peat, and grown in a glasshouse under natural daylight for 4-5 weeks. Mean daily temperature was 14°C and ranged from 10°C up to 22°C for a few hours on sunny days.

In addition, seeds of *A. thaliana* of the ecotypes Columbia (Col) and Wassilewskija (Ws) were harvested in 2018 from a culture in a growth chamber in a substrate of 75% blond peat and 25% black peat, under controlled conditions (16h/8h light/dark, 22°C/19°C light/dark) for 3 months. Seeds were sown in a substrate of 75% blond peat and 25% black peat and grown in a growth chamber under controlled conditions (8h/16h light/dark, 22°C/19°C light/dark) for 4 weeks.

Before harvest, a subsample of rosettes (at least 5 per genotype) from *A. thaliana* natural population, Col and Ws were inoculated on five leaves per plant with one 5 µL drop of an *A. brassicicola* (previously described Abra43 isolate [3]) conidial suspension (10^5 conidia.mL⁻¹ in water). Other plants as controls (at least 5 per genotype), were treated with one 5 µL drop of water on 5 leaves per plant. All plants were then maintained for two days in saturating humidity (100% relative humidity) and collected seven days after inoculation.

For all samples (seeds from natural populations and ecotypes and leaves from plants that had been inoculated or not), material was immersed and grinded in liquid nitrogen upon harvest, freeze-dried then stored at -20°C prior to analysis.

2.4. Extraction procedure

Extraction was carried out following a protocol previously published by Grosser *et al.* [22] with minor modifications. One Pasteur pipette (150 mm) per sample and sinigrin reference was filled with approximately 1 cm of glass wool, then set up on a homemade rack. Each column was filled with 0.5 mL of resin (40 mg dry weight equivalent) then washed with 1 mL of H₂O. Finally, 15 mL vials were placed on a rack to collect the column washes including camalexin.

Dry samples were weighed in round-bottom microtubes and two metal balls (3 mm in diameter) as boiling retardants were added. In parallel, seven calibration standards of sinigrin were prepared in the concentration range of 50 to 3500 µM by diluting the stock solution in H₂O.

A volume of 1 mL of 70% MeOH (not pre-heated) was added on each sample, vortexed and heated 5 min at 91°C. Then, the microtubes were placed in an ultrasonic bath during 15 min before centrifugation at 2700 *g* for 10 min at room temperature. The supernatants and the calibration standards were deposited on separate columns. Another 1 mL of 70% MeOH was added to the remaining pellets and to the calibration standards microtubes. The samples were vortexed and placed in an ultrasonic bath during 15 min then centrifugated at 2700 *g* for 10 min at room temperature. The supernatants and the 7 calibration standards were added to their respective columns.

Each column was sequentially washed with 2 x 1 mL of 70% MeOH, 1 mL of H₂O and 2 x 1 mL of 20 mM NaOAc buffer waiting for the column to run dry between each washing. At this moment, 15 mL vials (camalexin fractions) were replaced by 2 mL microtubes and placed overnight under an air stream to remove MeOH. A volume of 20 µL of sulfatase solution was added onto each column followed by 50 µL of 20 mM NaOAc buffer. The columns were covered with aluminium foil and let stand overnight.

The next day, the desulfo-glucosinolates were eluted in 2 mL microtubes by pipetting 2 x 0.75 mL of H₂O onto each column (desulfo-glucosinolates fractions).

Camalexin and desulfo-glucosinolates fractions were frozen in a -80°C freezer for 4 hours then freeze-dried for 16 hours (8h/8h, primary/secondary desiccation) to remove water.

2.5. Liquid chromatography

2.5.1. HPLC-UV-MS conditions for desulfo-glucosinolates

Chromatographic analyses were performed on a 2695 separation module coupled to a 2489 UV/Visible detector (Waters, Saint-Quentin-en-Yvelines, France) and to an Esquire 3000 Plus ESI-IT-MS (Bruker, Wissembourg, France) assisted by Empower (Waters) and Data Analysis software (Bruker).

After the freeze-drying process, the desulfo-glucosinolates fractions were dissolved in 1 mL of H₂O (high content samples, *e.g.* seeds) or 0.2 mL of H₂O (low content samples, *e.g.* leaves). The injection volume was 10 µL.

Samples were injected onto a Gemini C18 column (150 mm x 4.6 mm, 3 µm, Phenomenex, Le Pecq, France) heated at 40°C. The analytes were separated by a gradient elution with H₂O (A) and MeCN (B) at a flow rate of 0.75 mL.min⁻¹ (2% to 35% B in 35 min). The UV detection was monitored at 229 nm and MSⁿ detection was monitored in positive and negative mode.

2.5.2. HPLC-FLD conditions for camalexin

Chromatographic analyses were performed on a 2695 separation module coupled to a 2475 multi λ fluorescence detector assisted by Empower software (Waters).

After freeze-drying process, the camalexin fractions were dissolved in 2 mL of 70% MeOH. Eight calibration standards of camalexin were prepared in the concentration range of 0.025 to 5 µg.mL⁻¹ by diluting the stock solution in 70% MeOH. The injection volume was 10 µL.

Samples were injected onto a Gemini C18 column (150 mm x 4.6 mm, 3 μ m, Phenomenex) heated at 40°C. The analytes were separated by a gradient elution with H₂O (A) and MeCN (B) at a flow rate of 0.75 mL.min⁻¹ (2% to 100% B in 35 min). The fluorescence detection was monitored at 318 and 385 nm as excitation and emission wavelengths, respectively.

2.6. Method validation

The calibration curves were set to the concentration range expected for each compound. The linearity of the calibration curves was assessed by injecting calibration standards of sinigrin (7 levels) or camalexin (8 levels) in three replicates. Residuals (difference between nominal concentration and calculated concentration by the linear model) and their distribution (normally distributed around the mean) were monitored. LOD and LOQ were defined as 3 x and 10 x SD of five replicate measurements of analyte close to the detection limit, respectively.

Precision was evaluated by repeated analysis of both, standards and samples, within different analytical batches. For glucosinolates, accuracy was assessed using three certified rapeseed reference materials with three different levels of glucosinolates. Some samples were spiked with different levels of camalexin at the start of extraction procedure in order to evaluate the recovery. All statistical work was conducted on Microsoft Excel 365® and the Minitab 19® software.

2.7 Method calculation

2.7.1 Glucosinolates analysis

The HPLC-UV-MS chromatograms at 229 nm were integrated respecting the defined LOQ and glucosinolates were identified by MS data, retention time and comparison with literature data.

Peak areas of desulfo-glucosinolates were corrected with an UV response factor at 229 nm compared to desulfo-sinigrin provided reference data were available [12,22] (Table A1). For all other cases including unidentified glucosinolates the response factor was set to 1. Results were expressed in

mmol.kg⁻¹ of DM (following the standard) and calculated according to the formula presented in appendix (Eq. A1).

2.7.2 Camalexin analysis

The HPLC-FLD chromatograms acquired at an emission wavelength of 385 nm and an excitation wavelength of 318 nm were integrated according to the defined LOQ. The results were expressed in µg.g⁻¹ of DM following the formula presented in appendix (Eq. A2).

3. Results and discussion

3.1. Glucosinolates analysis validation

The evaluation of the modified method [22] was the first step of this study. For that, three samples of certified rapeseed reference material containing the three classes of glucosinolates, aliphatic (progoitrin, epiprogoitrin, glucoalyssin, gluconapoleiferin, gluconapin and glucobrassicinapin), benzenic (gluconasturtiin) and indolic (4-Hydroxyglucobrassicin, glucobrassicin and neoglucobrassicin) [36] and with three levels of glucosinolates were taken as references: BCR-366R at 11.9 ± 1.3, BCR-190R at 23 ± 4 and BCR-367R at 99 ± 9 mmol.kg⁻¹ [37].

Previously, the linear relationship between area under the curve at 229 nm and the concentration of sinigrin was validated in the concentration range evaluated for glucosinolates with a correlation coefficient higher than 0.999 (Fig. A1) and was repeated for different batch analyses at different times of the study. The range of linearity from 50 to 3500 µM was assessed after control of the standardized residuals. LOD and LOQ were statistically calculated at 3.8 and 12.6 µM, respectively (Table A2).

Next, each certified rapeseed sample was tested in triplicate at about 80 mg following the protocol described in the experimental section and the results were calculated with the calibration curve of sinigrin and UV response factor at 229 nm of identified glucosinolates (Fig. 2). The results (total and individual glucosinolates contents) were in accordance with the certification report [37] (Fig. 3).

Finally, the procedure was down scaled to 10 mg of DM for the three certified rapeseed samples. Fig. 4 shows that results were similar in a mass range of 10-80 mg for the three certified rapeseeds for total and individual glucosinolates contents.

The method was precise and accurate and can therefore be used from 10 to 80 mg samples with high or low glucosinolates contents.

3.2. Camalexin analysis validation

To verify whether the quantitative extraction of camalexin and glucosinolates together with desulfation can be performed simultaneously, rapeseed samples were spiked with camalexin. Indeed, the absence of camalexin in the rapeseed samples was checked beforehand.

Previously, the linear relationship between the area under the curve at 385 nm (after excitation at 318 nm) and the concentration of camalexin was validated in the concentration range evaluated for the samples with a correlation coefficient higher than 0.999 (Fig. A2) and was repeated for different batch analysis at different times of the study. The range of linearity was assessed after control of the standardized residuals. LOD and LOQ were statistically calculated at 0.014 and 0.046 $\mu\text{g}\cdot\text{mL}^{-1}$, respectively (Table A3).

Next, different rapeseed samples were supplemented with camalexin from 0.05 to 2 μg before the extraction procedure. The extracts containing camalexin and glucosinolates were deposited on anion-exchange columns. Columns were sequentially washed with some eluents to prepare them to the desulfation process of glucosinolates. All these columns washes were collected then freeze-dried giving fractions containing camalexin. These fractions were analysed in HPLC-FLD (Fig. 2) and the percentage of recovery was calculated with the calibration curve between 98 and 117% with an RSD between 3 and 21% (Table 1).

Finally, a down scaled was carried out from 50 to 10 mg of DM of one of the *A. thaliana* natural population leaf samples containing a medium quantity of camalexin. Quantification of camalexin were

homogeneous for starting materials from 50 to 20 mg while below 20 mg the sampling appeared too small to be reproducible (Fig. 5).

This method was linear from 0.025 to 5 $\mu\text{g}\cdot\text{mL}^{-1}$ with an LOD and LOQ of 0.014 and 0.046 $\mu\text{g}\cdot\text{mL}^{-1}$, respectively. It was precise and true and can therefore be used from 20 to 50 mg of samples.

3.3 Application of the method

The applicability of the method was evaluated by screening for glucosinolates and camalexin in different *Brassicaceae* genera (*Arabidopsis*, *Barbarea*, *Brassica*, *Capsella*, *Cardamine*, *Coincya*, *Sinapis* and *Sisymbrium*), genotypes of *A. thaliana* (natural population, ecotypes Col and Ws) and organs (leaves and seeds) and with plants inoculated or not with *A. brassicicola*.

According to previous results (see 3.1 and 3.2), these investigations were performed on 20 to 50 mg of DM as starting material and each sample was tested 2 to 11 times (depending on the sample availability). Glucosinolates were identified by MS data and retention times compared to the references, considering both rapeseed reference materials and *A. thaliana* Col seeds as authentic references (Table A4). Apparent glucosinolates not available as references were tentatively identified from MS data, reasonable retention times compared with the literature [38], and comparison with previous investigations of these species, as discussed below. Concentrations (total and individual) were calculated with the calibration curve of sinigrin and the UV response factor acquired at 229 nm. Camalexin was identified according to its retention time and its concentration was calculated with the calibration curve.

The range in total glucosinolates content was quite large from 0.2 to 92.4 $\text{mmol}\cdot\text{kg}^{-1}$ with an average RSD of 7% (Fig. 6). As expected [39], the total concentration of glucosinolates was higher in seeds (18.4 to 92.4 $\text{mmol}\cdot\text{kg}^{-1}$) compared to leaves (0.2 to 18.2 $\text{mmol}\cdot\text{kg}^{-1}$). For a given *Brassicaceae* species, seeds contained 2 to 20 times more glucosinolates than leaves. *Brassica nigra* was the species with the highest concentration of glucosinolates in the leaves (18.2 $\text{mmol}\cdot\text{kg}^{-1}$) and *C. monensis* the species with the highest concentration in the seeds (92.4 $\text{mmol}\cdot\text{kg}^{-1}$). Similarly, the seeds of the two *A. thaliana*

ecotypes Col and Ws, showed a 100-fold higher concentration of glucosinolates than leaves, while the concentration in the seeds was similar between the two ecotypes, Col (51.1 mmol.kg⁻¹) and Ws (58.6 mmol.kg⁻¹).

From 89 to 100% of the individual glucosinolates were identified or tentatively identified in different samples (Table 2). More precisely, 40 glucosinolates were (tentatively) identified and quantified in 21 samples including 31 response factors. In accordance with previously published reports, experiment data confirmed that leaves and seeds of *S. arvensis* mostly contained sinalbin and long chained methionine derived glucosinolates [39,40], while sinigrin was predominant in leaves and seeds of *B. nigra* [39,40]. Similarly, seeds of *C. bursa-pastoris* mostly contained glucoarabin and glucocamelinin [41], seeds of *S. officinale* exhibited high yields of glucopturanjivin [39], and leaves of *C. hirsuta* mostly gluconapin, glucobrassicinapin and glucotropaeolin [42]. Leaves and seeds of *B. vulgaris* contained mostly one glucosinolate. Four isomers with the side chain formula C₈H₉O have been reported from *B. vulgaris* [42], but usually the dominant isomer is glucobarbarin [43,44]. However, for some samples, the composition in glucosinolates differed from results reported in previous studies. Mostly sinigrin was found in the leaves of *C. bursa-pastoris* as it is sometimes referenced in seed samples [45]. Leaves of *S. officinale* mostly contained glucobrassicin and not a 65/35 ratio of glucopturanjivin and glucobrassicin as previously described [43]. In a previous study, on leaves of *C. monensis*, sinalbin, gluconapin, progoitrin, epiprogoitrin and sinigrin were detected [40]. Our seed samples mostly contained progoitrin and epiprogoitrin while in the leaves, besides small quantities of sinalbin and gluconapin, the main glucosinolate was tentatively identified as glucoaubrietin generally found in the genera *Tropaeolum* and *Aubretia* [39,40].

Unlike the previously discussed species, seeds of *A. thaliana* exhibit a diverse array of individual glucosinolates, with principally sulfur-containing side-chains [46–48]. The main compound, glucoerucin or glucoibervirin, representing 40% of the total glucosinolates content was different in the ecotypes Col and Ws, respectively. Although in trace quantities, presence of late-eluting acylated glucosinolates is well-established in seeds of *A. thaliana* [49] and *B. vulgaris* [50]. We searched for the

expected peaks using extracted ion chromatograms with negative result. We concluded that either our material did not contain these glucosinolates, or our conditions were not suitable for detection of acylated glucosinolates, which are known to require higher elution volumes from anion-exchange columns after desulfation process than other glucosinolates.

Leaves of *A. thaliana* natural population showed a high diversity of glucosinolates with indol-3-ylmethyl backbone, methylthio and methylsulfinylalkyl side-chains. Glucoraphanin was the main compound in all samples (50%) [46,48,50]. There was no significant change in the total glucosinolates content nor in the proportion of the 12 identified glucosinolates, following inoculation with *A. brassicicola*. The concentration of glucosinolates in the ecotypes Col and Ws remained extremely low after inoculation and did not differ from non-inoculated samples. This was a quite interesting observation as the pathogen was reported to trigger glucosinolates production [51]. However, this is in line with some reports mentioning no accumulation of glucosinolates after fungal inoculation. The effect might be linked to several factors, such as the kinetics of induction, environmental conditions, and the pathogen [52,53].

Camalexin was not detected in any seed sample, which is in line with results from by Rauhut *et al.* [54]. Likewise and confirmed by previous reports for some, camalexin was not found in the leaves of *B. vulgaris*, *B. nigra*, *C. bursa-pastoris* [55], *C. hirsuta* [56], *C. monensis*, *S. arvensis* and *S. officinale*. Conversely, all leaf samples of *A. thaliana* (except for the non-inoculated leaves of ecotype Ws) contained some camalexin. As expected, the inoculation with *A. brassicicola* triggered camalexin production, increasing its overall content from less than 2.8 $\mu\text{g}\cdot\text{g}^{-1}$ to 30.9 $\mu\text{g}\cdot\text{g}^{-1}$ (Fig. 7). With regards to the natural population and the ecotype Col of *A. thaliana*, non-infected samples showed very similar basal concentrations of camalexin of 2.7 and 2.8 $\mu\text{g}\cdot\text{g}^{-1}$, respectively. After inoculation, the concentration increased 11-fold to 30.9 $\mu\text{g}\cdot\text{g}^{-1}$ for the natural population and 7-fold to 20.2 $\mu\text{g}\cdot\text{g}^{-1}$ for ecotype Col, respectively. In the Ws ecotype, no camalexin was detected before inoculation. Inoculated samples then showed 3.3 $\mu\text{g}\cdot\text{g}^{-1}$ of the compound, which is in the range of the basal level of the two other ecotypes. The constitutive presence of camalexin in some ecotypes of *A. thaliana* has

been documented [57] as well as the lower camalexin content for Ws compared to the Col ecotype [58]. In addition to the variability between genotypes, the levels of camalexin measured in this study and the increase after infection were consistent with data from the literature [33,59,60]. It should nevertheless be kept in mind that the production of camalexin seems greatly dependent on a number of factors, including plant development stage, time of harvest after inoculation, and environmental conditions [54,58,61].

4. Conclusion

The present study proposes a quantification method for the simultaneous routine analysis of glucosinolates (total and individual) and camalexin adapted to small-sized samples from *Brassicaceous* plants. The protocol is based by simultaneous liquid extraction and combined purification prior to HPLC-UV-MS and HPLC-FLD approach. The linearity of the method for glucosinolates and camalexin were in the range of 50 to 3500 μM and 0.025 to 5 $\mu\text{g}\cdot\text{mL}^{-1}$, respectively. LOD/LOQ based on UV absorption and fluorescence detection were 3.8/12.6 μM and 0.014/0.046 $\mu\text{g}\cdot\text{mL}^{-1}$. This method was proven precise, accurate and true and can therefore be used from 20 to 50 mg of DM starting material, requiring as little as 60 mg for a triplicate analysis. The applicability of the proposed method was evaluated by the identification and quantification of glucosinolates and camalexin in different *Brassicaceae* genera, *A. thaliana* genotypes, organs (leaves and seeds), and after pathogen inoculation. The results were comparable to those found in the literature, which confirms the precision of our method.

The proposed concept not only allows characterizing glucosinolates and camalexin from single small-sized samples it also significantly reduces experimental time and the overall costs of analysis. Obtaining qualitative and quantitative information on phytoalexins and phytoanticipins from a single small-sized sample is a crucial element for a better understanding of the role of these compounds in the *A. brassicicola*-*Brassicaceae* interactions. This particularly concerns early developmental stages of the black spot cycle, in which important changes in the content of glucosinolates and camalexin are

expected. Such analyses are essential for all *Brassicaceae* species including those with small seeds, or with high concentrations of seed reserves.

CRedit authorship contribution statement

Dimitri Bréard: Conceptualization, Methodology, Validation, Investigation, Writing - Original Draft Preparation, Visualization. **Thibault Barrit:** Resources. **Daniel Sochard:** Resources. **Sophie Aligon:** Resources. **Elisabeth Planchet:** Writing - Review & Editing, Supervision. **Béatrice Teulat:** Writing - Review & Editing, Supervision. **Josiane Le Corff:** Resources, Writing - Review & Editing, Supervision. **Claire Campion:** Writing - Review & Editing, Supervision, Project administration, Funding acquisition. **David Guilet:** Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Appendix A. Supplementary data

Supplementary data to this article can be found online.

Acknowledgments

This research was funded by SFR Quasav (BRAf project 2018-2020). The authors also acknowledge Michaël Anjuere for manufacturing the columns rack, Pascale Satour for use of the freeze-dryer, PT Phyto (SFR Quasav) and PT Astral (SFR Matrix) for instruments, Andreas Schinkovitz and Fanny Schurrer for English corrections as well as Elza Neau and Marie-Anne Portela for inspiring the graphical abstract.

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Figures and Tables

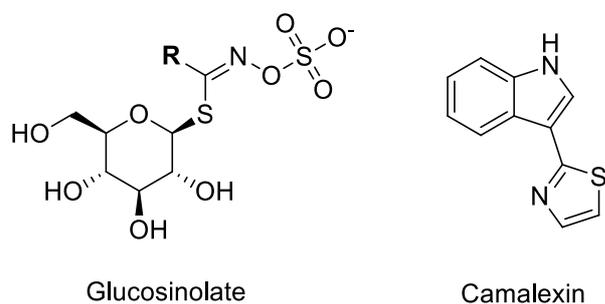


Fig. 1. General structure of glucosinolates with biological variation predominantly due to a substitution of side group R but derivatives at the thioglucose moiety are also known (137 identified whose 88 conclusively characterized) [24] and camalexin.

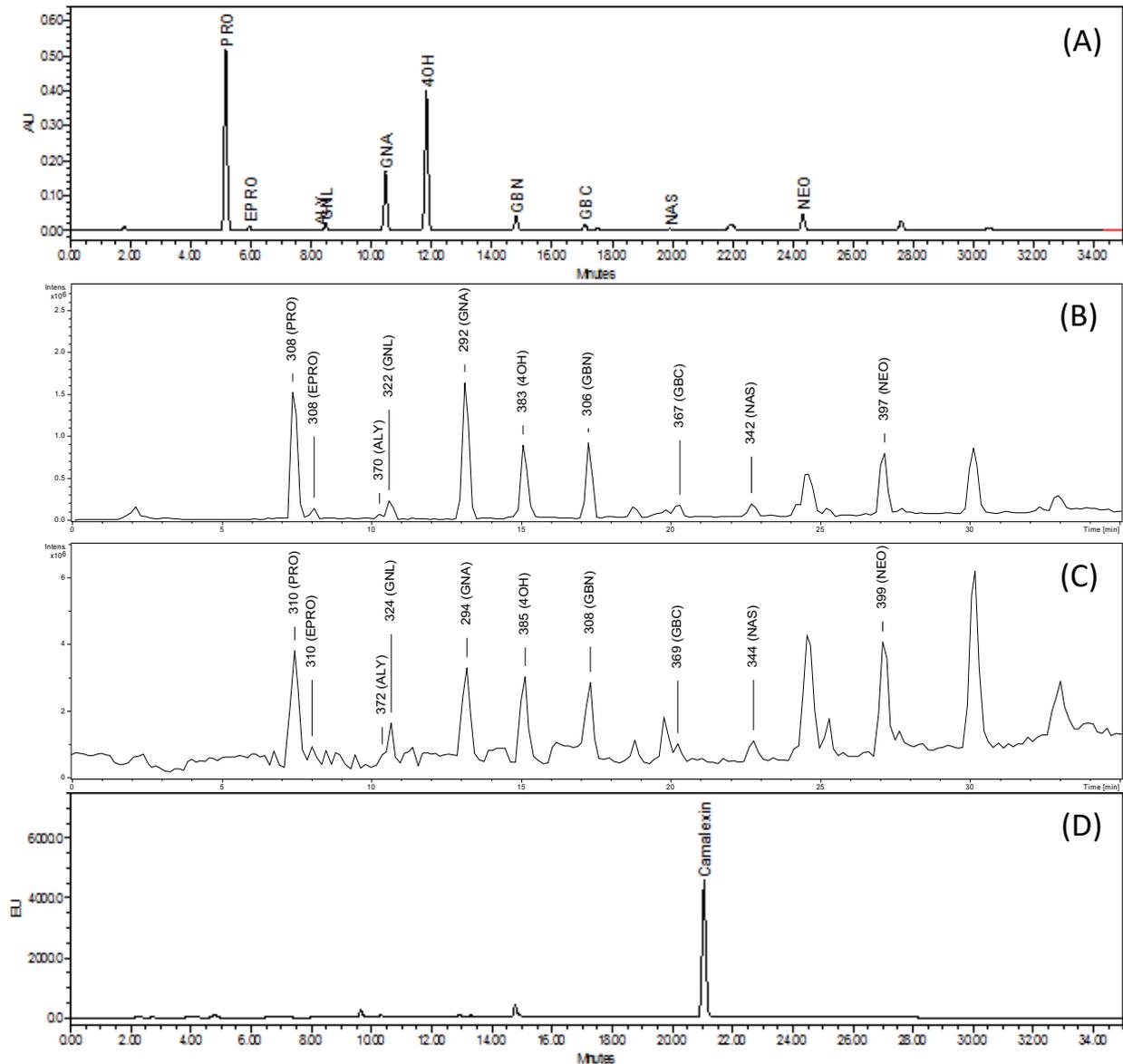


Fig. 2. Examples of HPLC analysis of desulfo-glucosinolates and camalexin fractions. (A) HPLC-UV chromatogram at 229 nm with corresponding glucosinolate, (B) total ion chromatogram in negative mode and (C) total ion chromatogram in positive mode with corresponding m/z and glucosinolate of a certified rapeseed sample. Progoitrin (PRO), epiprogoitrin (EPRO), glucoalyssin (ALY), gluconapoleiferin (GNL), gluconapin (GNA), 4-Hydroxyglucobrassicin (4OH), glucobrassicinapin (GBN), glucobrassicin (GBC), gluconasturtiin (NAS), neoglucobrassicin (NEO). (D) HPLC-FLD chromatogram at 318/385 nm as excitation/emission wavelengths of an *A. thaliana* natural population sample containing camalexin.

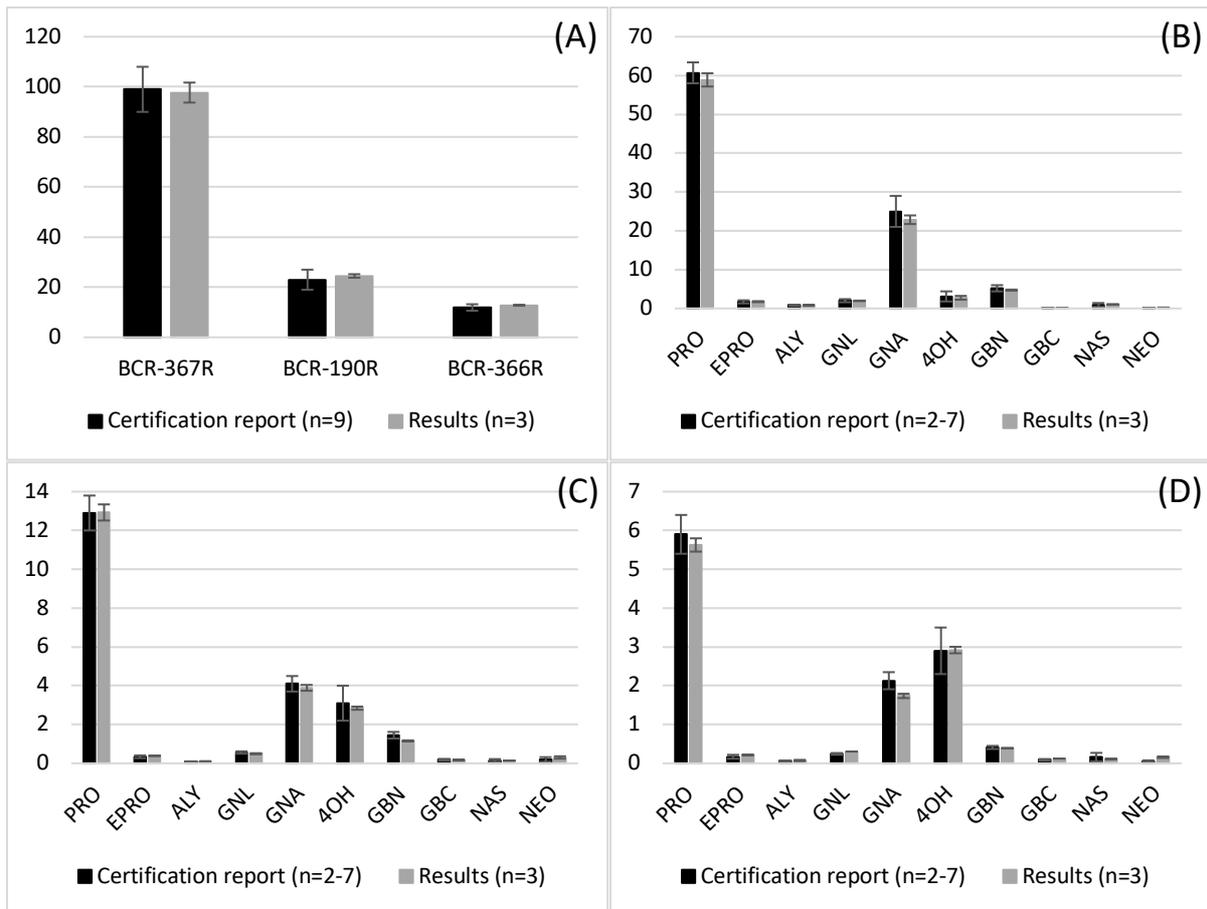


Fig. 3. Comparison of glucosinolates content in mmol.kg⁻¹ of DM between certification report of European method and our results with 80 mg of starting material. (A) Total glucosinolates content for BCR-367R (rapeseed with high level of glucosinolates), BCR-190R (medium level) and BCR-366R (low level). (B) Individual glucosinolates content for BCR-367R. (C) Individual glucosinolates content for BCR-190R. (D) Individual glucosinolates content for BCR-366R.

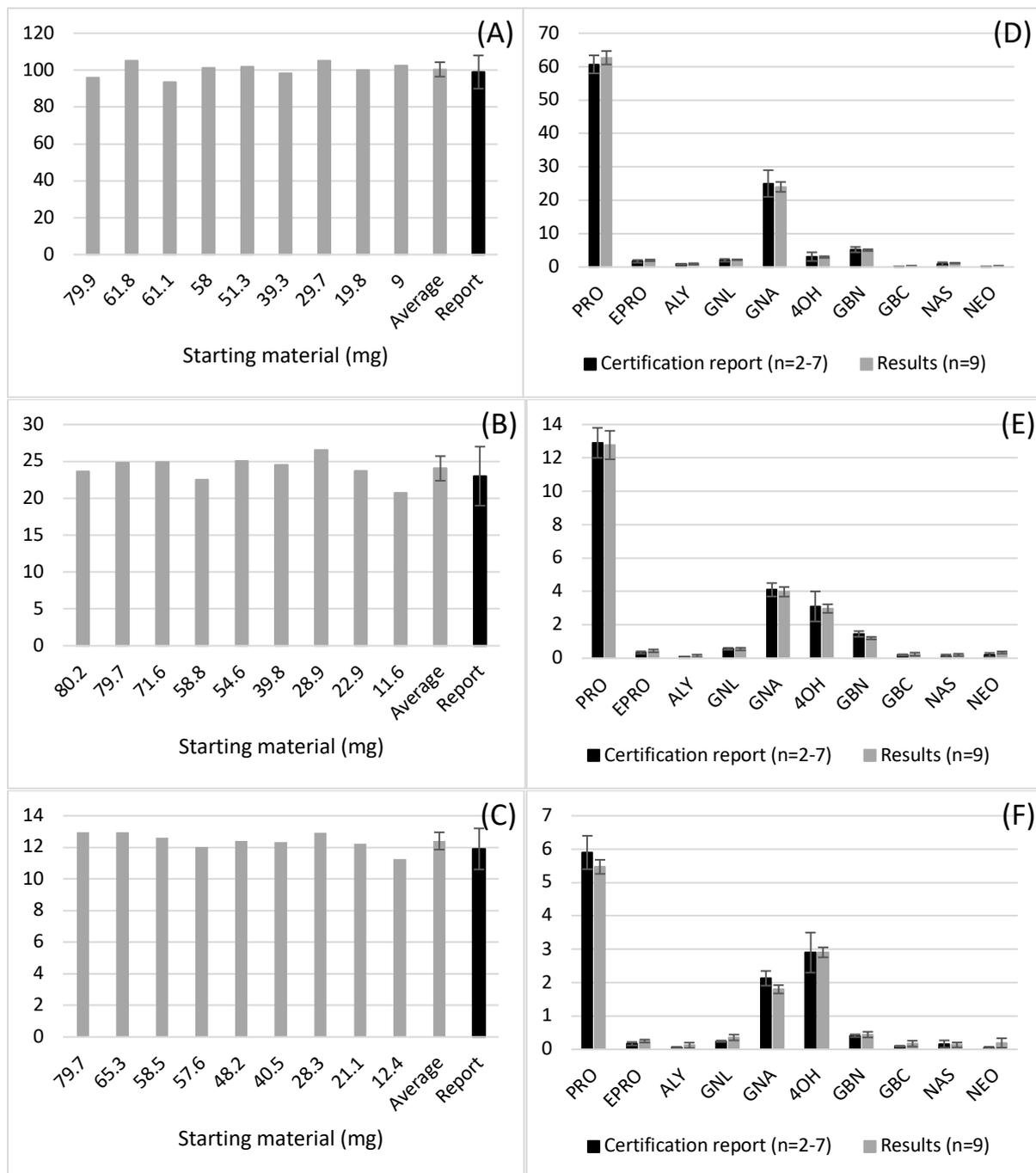


Fig. 4. Down scaled results in mmol.kg^{-1} of DM and comparison with certification report of European method. Total glucosinolates content for (A) BCR-367R, (B) BCR-190R, (C) BCR-366R with starting material from 10 to 80 mg. Individual glucosinolates content for (D) BCR-367R, (E) BCR-190R, (F) BCR-366R with down scaled process.

Table 1

Recovery of camalexin from rapeseed samples spiked at different levels (n=3).

Spiked quantity (μg)	Recovery (%)	RSD (%)
2.00	110	18
1.50	101	3
1.03	99	8
0.77	104	9
0.39	117	9
0.26	108	4
0.10	98	21
0.05	100	4

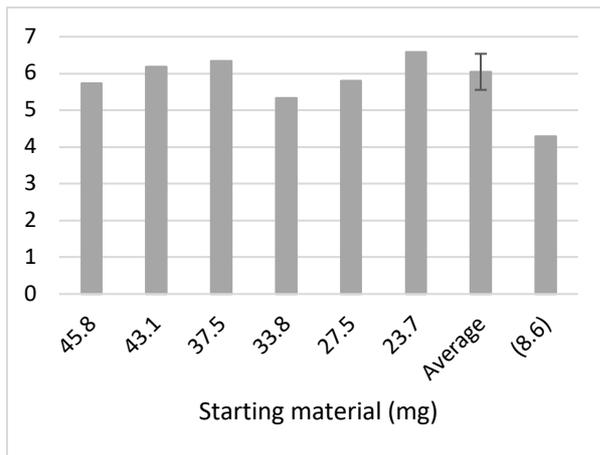


Fig. 5. Down scaled results in $\mu\text{g.g}^{-1}$ of DM for *A. thaliana* natural population leaf samples containing a medium quantity of camalexin. Camalexin content with starting material from 8 to 45 mg. The value between brackets was not included in the average.

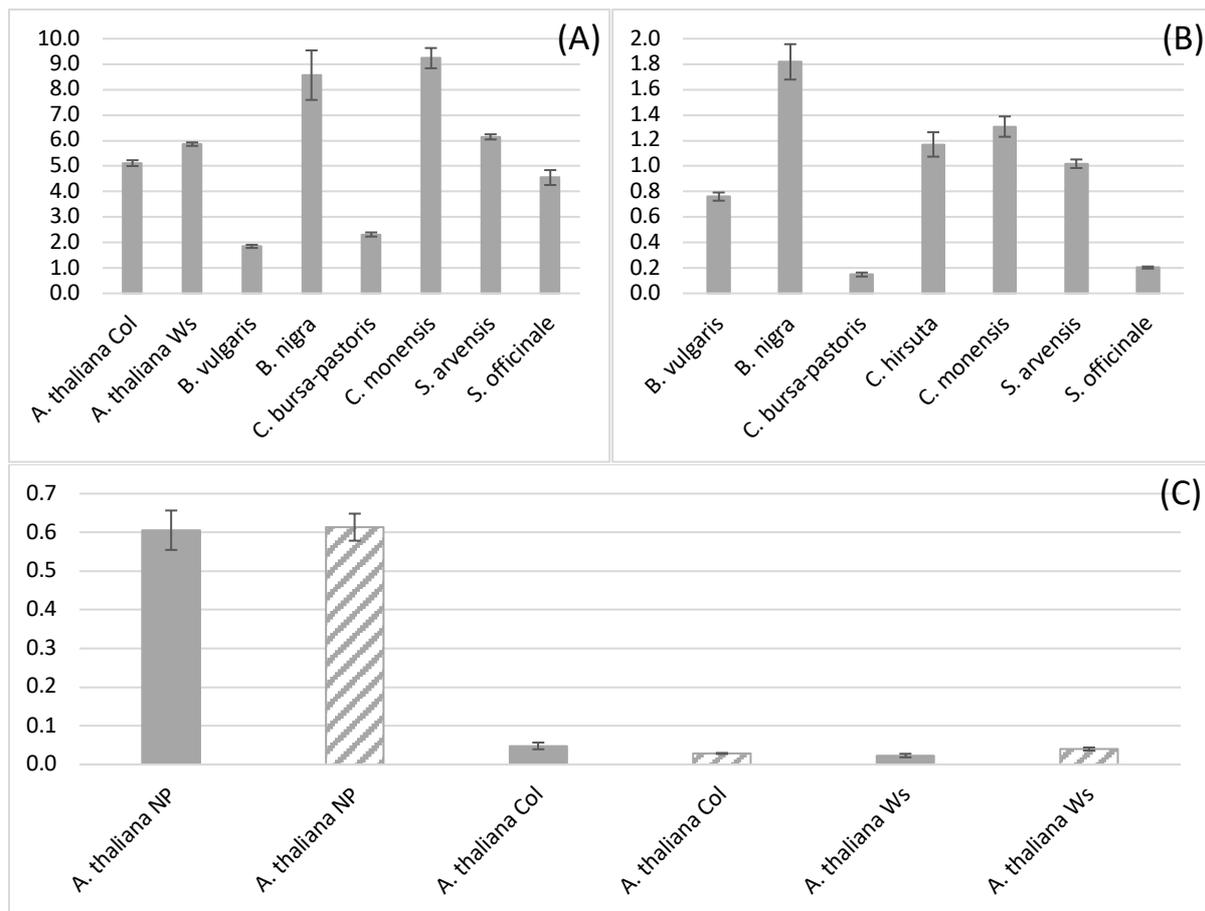


Fig. 6. Total glucosinolates content in mmol.kg⁻¹ of DM in our sample panel. (A) Comparison of seed samples. (B) Comparison of leaf samples. (C) Comparison of leaf samples inoculated (hatched bar plots) or not (full bar plots) with *A. brassicicola*.

Table 2

Individual glucosinolates content and percentage of identification of glucosinolates content in each sample of our panel (ranged by retention time).

	<i>A. thaliana</i> NP		<i>A. thaliana</i> Col			<i>A. thaliana</i> Ws			<i>B. vulgaris</i>		<i>B. nigra</i>		<i>C. bursa-pastoris</i>		<i>C. monensis</i>		<i>S. arvensis</i>		<i>S. officinale</i>		<i>C. hirsuta</i>	
	L	IL	L	IL	S	L	IL	S	L	S	L	S	L	S	L	S	L	S	L	S	L	
3-Hydroxypropyl GSL	-	-	-	-	0.5	-	-	3.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucobriferin (*)	0.5	0.5	-	blq	-	blq	blq	2.9	-	-	-	-	blq	-	-	-	-	-	-	-	-	-
4-Hydroxybutyl GSL	-	-	-	-	2.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Progoitrin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	37.9	-	-	-	-	-	-	-
Glucoraphanin	3.1	3.0	-	-	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Epiprogoitrin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	49.0	-	-	-	-	-	-	-
Sinigrin	-	-	-	-	-	-	-	-	-	-	17.5	82.3	1.3	-	-	-	-	-	-	-	-	-
Glucosylsin	0.1	0.1	-	-	blq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucanapoleiferin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	blq	-	-	-	-	-	-	-
Glucoputranjivin (*)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	42.7	-	-
Sinalbin (*)	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	blq	8.0	47.3	-	-	-	-	-
Glucanapin	-	-	-	-	-	-	-	-	-	-	blq	-	-	0.1	-	-	-	-	-	-	-	1.5
Glucosylsin	0.1	0.1	-	-	blq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4-Hydroxyglucobrassicin	-	-	-	-	-	-	-	-	-	-	blq	-	-	blq	4.6	-	blq	-	-	-	-	-
Glucobervirin	-	-	-	-	blq	-	-	23.4	-	-	blq	-	-	-	-	-	-	-	-	-	-	-
Glucocochlearin (*)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	blq
Epiglucoabarin (*)	-	-	-	-	-	-	-	-	0.2	0.4	-	-	-	-	-	-	-	-	-	-	-	-
2-Methylpropyl GSL (*)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.3	-
Glucobarbarin (*)	-	-	-	-	-	-	-	-	6.6	17.3	-	-	-	-	-	-	-	-	-	-	-	-
(R)-7-(Methylsulfinyl)heptyl GSL	0.1	0.1	-	-	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucobrassicinapin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.5
Glucotropaeolin (*)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.7
Glucosylsin	0.3	0.4	-	-	21.0	-	-	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucosylsin	-	-	-	-	-	-	-	-	-	-	-	-	-	12.7	-	-	-	-	-	-	-	-
Glucobrassicin	0.5	0.5	blq	blq	1.0	blq	blq	-	0.4	blq	blq	-	-	-	blq	blq	blq	1.8	-	-	-	0.7
Glucosylsin	0.5	0.6	-	-	4.1	-	-	3.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8-(Methylsulfonyl)octyl GSL (*)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5	3.1	-	-	-	-
4-Methoxyglucobrassicin (*)	0.6	0.7	0.5	0.2	-	0.2	0.3	-	blq	-	blq	-	-	-	blq	blq	-	blq	-	blq	-	-
Glucoberteroin	-	-	-	-	1.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucosylsin	-	-	-	-	-	-	-	-	0.4	0.4	blq	-	-	-	-	-	-	-	-	-	-	0.7
Glucosylsin	-	-	-	-	-	-	-	-	-	-	-	-	blq	11.3	-	-	blq	0.6	-	-	-	-
9-(Methylsulfonyl)nonyl GSL (*)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.2	8.6	-	-	-	-
Neoglucobrassicin	0.1	0.1	-	-	-	-	-	-	-	-	blq	-	-	-	-	-	-	-	-	blq	-	-
Glucosylsin	-	-	-	-	3.6	-	-	10.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucosylsin	-	-	-	-	-	-	-	-	-	-	-	-	blq	11.3	-	-	blq	-	-	-	-	-
10-(Methylsulfonyl)decyl GSL (*)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	1.2	-	-	-	-
4-(Benzoyloxy)butyl GSL	-	-	-	-	6.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7-(Methylsulfonyl)heptyl GSL	0.1	0.1	-	-	2.6	-	-	1.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5-(Benzoyloxy)pentyl GSL	-	-	-	-	blq	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8-(Methylsulfonyl)octyl GSL	0.1	0.1	-	-	3.6	-	-	8.8	-	blq	-	-	-	-	-	-	-	-	-	-	-	-
GSL identification (%)	99	99	100	100	92	94	92	94	100	98	96	96	97	98	100	99	99	99	89	99	97	97
Total GSL content (mmol.kg ⁻¹)	6.1	6.1	0.5	0.3	51.1	0.2	0.4	58.6	7.6	18.4	18.2	85.7	1.5	23.1	13.1	92.4	10.2	61.5	2.0	45.5	11.7	11.7
SD (mmol.kg ⁻¹)	0.5	0.3	0.1	0.0	1.1	0.0	0.0	0.7	0.3	0.6	1.4	9.7	0.2	0.8	0.8	4.0	0.3	1.0	0.1	2.9	1.0	1.0
RSD (%)	8	6	18	4	2	20	10	1	4	3	8	11	11	4	6	4	3	2	4	6	8	8

Glucosinolate (GSL). Below limit of quantification (blq) is between LOD and LOQ. Tentatively identified

(*)

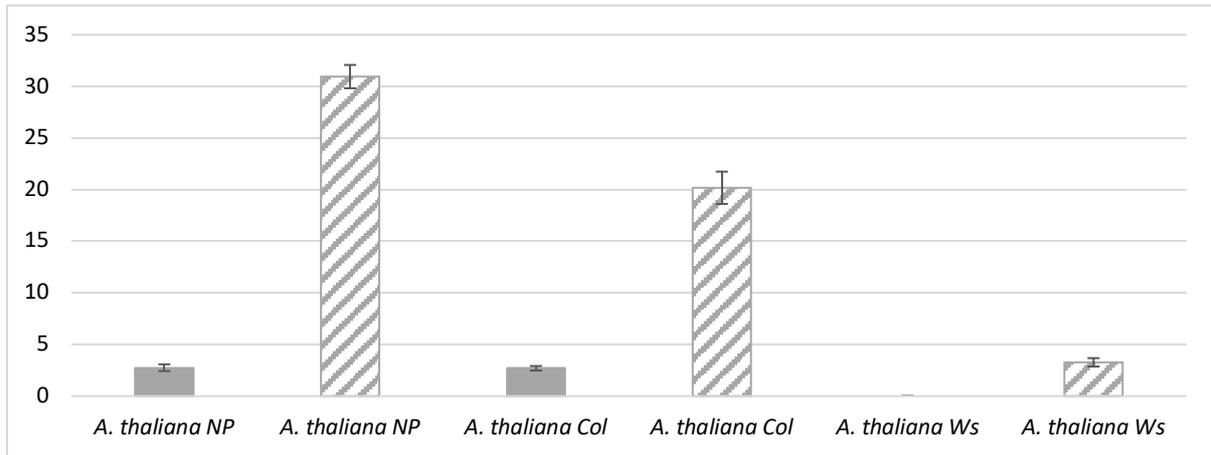


Fig. 7. Comparison of camalexin content in $\mu\text{g.g}^{-1}$ of DM in leaf samples inoculated (hatched bar plots) or not (full bar plots) with *A. brassicicola*.