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RESEARCH ARTICLE

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Analysis of 3-nitropropionic acid in Fabaceae plants by HPLC-MS/MS

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Abstract

Introduction: 3-Nitropropionic acid (3-NPA) is a toxic compound that can accumulate in esterified form in the Fabaceae family. In the Lotae tribe, many species have been identified as 3-NPA producers (e.g., *Securigera varia*), while some of the genetically close Lotae plants were formerly reported as 3-NPA-free (e.g., *Lotus corniculatus* and *Anthyllis vulneraria*). These plants are used as forage and have a tradition in ethnomedicine, also, the extracts of *A. vulneraria* are used in cosmetics.

Objectives: Our aim was to investigate the 3-NPA content of these selected Fabaceae species and to develop a validated quantitative method to evaluate 3-NPA concentrations in extracts of different herbal parts and cosmetic products.

Materials and Methods: A UHPLC-ESI-Orbitrap-MS/MS method was applied for detection and identification of 3-NPA derivatives in the form of glucose esters. For the quantitative analysis, an optimized sample processing method was developed. The free 3-NPA content was determined using HPLC-ESI-MS/MS.

Results: 3-NPA esters could be detected in all three species, but their quantity showed a high variation. *S. varia* contained 0.5–1.0 g/100 g of 3-NPA, while in *L. corniculatus* samples only trace quantities were detectable, below the LOQ (25 ng/ml). Most of the *A. vulneraria* samples showed similarly low concentrations, but one sample had 3-NPA levels comparable to *S. varia*. 3-NPA could not be detected in the tested cosmetics containing *A. vulneraria* extracts.

Conclusions: Using highly sensitive analytical methods, new 3-NPA-containing species were identified. The developed validated quantitative method is suitable for the determination of 3-NPA concentrations in herbal samples.

KEYWORDS
3-nitropropionic acid, Fabaceae, HPLC, MS/MS, quantitative determination

1 | INTRODUCTION

3-Nitropropionic acid (3-NPA) is a suicide inactivator of succinate dehydrogenase, blocking the respiratory electron transfer chain and causing misfunctioning of mitochondria.[1] Since neurons are the most sensitive cells to this effect, intoxication can cause damage to basal ganglia, the hippocampus, spinal tracts, and peripheral nerves.[2] This effect is most prominent in the neurons of the striatum.[2–4] As a consequence, 3-NPA is widely used as a neurotoxin to model the symptoms and neuronal lesions of Huntington's disease in animal models.[5,6] The no observed adverse effect level (NOAEL) of NPA is 2.5 and 3.75 mg/kg/day for male and female rats, respectively. Based on the NOAEL of the chronic rodent bioassay, an acceptable daily intake (ADI) of 25 μg/kg/day, or 1.750 mg/day for a human weighing 70 kg, was defined. The central nervous system can suffer irreversible changes upon receiving a threshold dose. NPA is not accumulated by the body and sub-threshold doses do not cause irreversible damage.[7]

3-NPA is produced by species of all four kingdoms of life – bacteria, fungi, animals, and plants. It has a general protective role against herbivores (in plants) and predators (in insects), although no studies are available investigating the evolution of the interactions between the organisms producing 3-NPA.[8] As a mycotoxin, it is synthesized by the genera *Aspergillus*, *Arthrinium*, *Diaporthe*, *Phomopsis*, and *Penicillium*,[8] causing severe and sometimes fatal human intoxications.[9] Most frequently, poisoning from 3-NPA as a mycotoxin happens after the consumption of moldy sugarcane,[10,11] but its presence could also be confirmed in hazelnuts, peanuts,[12] and coconut.[13] 3-NPA can be found in most fungus species in a free form, but an ester derivative (4-hydroxyphenethyl 3-nitro-propanoate) is also known from the endophytic fungus *Phomopsis* sp.[8]

Regarding plants, the occurrence of 3-NPA derivatives is most frequent in the Fabaceae family, as it can be found in the genera *Astragalus*, *Coronilla*, *Hippocrepis*, *Indigofera*, *Lotus*, *Securigera*, and *Scorpiurus*. *Hiptage madablota* (Malpighiaceae) and *Corynocarpus laevigata* (Corynocarpaceae) are well-known sources too.[8] Previous studies indicated that 3-NPA derivatives are localized specifically in the aerial parts of the plants[14,15]; however, they have been detected in roots as well.[16] The majority of 3-NPA can be found as esters, as it forms mono-, di-, tri-, and tetraesters with a glucose moiety.[17–19] This is a protective and neutralizing mechanism of the plant, defending itself from the produced 3-NPA.[8] Another source of 3-NPA is miserotoxin,[20] which is a glycoside of 3-nitropropanol. The esters and glycosides on their own are not inhibitors of succinate dehydrogenase; however, during digestion, the ester and glycosidic bounds are hydrolyzed and 3-NPA is released, causing poisoning in many species, e.g., horses, sheep, and cattle.[21–23] Due to differences in the gastrointestinal system between different species (ruminants vs. non-ruminants), the intoxication caused by these esters and glycosides can be of varying severity.[9]

The presence of 3-NPA esters in crown vetch (*Securigera varia* (L.) Lassen syn. *Coronaria varia* L.) has been known for a long time. Coronillin, coronarian, and coronillin were isolated from the plant in 1976.[18] *S. varia* is a perennial, creeping plant, widespread on the pastures and meadows of Europe and Asia.[17] As a nitrifying plant, it could be a valuable member of pasture communities; however, excessive consumption by grazing animals causes symptoms of poisoning.[24] In ethnomedicine, its seeds were used as a cardiotonic based on their cardenolide content,[25,26] while other research groups identified flavonoids in aerial parts of the plant.[17,26] The genus *Securigera* is in the same tribe (Lotae) as the genera *Lotus* and *Anthyllis*. This fact foreshadows the possibility of the occurrence of 3-NPA derivatives in these taxa, too. Previous studies investigated the 3-NPA content in species of the genera *Lotus* and *Anthyllis* and found 3-NPA derivatives in some *Lotus* species (i.e., *Lotus angustissimus*, *Lotus pedunculatus*, and *Lotus uliginosus*), but not in kidney vetch (*Anthyllis vulneraria* L.) or other *Lotus* species.[15] Birdsfoot trefoil (*Lotus corniculatus* L.) is a perennial plant native to grasslands of Europe and Asia and can be found on fields or even at roadsides and used as a forage plant.[27] Based on phytochemical analysis, it contains condensed tannins,[28] isoflavonoids,[29] and flavonoids.[30,31] 3-NPA derivatives have not been identified in this taxon before. *A. vulneraria* is widespread in Europe and North Africa; however, it is not as common as *S. varia* as it can be found in undisturbed grasslands and on calcareous soils. Former studies identified isoflavonoids,[29] flavonoids, and saponins in the extracts made from the aerial parts[32]; however, no 3-NPA derivatives have been identified to date. The common names of the plant (woundwort, *Wundklee*) indicate its medicinal use as it was used mainly as a wound-healing agent. In ethnomedicine, it is used internally to treat kidney problems, hence the name "kidney vetch."[33] Based on its traditional wound-healing properties, extracts of the aerial parts are used in various cosmetic products nowadays.

Until the 1980s, 3-NPA derivatives were quantified in biological samples using spectrophotometry after derivatization. In alkali media 3-NPA decomposes, resulting nitrite ions; then, Griess reagent (sulfanilic acid and 1-naphthylamine hydrochloride) can be applied to form a colorful azo dye. The method was optimized by Matsumoto et al. (1961) to be applicable for 3-NPA esters found in plant samples.[34] Later, 3-NPA was measured in various biological samples using HPLC. Since the UV absorbance of 3-NPA is poor, a wavelength of 210 nm was used for detection, which makes the development of the chromatographic method more complicated.[20,21,23,35–38] In light of the aforementioned, it seems obvious to use MS as a detection method. LC-MS/MS was applied as a screening method for detection of hundreds of mycotoxins, including 3-NPA.[12,39] Most of the methods investigate the free 3-NPA (and 3-nitropropanol) content (e.g.,[23,36,37]), although the plants of interest produce 3-NPA in the form of glucoside esters, which are cumbersome to analyze. Liu et al. (2017) investigated the liberation of 3-NPA from its esters after reactivation of endogenous enzymes in the plant, but the yield of the hydrolysis and extraction method was not verified.[38] Other research groups applied acidic hydrolysis in order to release 3-NPA from the bound form. However, it resulted not only in the disruption of ester bonds, but also in the partial degradation of 3-NPA.[35]

Our aim was to investigate the 3-NPA contents of *S. varia*, *L. corniculatus*, *A. vulneraria*, and cosmetic products containing *A. vulneraria*. We intended to analyze the presence of 3-NPA glucose

esters in the plant samples. Furthermore, we aimed to develop, optimize, and validate a suitable sample preparation and HPLC-MS/MS-based quantitative method, which enables us to detect 3-NPA with high sensitivity in the previously mentioned Fabaceae species, facilitating their safe cosmetic and medicinal use.

2 | EXPERIMENTAL PROCEDURES

2.1 | General and plant material

Gradient-grade acetonitrile was obtained from VWR International, and LC-MS-grade formic acid was obtained from Fischer Chemicals. Purified water prepared by Millipore Milli-Q equipment was used throughout the study. 3-NPA (purity ≥ 97%) was purchased from Sigma-Aldrich. All other chemicals were of analytical grade. *S. varia* samples were collected from Budapest, Csepel (N 47.392835, E 19.037363), September 2019; Budapest, Hűvösvölgy (N 47.555845, E 18.982506), June 2022; and Dunaegyháza (N 46.833061, E 18.941606), June 2022. *L. corniculatus* samples were collected from Budapest, Soroksár (N 47.392835, E 19.037363), September 2019; Budapest, Hűvösvölgy (N 47.555845, E 18.982506), June 2022; and Dunaegyháza (N 46.833061, E 18.941606), June 2022. *A. vulneraria* samples were collected from Budapest, Hűvösvölgy (N 47.555845, E 18.982506), June 2022; and generously provided by the Botanical Garden of the Hungarian University of Agriculture and Life Sciences (MATE), Soroksár (Hungary, 47.39754, 19.15214), September 2019 and July 2022. Voucher specimens of collected samples were deposited in the Department of Pharmacognosy. The roots were washed to remove soil, and the dried roots were ground. The aerial parts were dried at room temperature and ground without further separation of leaves and stems. Cosmetic products (Dr. Hauschka Facial Toner and Clarifying Toner) were manufactured by Wala GmbH.

2.2 | Preparation of in vitro samples of *A. vulneraria*

Seeds were obtained from Szentesi-Mag Ltd. The seeds were mixed with 0.01% (v/v) Tween 80 solution and then washed under running water for 10 min. Surface sterilization was conducted using 75% solution of commercial sodium hypochlorite, shaking the samples for 10 minutes. The seeds were rinsed with sterile distilled water three times. The seeds were planted onto Murashige–Skoog medium solidified with 0.7% agar.[40] The plants were transferred to fresh medium every 4 to 5 weeks. At harvest, their total age was 6 months (counted from germination) and 4 weeks passed from the last transfer. Residual medium was removed from the samples, then the roots and aerial parts were separated and frozen. Before analysis, the samples were dried at room temperature and ground.

2.3 | Preparation of samples for screening

From the ground plant material, 0.500 g was mixed with 30 ml of 70% aqueous methanol and extracted in an ultrasonic bath for 10 minutes at 25°C. After filtration, the sample was dried under vacuum with a rotary evaporator (Heidolph Instruments) at 60°C. The resulting residue was redissolved in 2 ml of 70% aqueous methanol. In case of aerial parts rich in chlorophyll, the samples were further purified on a C18-E (Strata, Phenomenex, 3 ml/200 mg) SPE column. SPE microcolumns were activated with 3.0 ml methanol and water before the sample was loaded. After air drying the cartridge 1.5 ml methanol was applied to achieve complete elution of the molecules of interest. Prior to HPLC analysis the eluted extract was filtered through a 0.22 μm PTFE filter (Nantong FilterBio Membrane Co., Ltd.).

2.4 | UHPLC-ESI-Orbitrap-MS/MS conditions

For obtaining high-resolution MS data of the root and aerial part extracts, a Dionex Ultimate 3000 UHPLC system (3000RS diode array detector, TCC-3000RS column thermostat, HPG-3400RS pump, SRD-3400 solvent rack degasser, WPS-3000TRS autosampler) hyphenated with a Orbitrap Q Exactive Focus Mass Spectrometer equipped with electrospray ionization (Thermo Fischer Scientific) was used. An Acquity UPLC BEH C18 column (30 × 2.1 mm i.d., 1.7 μm; Waters Corporation) was used, and the mobile phase consisted of 0.1% formic acid in water (eluent A) and a mixture of 0.1% formic acid in water and acetonitrile (20:80, v/v) (eluent B). For chromatographic separation, the following gradient was used: 0–20 min, 5–40% B; 20–21 min, 40–100% B; 21–23 min, 100–5% B. The flow rate was 0.3 ml/min, the column had a temperature of 25°C, and the injected volume was 1.0 μl. DAD spectra were recorded between 250 and 600 nm. The electrospray ionization source was operated in positive and negative ionization mode, and operation parameters were optimized automatically using the built-in software. The working parameters were as follows: spray voltage, 3,500 V; capillary temperature, 256.25°C; sheath gas (N_2), 47.5°C; auxiliary gas (N_2), 11.25 arbitrary units; and spare gas (N_2), 2.25 arbitrary units. The resolution of the full scan was 70,000, and the scanning range was from m/z 100 to 900. The presence of 3-NPA derivatives was confirmed by analyzing the extract ion chromatograms of the esters' ammonium adducts.

2.5 | Sample preparation for quantitative analysis

Powdered plant sample (0.150 g) was mixed with 5 ml of purified water and set aside for 24 hours to activate the endogenous hydrolyses. After centrifugation, the supernatant was collected, 5 ml of acetonitrile was added to the samples, and samples were sonicated for 25 minutes. The extraction cycles were repeated multiple times during method optimization, and the final samples were extracted twice. The collected supernatants were completed to 25.0 ml 1:1 water:acetonitrile and

homogenized. *S. varia* samples were diluted 20-fold. The samples were set aside for 2 weeks at −18°C before analysis. Each sample was analyzed in triplicate. The stability during the extraction cycles and sample preparation was investigated using 150 µl 3-NPA standard solution of 10 µg/ml instead of plant material. Optimization studies were conducted using *S. varia* root samples, as this is the richest source of 3-NPA derivatives. Cosmetic products were diluted with acetonitrile to double the volume and filtered before analysis.

2.6 | HPLC-ESI-MS/MS conditions

For chromatographic separation and determination an Agilent 1100 HPLC system (degasser, binary gradient pump, autosampler, column thermostat, and diode array detector; Agilent Technologies) was used hyphenated with an API 2000 Triple Quad system equipped with ESI ion source (Sciex). For quantitative determination of 3-NPA, a Luna C8 column was applied (250 × 4.6 mm, 5 µm; Phenomenex). The mobile phase consisted of 0.1% HCOOH (A) and acetonitrile (B). The following elution gradient was used: 0–8 min, 20–100% B; 8–10 min, 100% B, at a flow rate of 1 ml/min and using a splitter (3/7). The column temperature was set to 40°C. The injection volume was 10 µl. The MS/MS parameters were optimized for 3-NPA as follows: declustering potential, −40 V; focusing potential, 400 V; entrance potential, 7 V; collision energy, −10 V; collision cell exit potential, −4 V. Multiple reaction monitoring (MRM) was used in negative ion mode for the detection of the ions according to the following settings: Q1: 118.0 Da; Q3: 46.0 Da. Ion source parameters were set as follows: curtain gas, 45 psi; collision gas, 6 L/min; ion spray voltage, −4,000 V; temperature, 350°C; ion source gas 1, 50 psi; ion source gas 2, 50 psi.

2.7 | Preparation of stock solutions, calibration standards, and quality control samples, validation

Stock solutions of the standard were prepared dissolving 3-NPA in purified water, obtaining a 1 mg/ml solution. Calibration standards were prepared by diluting the stock solution with 1:1 water:acetonitrile. The 10-point calibration curve was prepared using concentrations of 6 µg/ml, 3 µg/ml, 1 µg/ml, 600 ng/ml, 300 ng/ml, 100 ng/ml, 60 ng/ml, 30 ng/ml, 10 ng/ml, and 6 ng/ml. Quality control (QC) samples were prepared separately from the stock solution at nominal concentrations of 5 µg/ml, 500 ng/ml, and 50 ng/ml. Each sample and concentration were analyzed in triplicate. Defining recovery values, extracts were made from a root and an aerial sample of *S. varia* (see above) and spiked with 10 µl of 1 mg/ml 3-NPA standard solution.

3 | RESULTS AND DISCUSSION

3.1 | Screening of 3-NPA derivatives

The collected plant samples were screened using UHPLC-MS/MS to investigate the presence and type of 3-NPA esters. In the chromatogram of *S. varia* extract, using positive ionization mode, the ammonium adducts of 3-NPA esters were the dominant peaks, as expected. However, the esters could be detected both in aerial parts and in the root (Figure 1), which contradicts the theory of Hipkin et al. (2004), who presumed that 3-NPA derivates are only synthesized and stored in the aerial parts.[15] In both samples, mono-, di-, and triesters of 3-NPA with glucose (Figure 2) could be detected in isomeric peaks (Table 1). In the root, denitrated triesters could be detected (Figure 2), which were present in smaller amounts in the aerial parts (Figure 1). The exact structural analysis, i.e., identification of connecting hydroxy groups and their stereochemistry, lies beyond the scope of our work, so only the number of attached 3-NPA groups was determined by MS/MS experiments. *L. corniculatus* has been reported previously to be free of 3-NPA derivatives,[15] but we could detect some 3-NPA esters, although their abundance was orders of magnitudes lower than that in *S. varia* extracts. To our surprise, one of the *A. vulneraria* samples was also found to be a rich source of 3-NPA esters, despite the fact that previous studies could not detect such molecules.[15,32] All compounds listed in Table 1 could be detected in the roots and in the aerial parts of this specimen.

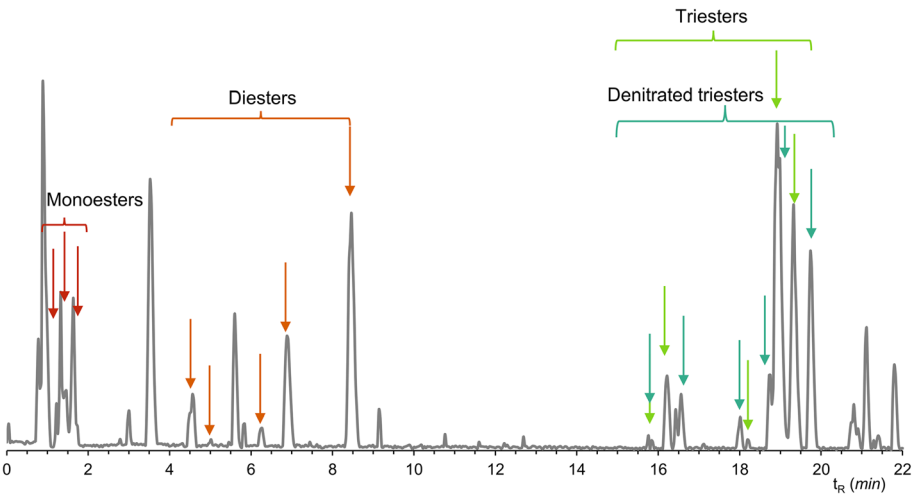

FIGURE 1 Base peak chromatogram of *S. varia* root extract recorded in positive ionization mode, showing the isomeric NPA monoesters (red arrows), diesters (orange arrows), triesters (green arrows), and monodenitrated triesters (turquoise arrows)

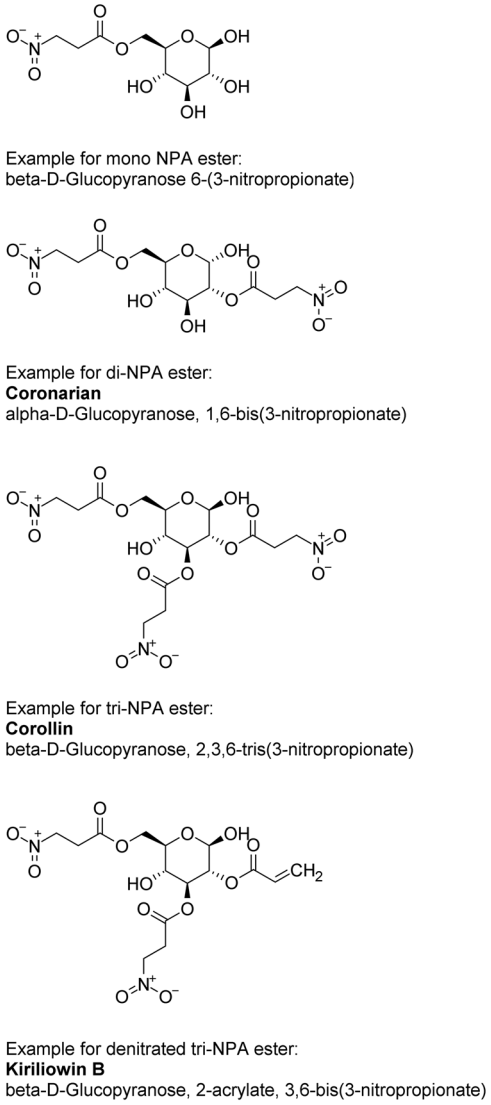

FIGURE 2 Example structures for mono-, di-, and trinitropropanoate esters and a monodenitrated triester

3.2 | Sample preparation for quantitative measurement

3.2.1 | Ester hydrolysis

As 3-NPA can be found in various esterified forms, resulting many isomeric peaks, the first step of the quantitation was to hydrolyze the samples and liberate free 3-NPA. This has not only simplified the chromatographic process, but also models better the toxicity of the plants, for which the released 3-NPA is responsible. Based on the work of Macaskill et al.,[35] using acid-catalyzed hydrolysis, not only the ester bond, but also partially 3-NPA is cleaved. Therefore, alkali-based hydrolysis was tested first, which was applied successfully in our previous work for ester hydrolysis.[41] In the work of Matsumoto et al.,[34] alkalis were used for the liberation of nitrite ions from 3-NPA derivatives, and they concluded that a higher pH and lower temperature result in less denitration. Thus, we used ammonia solution in aqueous medium above pH 12 at room temperature to selectively cleave ester bonds without the further degradation of 3-NPA. Unfortunately, this method was not selective enough, and we observed a major decrease in the level of 3-NPA at the end of the extraction cycles. Therefore, we followed the method of Liu et al.,[38] who exploited the endogenous enzymes of the plant samples for ester hydrolysis, regenerating them in aqueous medium. In their work, they found that a hydrolysis time of 24 h was optimal, as further soaking in water resulted in decreased amounts of 3-NPA due to decomposition. Testing their protocol, we found that aqueous treatment for 24 h significantly decreased the levels of the esters; however, the hydrolysis was still incomplete (Figure 3A and B). As a consequence, quantitative determination after enzyme treatment for 24 h would give an underestimation of the 3-NPA levels in plant samples. Further water treatment (48 h) was not a solution, as the levels of esters did not change significantly compared to 24-h treatment (Figure 3C), and 3-NPA started to show decomposition in water after 72 h. However, we discovered that if our samples in extraction medium (water:acetonitrile 1:1) were kept in the freezer (−18°C) for 2 weeks and re-analyzed, the residual ester peaks disappeared (Figure 3D) without the decomposition of 3-NPA.

3.3 | Extraction

Beside the completeness of ester hydrolysis, we also investigated the efficacy of the extraction methods. We tested the effect of extraction solvent in multiple sonication cycles. Using a pure standard solution, 3-NPA resulted stabile in both water and acetonitrile during five - cycles of extraction. Comparing the two solvents, there was no difference in absolute extractable 3-NPA after five cycles. Acetonitrile was slightly more efficient than water, as it extracted more than 95% of 3-NPA in two cycles (Figure 4). Based on our results, the samples were mixed with water and soaked for 24 h, and then they were extracted with acetonitrile twice. Filtered, diluted solutions were frozen and analyzed after 2 weeks.

3.4 | Optimization and validation of the chromatographic method

As 3-NPA is a small and polar molecule (predicted log P −0.26 by ChemAxon), a general reversed phase C18 stationary phase is not optimal for its chromatographic separation as it shows a very low retention time.[42] Other research groups used an ion exclusion stationary phase[35] or a reversed phase stationary (fluorophenyl) phase using only aqueous eluent with buffer and isocratic run.[38] Our research group applied a C8 reversed phase stationary phase. 3-NPA showed an optimal retention and excellent peak shape on it, eluting around 5 min using a gradient of 0.1% formic acid and acetonitrile (see Figure 3D). This method has the advantage of using an organic solvent throughout the run, which enables coupling with MS and eluting more apolar compounds from the sample without excessive and long washing time, as the total length of the method was 15 min. For the

TABLE 1 Identified 3-NPA derivatives with their observed *m/z* values, formulas, and proposed structures

t_R (min)	$[M + NH_4]^+$ (*m/z*)	Calculated formula	Calculated molecular mass (Da)	Fragment ions (*m/z*)	Structure
1.46	299.1075	$C_9H_{15}NO_9$	281.22	264.0697 (M + H-H_2O), 199.0587 (M + H-2H_2O-HNO_2), 145.0496 (M + H-3H_2O-HNO_2), 127.0396	Glucopyranose, 3-nitropropionate
1.60	299.1075				
1.67	299.1076				
4.56	400.1187	$C_{12}H_{18}N_2O_{12}$	382.09	281.0969 (M + NH_4-NPA), 264.0697 (M + H-NPA), 246.0597 (M + H-NPA-H_2O), 199.0593 (M + H-NPA-H_2O-HNO_2), 127.0397	Glucopyranose, bis (3-nitropropionate)
4.96	400.1190				
6.25	400.1186				
6.82	400.1187				
7.44	400.1187				
8.47	400.1183				
15.85	501.1295	$C_{15}H_{21}N_3O_{15}$	483.10	365.0812 (M + H-NPA), 347.0406 (M + H-NPA- H_2O), 300.0700 (M + H-NPA-H_2O-HNO_2), 246.0598 (M + H-2 NPA), 181.0487 (M + H-2 NPA-HNO_2-H_2O), 127.0387	Glucopyranose, tris (3-nitropropionate)
16.22	501.1296				
18.02	501.1298				
18.92	501.1294				
19.32	501.1294				
15.85	454.1288	$C_{15}H_{20}N_2O_{13}$	435.10	365.0812 (M + H-C_3H_3O), 347.0406 (M + H-C_3H_3O-H_2O), 300.0700 (M + H-C_3H_3O-H_2O-HNO_2), 246.0598 (M + H-C_3H_3O-NPA), 181.0487 (M + H-C_3H_3O-NPA-HNO_2-H_2O), 127.0387	Glucopyranose, monoacrylate, bis (3-nitropropionate)
16.56	454.1288				
17.95	454.1288				
18.72	454.1288				
19.18	454.1288				
19.73	454.1288				

MS/MS method, negative ionization was used, detecting the transition of *m/z* 118 → *m/z* 46, corresponding to the pseudo-molecular ion and the produced nitrite ion. Due to ion source fragmentation, the peaks of 3-NPA esters could be observed too (Figure 3A–C). During sample preparation, no internal standard was used, because the spatial structures of the molecules of interest were hard to mimic. The closest 3-nitro acid commercially available is methyl 3-nitrobutirate, which has very different physicochemical parameters compared to the 3-NPA glucose esters or to 3-NPA itself. As the 3-nitro group is essential for MRM, other non-3-nitro compounds could result in different ionization mechanisms and would not give a parallel signal to 3-NPA.

The calibration was based on the triplicate analysis of each working solution at 10 concentration levels. The calibration curves were plotted using a 1/*x*-weighted polynomial model for the regression of peak area vs. analyte concentration. The determined calibration ranges can be seen in Table 1 along with the regression equations and the coefficients of determination. The limit of detection (LOD) and the limit of quantitation (LOQ) values were determined at 3 and 10 times the signal-to-noise ratio, respectively (Table 2). The concentrations of high, medium, and low QC samples can be seen in Table 3 with the intra- and inter-assay accuracy (deviation from nominal concentration) and precision (relative standard deviation [RSD]) values ($n = 3$). All results fulfilled the requirements of the FDA and EMEA guidelines of bioanalytical method validation, as the accuracy and precision values did not exceed ±15% (Table 2). The recovery value was 98.1 ± 4.6% for the root sample and 86.8 ± 2.4% for the aerial parts. The difference between the values can be a result of the presence of different molecules in the root and in the aerial part. These compounds can cause a minimal ion suppression effect, or as ballast materials, can absorb a fraction of NPA. Although, the standard deviation of the recovery samples correlated with that of the measured samples. These values strengthen the accuracy of the developed method and prove the lack of significant matrix effects.

3.5 | 3-NPA content of samples

The 3-NPA content in the investigated *S. varia* samples showed good correlation with the results of previous studies using spectrophotometry,[16,24] as they report 0.4–1.0 g/100 g values (see Table 4). In the case of the root samples, the variance is low, while in the results of the aerial parts there is an outlier sample (Budapest, Csepel) with a significantly lower value. A possible explanation for this difference can be that this sample was collected in September – however, still flowering – in contrast to the other samples, collected in the summer. Regional differences in the soil and microclimate also cannot be excluded.

Regarding *L. corniculatus* samples, the quantitative results confirmed our Orbitrap-MS/MS observations, since 3-NPA was present, but its concentration was very low, around the detection limit (7.71 ng/ml), meaning less than 0.2 mg/100 g dry weight 3-NPA

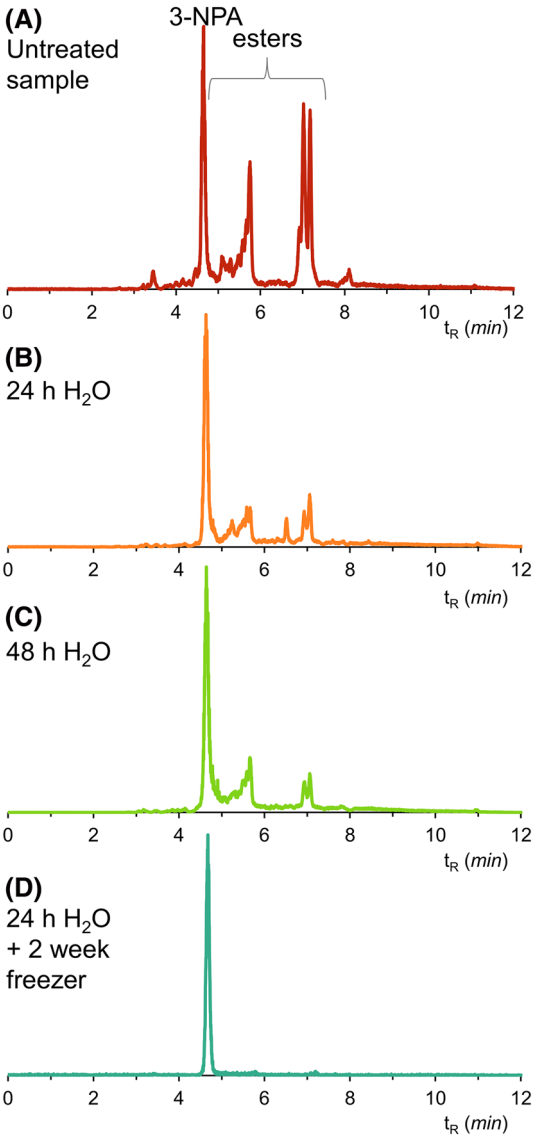

FIGURE 3 MRM chromatograms of NPA recorded from various pretreated extracts of *S. varia* root samples. NPA esters can be observed due to ion source fragmentation. (A) Aqueous extract analyzed immediately. (B) Sample pretreated with water for 24 h before extraction. (C) Sample pretreated with water 48 h before extraction. (D) Sample pretreated with water for 24 h before extraction and set aside for 2 weeks at 18°C

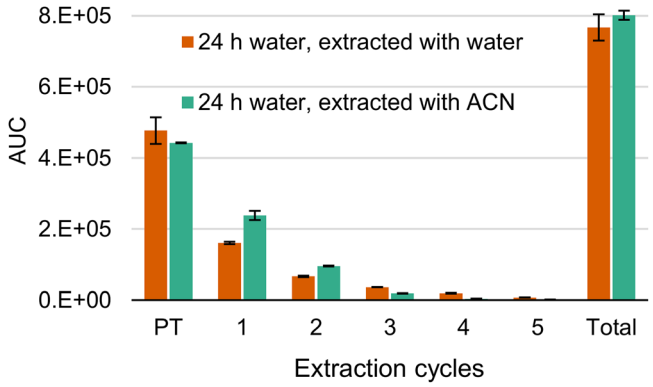

FIGURE 4 The effect of pretreatment (PT) and extraction cycles on the extracted amount of NPA

TABLE 2 Calibration curves of 3-NPA

Equation	$y = -0.0017x^2 + 35.065x + 193.89$
R^2	1
Range	6 ng/ml to 6,000 ng/ml
LOD	7.71 ng/ml
LOQ	25.71 ng/ml

TABLE 3 The intra- and inter-assay accuracy and precision values of 3-NPA ($n = 3$)

Accuracy	Intra-assay	Inter-assay
Low QC	−9.56%	4.95%
Medium QC	−1.97%	7.67%
High QC	2.20%	6.01%
Precision	**Intra-assay**	**Inter-assay**
Low QC	12.82%	9.78%
Medium QC	4.34%	4.47%
High QC	8.46%	5.48%

TABLE 4 The 3-NPA content (g/100 g) of different *S. varia* samples

	3-NPA content (g/100 g)	
Sample origin	**Roots**	**Aerial parts**
Budapest, Csepel	0.41 ± 0.03	0.26 ± 0.01
Budapest, Hűvösvölgy	0.58 ± 0.04	1.28 ± 0.25
Dunaegyháza	0.66 ± 0.12	1.57 ± 0.15

content. These results indicate that the species has the genetical background for the production of 3-NPA esters, similarly to other *Lotus* species, but this pathway is not as active as in, e.g., *L. uliginosus* or *L. angustissimus*.[15] Using these more sensitive methods, other species of the Fabaceae family can be identified as 3-NPA producers.

The first investigated *A. vulneraria* sample (collected at Soroksár, September 2019) showed a surprisingly high 3-NPA level during both the qualitative study and the quantitative measurements. The aerial part samples contained 0.052 ± 0.007 g/100 g 3-NPA, while the root samples had 0.46 ± 0.015 g/100 g 3-NPA, which is comparable with *S. varia* root samples (see Table 4). For the confirmation of these results, the sample collection was repeated from the same place (Soroksár, July 2022) and from another geographical location (Hűvösvölgy, June 2022). The sample from the same habitat had a 3-NPA level that was lower than the LOD; however, the sample from Hűvösvölgy contained around 0.43 ± 0.018 mg/100 g 3-NPA. To

investigate the effects of the environment, in vitro samples of *A. vulneraria* were also analyzed, and 3-NPA was present in detectable, but not quantifiable amount. Summarizing these results, *A. vulneraria* definitely possesses the genes responsible for the production of 3-NPA derivatives; however, in most cases, the plant synthesizes these protective molecules only in trace amounts. As two samples from the same geographical origin showed highly different levels of 3-NPA, regional effects such as soil composition could not explain this phenomenon. Currently, the reasons behind the high deviance of the samples are not clear; it can be caused by a local mutation, differences in climate, a possible infection, *etc*. 3-NPA could not be detected in cosmetics containing extracts of *A. vulneraria*.

In conclusion, we identified for the first time *L. corniculatus* and *A. vulneraria* as species containing 3-NPA. Their 3-NPA content was very low (below 0.2 mg/100 g), so considering the acceptable daily intake for humans (25 μg/kg/day),[7] their internal and external application (assuming rational use) is safe. However, local and/or temporal elevations in the 3-NPA concentrations of plants cannot be excluded.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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