



# The content and profile of biologically active compounds present in individual parts of nasturtium (*Tropaeolum majus* L.): comprehensive study

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## Abstract

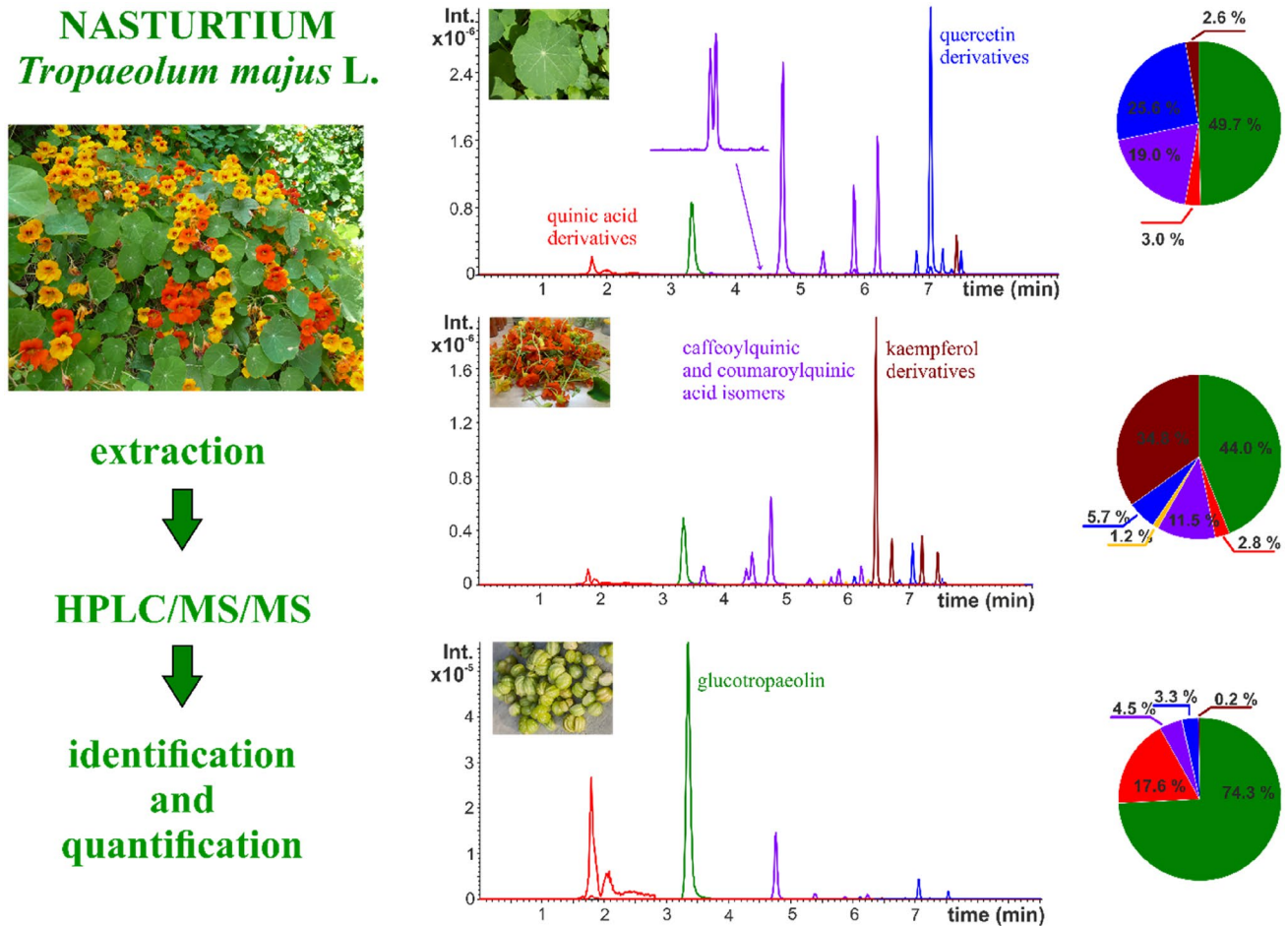
The nasturtium (*Tropaeolum majus* L.) contains many biologically active compounds with very promising effects on human health. Our attention was paid to glucotropaeolin and phenolic compounds that were simultaneously determined in different parts of nasturtium using rapid reversed-phase high performance liquid chromatography coupled to tandem mass spectrometry. Mainly isomers of hydroxycinnamic acid and derivatives of quinic acid, kaempferol, and quercetin were present. Moreover, many of them were identified for the first time. Their representation varied significantly depending on the part of nasturtium (flower, stem, seed, and leaf). Although the highest total concentration of the target compounds was found in leaves, all monitored compounds were present in flowers at concentrations higher than their limit of quantification. Furthermore, the effect of sample pre-treatment (drying and freezing) on their content was investigated. Surprisingly, frozen samples showed a considerable reduction in glucotropaeolin content. Finally, antioxidant capacity, total phenolic content, and total anthocyanin content were determined using spectrophotometric techniques and the results were compared to chromatographic data.

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## Graphical abstract



**Keywords** Nasturtium (*Tropaeolum majus* L.) · Phenolic compounds · Glucotropaeolin · Antioxidant capacity · HPLC/MS/MS

**Abbreviations**

ABTS	2,2'-Azinobis(3-ethyl-2,3-dihydrobenzothiazol-6-sulfonát)
DPPH	1,1'-Difenyl-2-pikrylhydrazyl
FW	Fresh weight
GAE	Gallic acid equivalent
GTL	Glucotropaeolin
PPs	Phenolic compounds
TAC	Total anthocyanin content
TEAC	Trolox equivalent antioxidant capacity
TPC	Total phenolic content

**Introduction**

Nasturtium (*Tropaeolum majus* L., *T. majus*) is an annual or perennial plant belonging to the family Tropaeolaceae that provide edible leaves, flowers, and green seeds. *T. majus* originates in the mountainous regions of South and Central America [1–4]. In Europe, it has been grown as an ornamental and medicinal plant since the seventeenth century [1].

The chemical composition of *T. majus* differs from the part of the plant, the colour of the flowers, and the method and place of its cultivation. In general, *T. majus* is a very rich source of many biologically active substances, especially phenolic compounds (PPs) and glucosinolates. Glucotropaeolin (GTL) and sinalbin are the main glucosinolate representatives [2, 5], while kaempferol and quercetin derivatives

together with hydroxycinnamic acid isomers are the most common PPs [2, 6–8]. Other important compounds include fatty acids, minerals, carotenoids, anthocyanins, terpenoids, benzyl isothiocyanate, and ascorbic acid [2, 4, 5, 8]. Thanks to this unique constitution, *T. majus* has natriuretic, diuretic, antihypertensive, antioxidant, antimicrobial, antiseptic, anti-inflammatory, antifungal, antidepressant, and expectorant properties [2–7, 9]. Therefore, it commonly serves as a raw material for the production of nutraceuticals used for the treatment of ophthalmic, respiratory, and urinary tract infections as well as skin, hair, and nail diseases [2, 10]. In addition to the pharmaceutical industry, *T. majus* has an exceptional place also in the food industry: leaves and flowers give the dish a particular pepper flavour attributed to the presence of glucosinolates [3], flowers can also be utilized as a natural dye due to the high concentration of anthocyanins [3, 4], and seeds contain important essential oils [2].

The pre-treatment of *T. majus* samples is a relatively time-consuming process involving several steps. First, the part of the plant intended for an analysis has to be thoroughly washed with water to remove impurities. Before crushing the sample into powder, various processes, such as freeze-drying [1, 6–8, 10, 11] or drying at room temperature/with gentle heating [1, 12], are usually carried out. For the isolation of target analytes, liquid–liquid extraction (LLE) with organic polar solvents is traditionally implied [1, 3, 6–9]. Phenolic compounds analysis is usually performed by reversed-phase high performance liquid chromatography (RP-HPLC) coupled to a spectrophotometric detector or/and a mass spectrometer [1, 3, 7, 8]. Glucosinolates are rarely analysed directly [13–15] and the desulfation step on an ion-exchange column in the presence of sulfatase is very often involved prior to their chromatographic analysis [16–18].

*Tropaeolum majus* L. is a very promising herb with therapeutic effects, but only a limited number of studies have been devoted to the analysis of phenolic compounds present in flowers or leaves. Only the total phenolic content or antioxidant capacity of the extracts has usually been studied, but quantitative analysis of individual phenolic compounds [6] and glucotropaeolin [14] has been rarely performed. Therefore, the aim of this work was to determine the representation of PPs and GTL in individual parts of the plant (flower, stem, seed, and leaf) and also to reveal possible changes in their content during freezing or drying, which are the most common procedures performed to preserve and store the plant. For this purpose, various parts of *T. majus* sample were subjected to a different sample pre-treatment procedure followed by rapid reversed-phase high performance liquid chromatography analysis coupled to a mass spectrometer with electrospray ionization and triple quadrupole analyser (RP-HPLC/MS/MS) as well as spectrophotometric analysis

revealing the total phenolic content and antioxidant capacity of the sample. In our study, a total of 30 biologically active compounds including glucotropaeolin were identified and quantified. To the best of our knowledge, this is the first time that GTL and PPs were analysed simultaneously. Moreover, it was found that pre-treatment of the plant before its storage has a high impact on the content of biologically active compounds.

## Materials and methods

### Chemicals and reagents

Standards of PPs, such as quinic acid, chlorogenic acid, neochlorogenic acid, kaempferol 3-O-glucoside (astragaline), and quercetin 3-O-glucoside (isoquercitrin), together with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid (purity of all  $\geq 98\%$ ), and 2 M Folin-Ciocalteu reagent, as well as acetonitrile, methanol, formic acid, and ammonium formate (all HPLC/MS grade) were purchased from Merck (KGaA, Darmstadt, Germany). Glucotropaeolin potassium salt ( $> 99\%$  purity) was purchased from PhytoPlan (Heidelberg, Germany). Other chemicals used, namely potassium persulfate, hydrochloric acid, sodium acetate, potassium chloride, and sodium carbonate (all p.a. grade), were purchased from Lach-Ner (Neratovice, Czech Republic). High purity water was prepared using a Milli-Q purification system (Merck Millipore, Germany).

### Standards and samples

The quantitative analysis of the PPs found was performed using standards of quinic acid, chlorogenic acid, glucotropaeolin, astragaline, and isoquercitrin. The calibration solutions of these standards were prepared by sequential dilution of their stock methanolic solutions ( $c = 1$  g/L for quinic acid, chlorogenic acid, and glucotropaeolin,  $c = 0.75$  g/L for astragaline, and  $c = 0.4$  g/L for isoquercitrin) with the mixture of acetonitrile:water (50:50, v/v; pH 2.5). The concentration ranges of the calibration solutions together with the calibration data are shown in Table S1.

Nasturtium sample was collected in November 2018 in Pardubice (Czech Republic). First, the individual parts of the fresh plant (orange flowers: FO, yellow flowers: FY, stems: St, seeds: Se, and leaves: Le) were weighted into portions of approximately 5 g. Further, the samples were either extracted immediately (fresh; FO-1, FY-1, St-1, Se-1, Le-1)

or were further processed before extraction by drying in an oven at 30 °C (FO-2, FY-2, St-2, Se-2, Le-2) or freezing at – 18 °C (FO-3, FY-3, St-3, Se-3, Le-3).

### Extraction procedure

Approximately 5 g of sample (fresh weight) was mixed with 40 mL of aqueous methanol solution (70%, v/v) and extracted using an ULTRA TURRAX T18 homogenizer (IKA, Germany) for 10 min. First, the samples were ground for one minute at a high speed (20,000 rpm), and then the speed was reduced (3000 rpm). Before the analysis, each extract was centrifuged for 10 min at 5000 rpm, diluted, and filtered through a 0.45 µm nylon filter (Agilent, Santa Clara, USA).

### HPLC/MS/MS analysis

The liquid chromatograph consisting of two LC-20AD pumps, a DGU 20A degasser, a SIL-20A autosampler (all Shimadzu, Kyoto, Japan), and an LCO 102 single-column thermostat (Ecom, Prague, Czech Republic) was coupled with a QTRAP 4500 mass spectrometer (AB Sciex, Framingham, USA) operating in electrospray ionization mode. The optimisation of separation was performed on Ascentis Express C18 (150×3 mm; 2.7 µm, Supelco, Bellefonte, USA); Kinetex Polar C18 (150×3 mm; 2.6 µm) and Luna Omega PS C18 (150×3 mm; 5 µm) columns (both Phenomenex, Torrance, USA).

The best separation was achieved on the Kinetex Polar C18 analytical column tempered at 30 °C using a gradient elution of a mobile phase composed of water acidified with formic acid to pH 2.5 (A) and acetonitrile (B) at a flow rate of 0.6 mL/min. The optimal gradient programme was following: 0 min–10% B; 1 min–15% B; 3 min–20% B; 5 min–36% B; 6 min–50% B; 7 min–10% B. The injection volume was 2 µL.

The mass spectrometer operating in the negative-ion mode was set to the following conditions: temperature 400 °C, curtain gas 20 psi, collision gas–medium, ion source gases 40 and 50 psi, ion spray voltage – 4500 V, and entrance potential – 10 V. The quantification of the target compounds was performed using multiple reaction monitoring (MRM) mode. Optimisation of the MRM transition parameters, such as declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP), was performed by direct infusion of the compounds with available standards into the mass spectrometer. In case of compounds with commercially unavailable standards, a comprehensive stepwise optimisation of individual MRM parameters was necessary. All optimised parameters are listed in Table 1.

### Statistical evaluation of experimental data and validation of method

The quantitative analysis of all identified PPs was performed by the external calibration curve method using structurally similar commonly available standards. Quinic acid was used for the quantification of its derivatives, chlorogenic acid was used for caffeoylquinic and coumaroylquinic acid isomers, astragalol for kaempferol derivatives, and isoquercitrin for quercetin and myricetin derivatives. GTL was quantified directly using an available standard. The method was validated in terms of linearity, detection limits, accuracy, precision, and repeatability [19, 20]. The calibration data were measured at nine concentration levels, each level three times ( $n=3$ ), and interpolated using the linear least squares regression (QC Expert 2.9, Trilobyte, Pardubice, Czech Republic). Jackknife residuals together with Pregibon, Williams, and L–R graphs were used to identify influential points. The linearity of the calibration curves was verified by residual plots and the significance of the straight-line regression intercept was tested using Student's  $t$  test. The regression parameters with standard deviations and coefficients of determination are given in Table S1. The coefficients of determination were in the range of 0.9994–0.9999 for all standards, demonstrating high linearity. The instrumental limits of detection (LOD) and quantification (LOQ) were calculated as the concentration yielded signal-to-noise ratio of  $S/N=3$  and  $S/N=10$ , respectively (Table S1). The accuracy and precision of the method were verified by measuring the calibration solutions at three concentration levels, each level with ten repetitions. Satisfactory mean recovery values in the range of 90–107% were observed for all concentration levels [21]. Intra-day and inter-day repeatability was assessed by measuring five extracts of one selected sample prepared in one day and 1 week later, respectively, and each extract was analysed 5 times ( $n=25$ ). The RSD value of intra-day and extra-day measurements was less than 7 and 12%, respectively, indicating good repeatability.

The analyses of all sample extracts were repeated three times ( $n=3$ ) and the final results were calculated and presented as confidence intervals  $\bar{x} \pm s \cdot t_{1-\alpha}$ , where  $\bar{x}$  is the arithmetic mean,  $s$  is the standard deviation, and  $t_{1-\alpha}$  the critical value of Student's  $t$  distribution for three repetitions (2.353) at a significance level  $\alpha$  of 0.05 (95% probability).

### Spectrophotometric analysis

Determination of antioxidant capacity and total anthocyanin and phenolic content was carried out with a UV-2450 spectrophotometer (Shimadzu) using a 1 cm S/G10 glassy

**Table 1** Summary of identified biologically active compounds with their retention times ( $t_R$ ), optimised parameters of MRM transitions—declustering potential (DP), collision energy (CE), and collision cell

exit potential (CXP), and the deprotonated molecules together with fragment ions recorded in the negative-ion tandem mass spectra

No	Time (min)	Compound	MRM transition	DP (V)	CE (V)	CXP (V)	[M–H] <sup>–</sup>	Fragment ions <sup>a</sup>
1	1.63	Quinic acid	191/85	– 85	– 30	– 7	191	127,93,87,85
2	1.11	Quinic acid derivative	191/111	– 35	– 16	– 8	191	111,87,85
3	2.04	Quinic acid derivative	191/111	– 35	– 16	– 8	191	111,87,85
4	3.34	Glucotropaeolin	408/97	– 100	– 50	– 6	408	275,259,166,97,95,80,75
5	3.45	3-caffeoylquinic acid ( <i>cis</i> )	353/191	– 70	– 30	– 7	353	191,179,135
6	3.63	3-caffeoylquinic acid ( <i>trans</i> )	353/179	– 75	– 28	– 5	353	191,179,135
7	4.36	3-p-coumaroylquinic acid ( <i>cis</i> )	337/163	– 65	– 20	– 14	337	191,173,163
8	4.45	3-p-coumaroylquinic acid ( <i>trans</i> )	337/163	– 65	– 20	– 14	337	191,173,163
9	4.74	5-caffeoylquinic acid ( <i>trans</i> )	353/191	– 70	– 30	– 7	353	191
10	5.38	5-caffeoylquinic acid ( <i>cis</i> )	353/191	– 70	– 30	– 7	353	191
11	5.39	4-p-coumaroylquinic acid ( <i>cis</i> )	337/173	– 60	– 20	– 14	337	191,173,163
12	5.60	Myricetin dihexoside	641/317	– 100	– 46	– 10	641	479,317,316
13	5.73	4-p-coumaroylquinic acid ( <i>trans</i> )	337/173	– 60	– 20	– 14	337	191,173,163
14	5.85	5-p-coumaroylquinic acid ( <i>trans</i> )	337/191	– 65	– 20	– 14	337	191,173,163
15	5.98	Myricetin acetyl dihexoside	683/317	– 70	– 50	– 10	683	641,503,317,316
16	6.10	Quercetin dihexoside (sophoroside)	625/301	– 150	– 48	– 10	625	445,301,300
17	6.21	5-p-coumaroylquinic acid ( <i>cis</i> )	337/191	– 65	– 20	– 14	337	191,173,163
18	6.33	Myricetin hexoside	479/317	– 110	– 40	– 11	479	317,316
19	6.37	Quercetin acetyl hexoside derivative	711/300	– 60	– 54	– 5	711	667,625,505,343,301,300
20	6.45	Kaempferol dihexoside (sophoroside)	609/285	– 120	– 50	– 9	609	429,285,284
21	6.70	Kaempferol acetyl dihexoside derivative	695/285	– 70	– 54	– 9	695	651,471,285,284
22	6.80	Kaempferol acetyl dihexoside derivative	695/285	– 70	– 54	– 9	695	651,471,285,284
23	6.83	Isoquercitrin	463/300	– 120	– 38	– 5	463	301,300
24	7.04	Quercetin acetyl hexoside derivative	549/505	– 60	– 18	– 5	549	505,301,300
25	7.19	Astragaln	447/285	– 110	– 38	– 5	447	285,284
26	7.23	Quercetin acetyl hexoside derivative	549/505	– 60	– 18	– 5	549	505,301,300
27	7.45	Kaempferol acetyl hexoside derivative	533/489	– 50	– 18	– 5	533	489,285,284
28	7.52	Methoxy quercetin acetyl hexoside derivative	563/315	– 70	– 22	– 5	563	519,315,301,300
29	7.57	Kaempferol acetyl hexoside derivative	533/489	– 50	– 18	– 5	533	489,285,284
30	7.70	Methoxy quercetin acetyl hexoside	519/315	– 100	– 18	– 5	519	315

<sup>a</sup>In case of quercetin, myricetin, and kaempferol derivatives, no fragment ions with  $m/z$  lower than aglycone are reported

cuvette from Fisher Scientific (Pardubice, Czech Republic). All experiments were repeated five times for each sample ( $n=5$ ) and the results are presented as confidence intervals  $\bar{x} \pm s.t_{1-\alpha}$ . In this case, the critical value of Student's  $t$  distribution was 2.015.

## Antioxidant capacity

The antioxidant capacity of the nasturtium extracts was assessed using two different spectrophotometric approaches, ABTS and DPPH methods.

**ABTS method:** the preparation of ABTS<sup>•+</sup> for the determination of ABTS radical cation scavenging activity was adopted from the literature [22]. Then, dark green working

solution of ABTS<sup>•+</sup> (3 mL) was thoroughly mixed with 40  $\mu$ L of sample extract or Trolox calibration solution, and after 30 min of reaction, the decrease in absorbance at 734 nm against water as a blank solution was measured. The optimal reaction time was tested by a kinetic study up to 180 min. The percentage decrease in absorbance was converted to an equivalent amount of Trolox per gram of fresh sample (Trolox equivalent antioxidant capacity; TEAC) using the calibration curve.

**DPPH method:** this procedure described by Rivero-Peréz [23] was applied with slight modification. DPPH was dissolved in methanol to a concentration of approximately 0.1 mmol/L and subsequently diluted to provide the solution absorbance of 0.8 at 515 nm. Further, 3 mL of this solution

was mixed with 50  $\mu\text{L}$  of sample extract or Trolox calibration solution. The decrease in absorbance measured after 30 min of reaction was recorded and converted to TEAC. The reaction time was again optimised by the kinetic study.

ABTS and DPPH calibration data were obtained at eight Trolox concentration levels. Each level was repeated 5 times ( $n=5$ ). The calibration solutions were prepared by sequential dilution of stock Trolox solution ( $c=0.01$  mol/L) with a mixture of water and methanol (50:50,  $v/v$ ). The amount of Trolox standard added to the working solution of ABTS and DPPH was in the range of 0.03–0.30  $\mu\text{mol}$  and the calibration equation parameters obtained were  $y=308.8(3.4)x-1.897(0.678)$ ,  $R^2=0.9995$  and  $y=248.5(1.6)x-0.829(0.311)$ ,  $R^2=0.9998$ , respectively. The calibration data were statistically processed as described in chapter of Statistical evaluation of experimental data.

### Total phenolic content

The total phenolic content (TPC) was determined using the Folin-Ciocalteu's method according to the procedure of Šilarová et al. [22] with slight modification. First, the 2 M Folin-Ciocalteu's reagent was diluted twenty times with water. Subsequently, 2 mL of the reagent was thoroughly mixed with 50  $\mu\text{L}$  of sample extract or gallic acid calibration solution, the mixture was incubated at room temperature for 5 min, and finally 1 mL of 7.5% aqueous solution of  $\text{Na}_2\text{CO}_3$  was added and mixed well. After another 30 min of incubation, an increase in absorbance was recorded at 750 nm and converted to an equivalent amount of gallic acid (GAE) per gram of fresh sample using the corresponding calibration curve.

The calibration data for the TPC method were measured at nine concentration levels of gallic acid ( $n=5$ ). The calibration solutions were prepared by sequential dilution of stock gallic acid solution ( $c=1$  g/L) with a mixture of water and methanol (50:50,  $v/v$ ). The amount of gallic acid was in the range of 2.5–30  $\mu\text{g}$  and the calibration equation parameters were  $y=32.02(0.33)x+0.034(0.006)$  and  $R^2=0.9996$ . The calibration data were statistically processed as described in chapter of Statistical evaluation of experimental data.

### Total anthocyanin content

The total anthocyanin content (TAC) was determined using a pH-differential method described by Gusti and Wrolstad [24]. A change in the absorbance of the samples in two buffers adjusted with HCl to different pH values was monitored. The first buffer was potassium chloride (0.025 mol/L) with pH 1 and the second buffer was sodium acetate (0.4 mol/L) with pH 4.5. Then 400  $\mu\text{L}$  of the sample solution was added

to 2.6 mL of both buffers and the corresponding absorbance at 700 and 496 nm was recorded against distilled water used as a blank. Each sample was measured five times. The wavelength of 496 nm corresponds to absorption maximum of pelargonidin-3-glucoside [24], which is the most similar analogue of the most dominant anthocyanin present in orange flower extracts (pelargonidin-3-sophoroside). This wavelength was confirmed by absorption spectrum of flower extracts (Figure S1).

The TAC (mg/L) was calculated according to the formula below and expressed as pelargonidin-3-glucoside equivalents:

$$\text{TAC (mg/L)} = \frac{[A_{496} - A_{700}]_{\text{pH1}} - (A_{496} - A_{700})_{\text{pH4.5}} \cdot MW \cdot DF \cdot 1000}{\epsilon \cdot l}$$

where  $A$  is the absorbance recorded at the given wavelength and buffer pH,  $MW$  is the molecular weight of pelargonidin-3-glucoside ( $MW=433.4$  g/mol),  $DF$  is the dilution factor (0.4 mL of sample is diluted to 3 mL,  $DF=7.5$ ),  $\epsilon$  is the molar absorption coefficient ( $15,600 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$  for pelargonidin-3-glucoside), and  $l$  is the pathlength (cm).

## Results and discussion

### Optimisation of HPLC/MS/MS method

The highest number of identified PPs was monitored in orange flower extract, and therefore this extract was used to optimise the HPLC separation. The aim of the optimisation was simultaneous separation of monitored PPs and GTL with the best possible resolution in the shortest time. Various combinations of chromatographic parameters, such as a type of analytical column, a type of organic solvent (acetonitrile or methanol) and its initial concentration, an amount of formic acid added to the water to achieve pH 2.3, 2.4, and 2.5, as well as different gradient elution profiles, were tested. The best separation was achieved on the Kinetex Polar C18 column (designed for the separation of polar compounds) combined with the mixture of acetonitrile and water as the mobile phase. Compared to acetonitrile, methanol provided slightly higher ionization efficiency of the monitored compounds; however, a significant tailing of the peaks was observed, especially for GTL. Concerning the pH, it was found that pH 2.5 was sufficient for satisfactory separation, and its further decrease played no role in ionization efficiency or peak resolution and selectivity. Although the addition of an ion-pairing agent improved the peak shape of GTL, the ionization efficiency of all PPs was rapidly

decreased. Therefore, only formic acid was added to the mobile phase.

Further, the parameters of very selective and sensitive MRM mode of mass spectrometric detection, such as DP, CE, and CXP, were optimised for quantification of all monitored compounds. For this purpose, the direct infusion of standard solutions of quinic acid, glucotropaeolin, chlorogenic acid, neochlorogenic acid, isoquercitrin, and astragalol to the mass spectrometer was performed. The MRM parameters of the compounds without commercially available standard were optimised by stepwise changes of individual parameters during the HPLC/MS/MS analysis, and the dependence of the peak area and intensity on the value of individual parameters was recorded. The DP ranged from  $-20$  to  $-200$  V with a step of 10 V. Then, the optimal DP was selected based on the maximum in the peak area and intensity (Figure S2A). CE was first optimised in the range of  $-50$  to  $-20$  V with the step of 10 V and the intensities of the main fragments were monitored (Figure S2B). For the most intensive fragments, the individual MRM transition was further tuned with the step of 2 V in the range of  $\pm 10$  V of the expected value. Finally, the CXP parameter ranged from  $-14$  to  $-5$  V for the individual MRM transitions and the CXP value with the highest peak intensity was selected (Figure S2C). The optimal values of DP, CE, and CXP for all compounds are listed in Table 1 and an example of MRM optimisation of the quercetin acetyl hexoside derivative (No.24) is shown in Figure S2.

## Qualitative analysis

The identification of PPs present in extracts prepared from different parts of nasturtium was performed according to their retention behaviour, full scan and tandem mass spectra as well as the information already reported in the literature [1, 6–8, 25]. The deprotonated molecules,  $[M-H]^-$ , were mainly observed in the negative-ion full scan mass spectra, while the protonated molecules,  $[M+H]^+$ , together with the adducts with sodium,  $[M+Na]^+$ , were observed in the positive-ion mode. Tandem mass spectrometry in both negative- and positive-ion modes was employed to obtain more detailed structural information. The ionization efficiency of most compounds was better in the negative-ion mode; therefore, it was further used for quantitative analysis.

In our approach, a total of 30 biologically active compounds were identified in nasturtium, while only a maximum of 16 non-anthocyanin phenolic compounds have been identified in previous studies [1, 6–8]. Although some hydroxycinnamic acid isomers have already been quantified in these studies, four new derivatives of hydroxycinnamic

acid together with derivatives of quinic acid were identified for the first time in our study. Kaempferol, quercetin, and myricetin derivatives were determined in nasturtium extracts as well (Table 2). In addition to these compounds, anthocyanins are also known components of nasturtium flowers [3, 6]; however, their presence was not monitored because they required different chromatographic conditions. Therefore, only the TAC was determined using the pH-differential method.

The compounds Nos. 1–3 are highly polar and belong to the family of quinic acid derivatives. Using an available standard, the compound No. 1 was identified as quinic acid ( $m/z=191$ ,  $[M-H]^-$ ), providing fragment ions with  $m/z=111$ , 97, and 85. The molar mass of the compounds Nos. 2 and 3 could not be distinguished due to the high content of polar matrix compounds. These two compounds differ from quinic acid (No. 1) in retention behaviour, while the fragmentation behaviour remains the same. Therefore, these two compounds were attributed to quinic acid derivatives.

The compound No. 4 was identified as glucotropaeolin ( $m/z=408$ ,  $[M-H]^-$ ) using an available standard.

The compounds Nos. 5 and 6 were identified as *cis* and *trans* 3-O-caffeoylquinic acid isomers (3-CafQ, neochlorogenic acid), respectively, and compounds 9 and 10 as *trans* and *cis* 5-O-caffeoylquinic acid (5-CafQ, chlorogenic acid) isomers, respectively. The identification was performed based on  $[M-H]^-$  ion ( $m/z=353$ ) and the main fragment ion  $m/z=191$  corresponding to the quinic acid moiety. In case of 3-CafQ, fragment ions  $m/z=179$  and 135 were also observed. While the *trans* isomers have already been determined in nasturtium [1, 6–8] and the corresponding standards are available (chlorogenic and neochlorogenic acids), the *cis* isomers have been identified in nasturtium for the first time.

In our study, six isomers (compounds Nos. 7, 8, 11, 13, 14, and 17) of *p*-coumaroylquinic acid (CouQ) were found in nasturtium extracts. Their identification was performed on the basis of  $[M-H]^-$  ion ( $m/z=337$ ) and the fragmentation and retention behaviour [1, 6–8, 25]. Three significant fragment ions,  $m/z=163$ , 173, and 191, were observed in tandem mass spectra, which intensities strongly depend on the binding of *p*-coumaric acid to the structure of quinic acid. The fragment ion  $m/z=163$  was the most intensive for 3-CouQ, the ion  $m/z=173$  corresponded to 4-CouQ, and the ion  $m/z=191$  was typical of 5-CouQ. The retention behaviour of *cis* and *trans* isomers of CouQ acids was distinguished based on the information from the literature [25, 26], where it is stated that *cis*-5-acyl-quinic acids are more hydrophobic and elute later than their *trans* counterparts, unlike the *cis*-3-acyl and *cis*-4-acyl derivatives.

**Table 2** The concentrations ( $\mu\text{g/g}$ ) of phenolic compounds and glucotropaeolin found in different parts of nasturtium plant (orange flowers – FO, yellow flowers – FY, stems – St, seeds – Se, and leaves – Le) that were subjected to different sample pre-treatment (fresh—1, dried—2 or frozen—3)

	Fresh			Dried			Frozen									
	Se-1	Le-1	St-1	FO-1	FY-1	Se-2	FO-2	St-2	Le-3	FY-2	Se-3	FO-3	St-3	Le-3	FY-3	
	4	769	1779	709	790	702	655	744	241	1332	241	827	1.5	331	3.5	480
<b>Quinic acids</b>																
1	2.3	1.8	4.4	5.2	6.8	1.87	3.23	1.37	2.3	1.37	5.4	2.9	3.6	4.6	6.1	7.8
2	116	84	90	35	42	142	98	17.6	163	17.6	92	198	109	103	43	45
3	18	23	21.9	10.8	12.2	72	34	5.6	66	5.6	50	66	49	50	22	25
	136	109	116	51.0	60.9	216	136	25	232	25	148	267	162	158	71	78
	Sum															
<b>Hydroxycinnamic acids</b>																
5	LOD	LOQ	0.43	1.54	1.63	LOD	LOQ	LOD	LOQ	LOD	2.19	LOD	LOQ	LOQ	1.73	2.6
6	LOQ	2.4	5.6	26.4	27.3	LOQ	3.8	0.76	3.8	0.76	26.5	LOQ	1.61	3.5	32	37.2
7	LOQ	0.31	1.25	22	29	LOQ	LOQ	LOQ	0.69	LOQ	17.6	LOQ	0.36	1.18	27	39
8	0.29	0.32	1.32	40	43	LOQ	36	LOQ	0.65	LOQ	61	LOQ	0.40	1.41	42	53
9	40	403	74.5	78	84	18.7	100	61	418	61	114	38	403	73.2	96	114
10	4.1	14.8	6.7	5.7	4.4	1.39	5.6	5.0	35	5.0	7.1	1.28	0.94	1.30	0.55	0.42
11	LOD	0.55	LOQ	1.97	1.52	LOQ	4.7	LOD	2.13	LOD	9.4	LOD	1.10	LOQ	1.65	2.2
13	LOD	0.44	LOD	2.5	2.8	LOQ	5.7	LOD	2.18	LOD	10.3	LOD	1.27	LOQ	1.75	2.33
14	0.92	107.8	2.17	15.5	26	0.36	16.9	1.19	144	1.19	23.7	1.11	208	2.13	14.6	25.2
17	1.3	151	3.72	12.5	24	0.85	14.2	2.33	165	2.33	28	1.33	264	3.85	12.9	27.5
	46.8	680	95.8	207	245	21	228	70	770	70	348	41	881	87	230	304
	Sum															
<b>Myricetin derivatives</b>																
12	1.10	0.49	5.5	8.5	7.4	LOD	9.3	LOD	LOD	LOD	9.8	0.90	0.12	2.5	9.6	10.5
15	LOQ	0.53	LOD	4.50	4.2	LOD	4.3	LOD	LOD	LOD	4.7	LOD	0.75	LOD	5.3	5.8
18	LOQ	0.34	0.47	8.2	9.1	LOD	8.7	LOD	LOD	LOD	9.5	LOD	LOD	LOQ	9.4	10.2
	1.10	1.36	5.97	21.2	20.7	–	22	–	–	–	24	0.90	0.87	2.5	24.3	26.5
	Sum															
<b>Quercetin derivatives</b>																
16	2.0	5.2	4.9	15.7	18.4	1.05	14.1	0.73	5.29	0.73	17.4	2.75	4.12	4.93	17.0	19.3
19	0.45	3.5	2.3	3.8	2.1	0.144	3.9	0.00	3.1	0.00	2.64	0.49	3.6	2.6	5.4	3.5
23	5.2	201	6.9	17.8	15.1	0.91	8.0	4.9	55.2	4.9	10.9	0.58	27	0.66	8.3	10.2
24	23	640	187	55	54	9.3	92	112	840	112	85	21.9	762	194	82	94
26	1.12	41	9.4	3.0	4.2	0.44	4.0	5.8	65	5.8	3.8	0.96	44	8.3	2.9	4.03
28	2.1	26	8.0	6.3	6.4	2.59	8.1	15.0	45.5	15.0	9.4	2.8	37	9.4	10.0	10.4
30	LOQ	0.93	0.86	0.22	LOQ	0.18	1.76	1.69	2.9	1.69	1.46	0.13	2.1	0.32	0.70	LOQ
	33.7	918	220	102	100	15	132	140	1012	140	131	30	879	220	126	141
	Sum															
<b>Kaempferol derivatives</b>																
20	1.9	2.06	3.58	438	163	0.57	423	0.232	3.7	0.232	151	0.73	0.89	1.18	456	183
21	0.21	0.54	0.19	70.9	16.5	LOQ	74	LOD	0.69	LOD	18.1	LOQ	0.67	LOQ	91	26



Table 2 (continued)

	Fresh			Dried			Frozen								
	Se-1	Le-1	St-1	FO-1	FY-1	Se-2	Le-2	St-2	FO-2	FY-2	Se-3	Le-3	St-3	FO-3	FY-3
	LOD	LOD	LOD	3.40	1.1	LOQ	LOQ	LOD	4.1	0.87	LOQ	LOQ	LOD	LOD	1.15
22	0.24	17.2	0.27	74	22	LOQ	3.2	0.100	64	20.6	0.14	2.6	0.18	77	25
25	0.25	70	1.4	36.7	30	LOD	89	0.9	51	35.9	0.31	119	2.1	59	50
27	LOD	4.2	LOD	2.1	1.9	LOD	5.28	LOQ	2.6	1.9	LOD	6.9	LOD	2.6	2.23
29	2.57	93.9	5.40	626	234	0.57	101	1.3	620	228	1.2	130	3.5	689	287
Sum	221	1802	443	1006	660	252	2115	236	1138	879	340	2053	470	1140	836
Total phenolic content	990	3581	1152	1795	1362	907	3447	478	1882	1705	382	2384	474	1617	907
Total amount of monitored compounds															

The compounds Nos. **12**, **15**, and **18** giving deprotonated molecules  $m/z = 641$ ,  $683$ , and  $479$  were identified as myricetin dihexoside, acetyl dihexoside, and hexoside, according to characteristic neutral losses of dihexoside ( $m/z = 324$ ), acetyl dihexoside ( $m/z = 366$ ), and hexoside ( $m/z = 162$ ), respectively. The main fragment ions  $m/z = 316$  or  $317$  corresponded to myricetin aglycone [6–8].

The other group of compounds present in the nasturtium extracts included quercetin glycosides and other quercetin derivatives (compounds Nos. **16**, **19**, **23**, **24**, **26**, **28**, and **30**). The compound No. **23** ( $m/z = 463$ ,  $[M-H]^-$ ) was identified as isoquercitrin (quercetin-3-O-glucoside) using an available standard. The main fragment ion  $m/z = 301$  corresponding to quercetin aglycone, which arose by the loss of dihexoside moiety ( $m/z = 324$ ), was observed in tandem mass spectra of the compound No. **16** ( $m/z = 625$ ,  $[M-H]^-$ ). Therefore, this compound was identified as quercetin dihexoside, probably sophoroside [8]. The ions of quercetin aglycone ( $m/z = 301$  and  $m/z = 303$ ) were also present in the tandem mass spectra of compound No. **19** giving  $m/z = 711$  and  $m/z = 713$  in negative- and positive-ion mode, respectively. According to fragment ions observed in tandem mass spectra recorded in the positive- and negative-ion mode, typical neutral losses of the acetyl dihexoside ( $m/z = 366$ ), acetyl hexoside ( $m/z = 204$ ), acetyl ( $m/z = 42$ ), and hexoside ( $m/z = 162$ ) moiety, and the presence of fragment ion  $m/z = 505$  (in negative-ion mode), which corresponds to quercetin acetyl hexoside [6, 8], we assume that it is a derivative of quercetin acetyl hexoside, probably quercetin di(acetylhexoside). Very intensive fragment ion  $m/z = 505$  with typical neutral loss of acetyl hexoside ( $m/z = 204$ ) was also observed in the negative-ion tandem mass spectra of compounds Nos. **24** and **26** ( $m/z = 549$ ,  $[M-H]^-$ ), which have the same molar mass and fragmentation behaviour, but differ in retention behaviour. Therefore, these compounds are supposed to be isomers of quercetin acetyl hexoside derivatives. The last two compounds derived from quercetin, No. **28** ( $m/z = 563$ ,  $[M-H]^-$ ) and No. **30** ( $m/z = 519$ ,  $[M-H]^-$ ), are derivatives of methoxy quercetin with the main fragment ion  $m/z = 315$ . Further, the fragment ion  $m/z = 519$  was also observed in tandem mass spectra of compound No. **28**. Since both compounds showed the typical neutral loss of the acetyl hexoside moiety ( $m/z = 204$ ), these were identified as methoxy quercetin acetyl hexoside (No. **30**) and its derivative (No. **28**).

The last group of compounds was the glycosides and derivatives of kaempferol (compounds Nos. **20–22**, **25**, **27**, and **29**). The compound No. **20** ( $m/z = 609$ ,  $[M-H]^-$ ), which provides a very intensive fragment ion corresponding to kaempferol aglycone,  $m/z = 284$  (285), was identified as kaempferol dihexoside. Due to the characteristic loss of the dihexoside moiety ( $m/z = 324$ ), the glycosidic part of the molecule is probably sophoroside [8]. Ion  $m/z = 651$  and ion corresponding to kaempferol aglycone ( $m/z = 285$  or  $284$ )

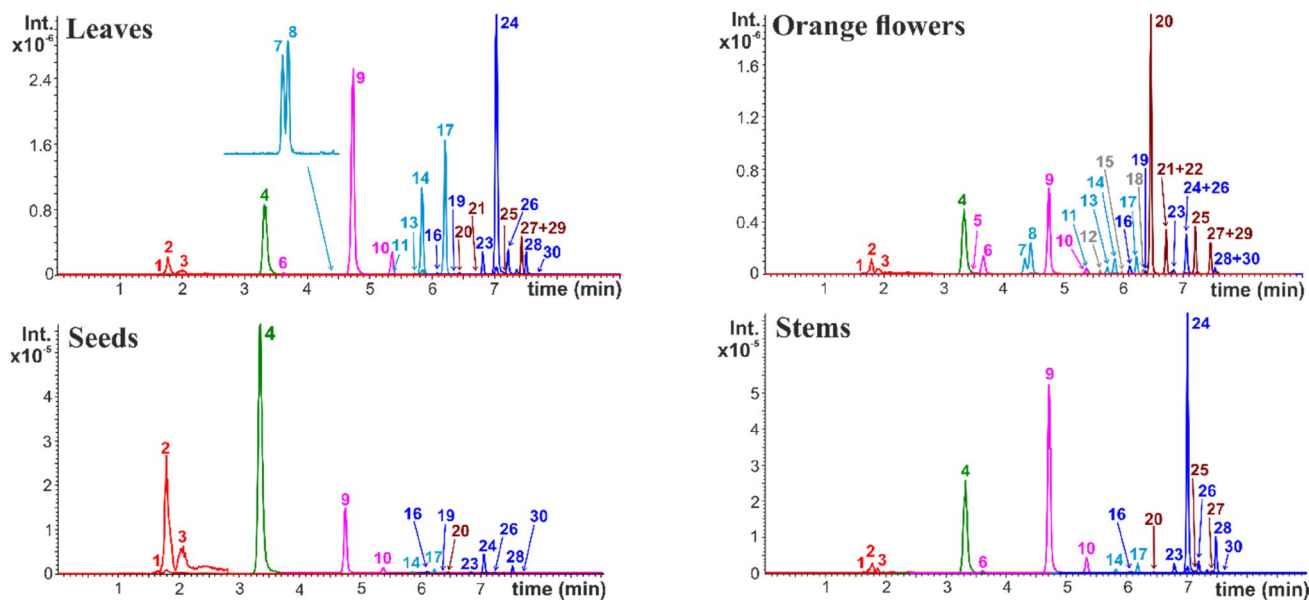
were the main fragment ions in negative-ion tandem mass spectra of compounds Nos. **21** and **22** ( $m/z=695$ ,  $[M-H]^-$ ). Other fragments ( $m/z=609$ , 591, and 471) were observed with lower intensities. According to the characteristic losses corresponding to the acetyl dihexoside ( $m/z=366$ ), dihexoside ( $m/z=324$ ), or acetyl ( $m/z=42$ ) moieties, we assume that these compounds can represent isomers of kaempferol acetyl dihexoside (sophoroside) derivatives. Although, the kaempferol acetyl dihexoside (sophoroside) has already been discussed by Garzón et al. [6] and Navarro-González et al. [8] as a molecule providing a deprotonated molecule  $m/z=651$ , Bazylo et al. [1] determined it with  $m/z=695$  and the ion  $m/z=651$  identified as its fragment ion, which also corresponds to our results. Furthermore, the molar mass of these compounds was confirmed in the positive-ion mass spectra, where only a protonated molecule  $m/z=697$  was observed. The compound No. **25** ( $m/z=463$ ,  $[M-H]^-$ ) was identified as astragalín (kaempferol-3-O-glucoside) using an available standard. The last compounds of the kaempferol group, Nos. **27** and **29** ( $m/z=533$ ,  $[M-H]^-$ ), have the same fragmentation behaviour. These provide fragment ions  $m/z=285$  and 489 corresponding to kaempferol aglycone and kaempferol acetyl hexoside, respectively [6, 8], and a typical neutral loss of acetyl hexoside ( $m/z=204$ ). Therefore, these two compounds are supposed to be isomers of kaempferol acetyl hexoside derivatives.

## Quantitative analysis

Quantitative analysis of the biologically active compounds monitored was performed using the external standard calibration method using commonly available standards or structurally similar analogues (see experimental part). The individual calibration parameters together with their standard deviations are shown in Table S1. The instrumental LODs and LOQs were in the range of 0.39–3.84 and 1.3–12.8  $\mu\text{g/L}$ , respectively. The natural water content of the plant plays an important role in the calculation and subsequent comparison of the analyte concentrations. The water content determined in leaves, flowers, seeds, and stems was approximately 85, 91, 87, and 91%, respectively, which is consistent with the results of Navarro-González et al. [8], reporting 89% moisture in flowers. For this reason, the individual parts of the plant were divided into portions immediately after collection, and the results are related to a gram of fresh material (Tables 2 and S2-S6).

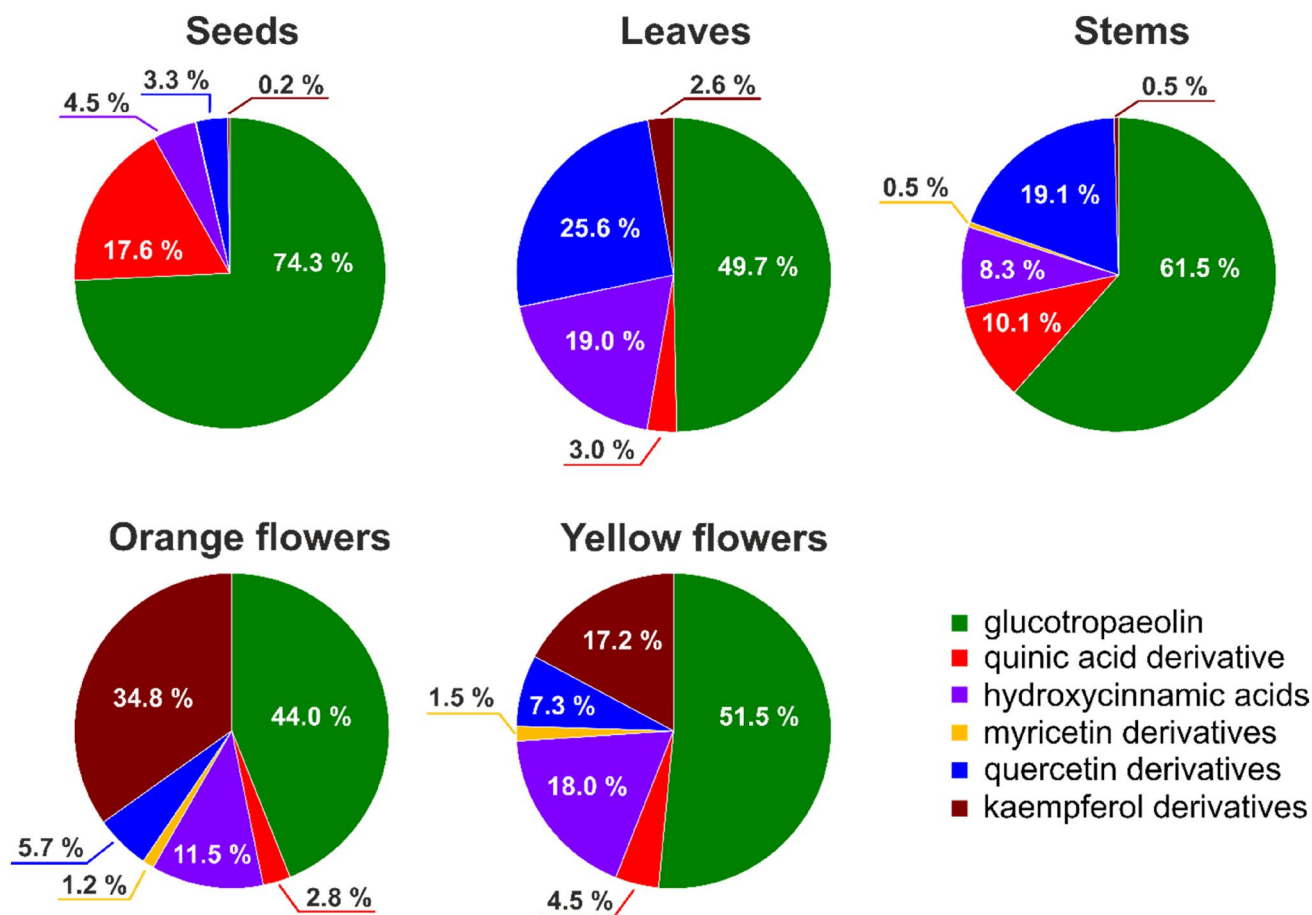
## The biologically active compounds in different part of nasturtium

The main biologically active compound of the nasturtium plant is glucotropaeolin, with the highest content in leaves. In terms of PPs, mainly quinic acid derivatives, CafQ and CouQ acid isomers as well as quercetin and kaempferol hexosides are present in various parts of the plant. On the other



**Fig. 1** HPLC/MS/MS separation of monitored compounds present in different parts of *T. majus*. The numbers of compounds correspond to those given in Tables 1, 2. The individual groups of compounds are colour coded: red (quinic acid derivatives, Nos. 1–3); green (glucotropaeolin, No. 4); pink (caffeoylquinic acid iso-

mers, Nos. 5,6,9,10); sky blue (coumaroylquinic acid isomers, Nos. 7,8,11,13,14,17); grey (myricetin derivatives, Nos. 12,15,18); blue (quercetin derivatives, Nos. 16,19,23,24,26,28,30); brown (kaempferol derivatives, Nos. 20,21,22,25,27,29)



**Fig. 2** Representation of six main groups (Table 2) of biologically active compounds in different parts of *T. majus*

hand, myricetin derivatives were observed at concentrations higher than LOQ only in flowers. Their concentrations were negligible in other parts of the plant. According to Garzón et al. [6], myricetin derivatives should be present mainly in red flower petals. The concentrations of all monitored compounds present in individual parts of the plant, including also their sum and sample pre-treatment used (freezing, drying), are listed in Table 2 and all results together with confidence intervals are present in supplementary materials in Tables S2-S4.

Examples of HPLC/MS/MS separations of extracts prepared from individual parts of dried samples of *T. majus* are shown in Fig. 1, from which it is evident that the occurrence and content of individual PPs varied considerably depending on the parts of the plant analysed. The corresponding representation of the main groups of compounds studied (GTL, quinic acid derivatives, hydroxycinnamic acids, myricetin derivatives, quercetin derivatives and kaempferol derivatives) is clearly depicted in Fig. 2. The greatest diversity of phenolic compounds was observed in flowers, where almost all identified compounds were detected at a concentration above LOQ. However, the highest total content of

PPs (1800 µg/g) and GTL (1780 µg/g) was found in leaf extracts (Table 2), in which GTL even occupied almost 50% of all biologically active compounds (Fig. 2). Assuming that the sample contains approximately 90% water, the determined GTL concentration agrees with the results of Kleinwächter et al. [14], who found concentrations ranging from 16 to 50 mg/g GTL dried weight (DW) in extracts prepared from freeze-dried leaves. Quercetin acetyl hexoside derivative (compound No. 24), *trans* 5-CafQ acid (No. 9), and isoquercitrin (No. 23) were the most abundant PPs in leaves. Quercetin derivatives even reached up to 25% of the total amount of monitored substances and 50% of total PPs content (Fig. 2). The second highest amount (38% of total PPs) was represented by the isomers of caffeoylquinic acids and coumaroylquinic acids. Quinic acid and its derivatives comprised approximately 10% of all PPs. The remaining 5% were kaempferol derivatives.

The seed extract contained the lowest PPs content, while the GTL content was similar to that of the stem and flowers. Therefore, GTL occupies almost 78% of all biologically active seeds compounds (Fig. 2). The distribution of PPs is similar in seed and stem extracts, containing mainly quinic

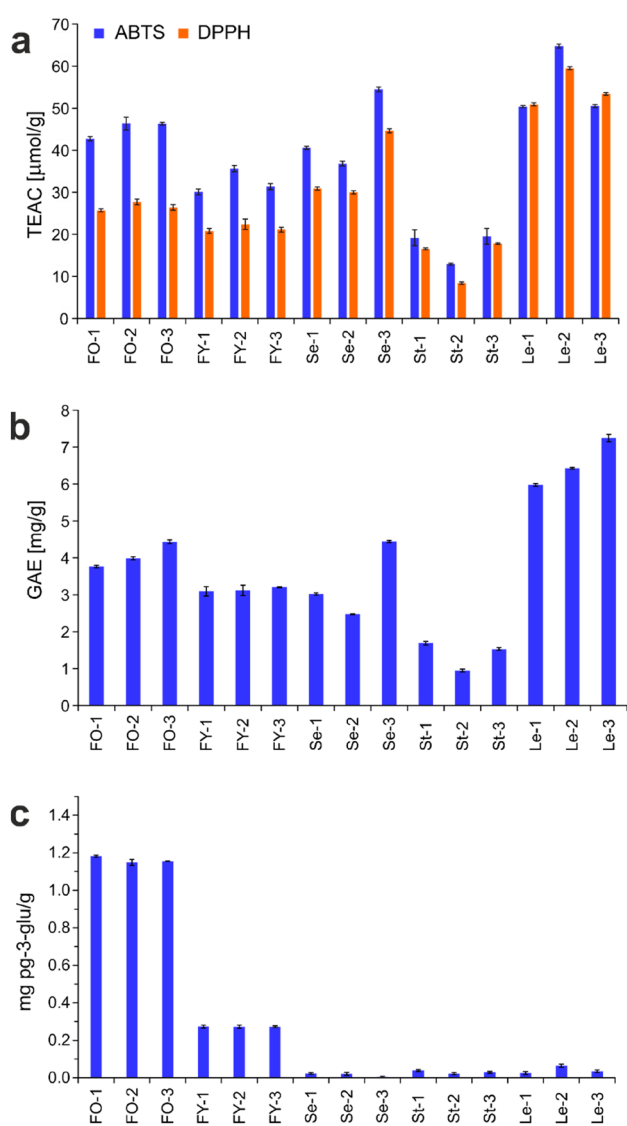
acid derivatives, *trans* 5-CafQ acid, and quercetin derivatives. However, their concentration is at least two times higher in the stem sample than in the seed sample. From the distribution and content of biologically active compounds present in stems, seeds, and leaves, it is evident that the stem forms a kind of “intermediator” between seeds and leaves.

While quercetin derivatives were significant compounds in leaves and stems, kaempferol derivatives along with hydroxycinnamic acid isomers were the dominant PPs in orange and yellow flowers. The concentrations of all monitored compounds are more or less similar in the extracts of orange and yellow flowers, except for the derivatives and isomers of kaempferol, which were almost three times higher in the orange flower. Kaempferol dihexoside was the most intensive PPs in orange flower, which is also consistent with the results published by Garzón et al. [6]. In the study by Barros et al. [11], rutin was introduced as the dominant compound of nasturtium flowers, although it should not occur there. Kaempferol dihexoside and rutin have the same molar mass, but their fragmentation and retention behaviour are different. Therefore, the results presented [11] are probably misinterpreted. Moreover, some other compounds, such as ferulic acid, caffeic acid, apigenin, kaempferol aglycone, etc., have also been quantified exclusively by Barros et al. [11], although no other research has shown their presence in this plant even using different mass spectrometric methods [1, 6–8]. In our work, the known retention and fragmentation behaviour of common PPs was compared with data obtained after analysis of nasturtium extracts, and the aglycones of flavonoids and basic phenolic acids were not found.

### Effect of plant processing on the content of biologically active compounds

The treatment of the plant before storage (drying or freezing) affects the content of biologically active compounds. In most cases, their concentration in the dried samples was comparable or slightly lower than in the fresh samples. Only a higher content of quinic acid derivatives in dried leaves, flowers, and seeds along with a higher content of hydroxycinnamic acids and quercetin derivatives in leaves and flowers was observed.

Interestingly, the GTL content rapidly decreased to almost a negligible value (1.5 µg/g) in all frozen samples, especially in frozen seeds. Thus, freezing has a strong negative effect on the GTL content. On the other hand, the freezing almost did not affect the concentration of PPs, which remained very similar to that of the fresh sample. Only the concentration of quinic acid derivatives and 5-CouQ acid isomers increased slightly. In the study by Kandil et al. [12], the highest concentration of GTL was observed in freeze-dried samples, while drying the sample on air or in an oven had a negative impact on the GTL concentration.



**Fig. 3** Antioxidant capacity determined by ABTS and DPPH method **a**, total phenolic content **b** and total anthocyanin content **c** of individual extracts prepared from different parts of *T. majus* after different sample pre-treatment

### Antioxidant capacity assessment

First, the amount of sample added to the reaction solutions and the reaction time were optimised using orange flower and stem extracts. Optimal conditions along with calibration data are given in the experimental part and the final results are shown in Fig. 3a. Significant differences were observed between the TEAC values found in individual parts of the plant, indicating a diverse proportion of PPs. The antioxidant capacity determined by the ABTS method is usually higher than that of the DPPH method, with the largest difference being in the case of flower extracts. The TEAC values of the orange flower extracts were in the range of 40.0–46.3 µmol/g

for the ABTS method, while ranged only between 25.7 and 29.9  $\mu\text{mol/g}$  for DPPH method. For yellow flowers, the TEAC values for the ABTS and DPPH methods were lower and ranged between 30.1–35.6 and 20.8–22.4  $\mu\text{mol/g}$ , respectively. Flowers probably contain compounds that reluctantly react with the DPPH radical, which is known for its higher selectivity [27]. Regardless of the method used, the stem extracts showed the lowest TEAC (on average around 14  $\mu\text{mol/g}$ ). On the other hand, the highest TEAC values were acquired in leaves (around 55  $\mu\text{mol/g}$ ), where both methods also provided the greatest similarity of the results. These findings are consistent with those reported in the study by Fukalova et al. [28], where the TEAC determined by the DPPH method was 48.7  $\mu\text{mol/g}$  fresh weight (FW). In contrast, even up to ten times higher TEAC values of orange flower extract were presented by Garzón and Wrolstad [3], who achieved the ABTS and DPPH radical scavenging activities of 458  $\mu\text{mol/g}$  FW and 91.9  $\mu\text{mol/g}$  FW, respectively. In the study by Navarro-González et al. [8], many times lower TEAC of flower extract determined by the ABTS method were reported (9.5  $\mu\text{mol/g}$ ). This disagreement in antioxidant capacity results may be caused by the use of various extraction solvents and techniques as well as various species of the nasturtium.

Treatment of the plant prior the storage mainly affected the antioxidant capacity of the stem and seeds, while it had a smaller impact on the leaves. Interestingly, the antioxidant capacity of the flower extracts remained similar (Fig. 3a).

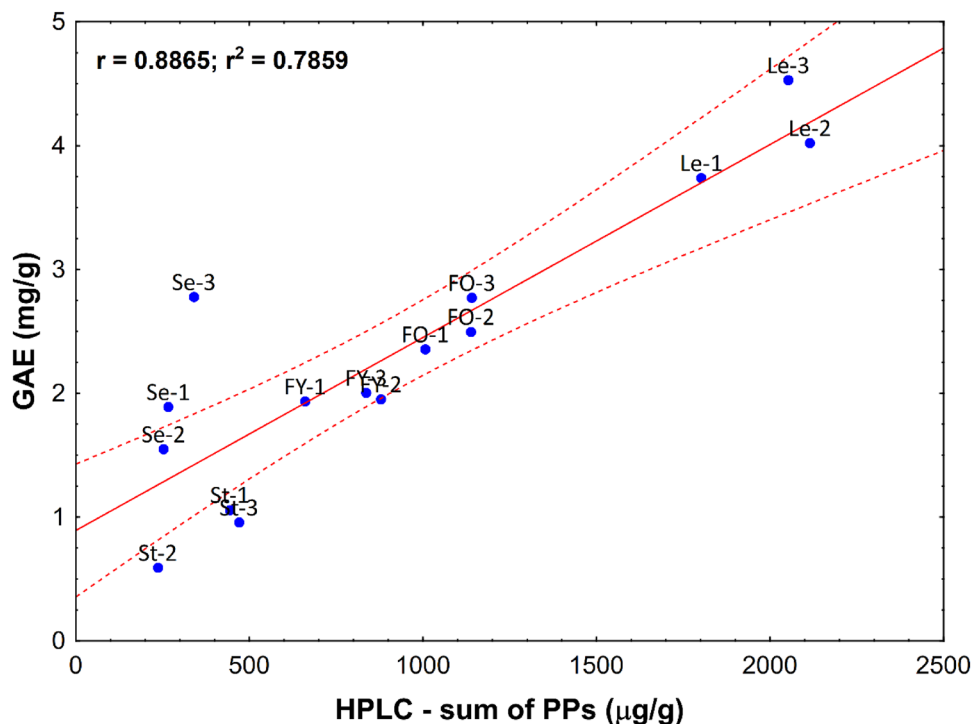
In general, the TEAC results determined by the ABTS method correlated perfectly with the total amount of PPs determined by HPLC/MS/MS (Figure S3a), except for seed extracts, which probably contain other antioxidants that do not belong to the PPs family.

### Total phenolic content assessment

First, the volume of sample added to the Folin–Ciocalteu reagent solution was optimised using orange flower and stem extracts. The final conditions together with calibration data are given in the experimental part.

The TPC values varied considerably between parts of the plant as illustrated in Fig. 3b. Among all measured samples, the lowest TPC was obtained in stem extracts, which agrees with HPLC/MS/MS analysis. In contrast, the highest TPC was found in leaves (3.4–4.0 mg GAE/g FW). Fukalova et al. [28] presented almost identical data, where extracts from fresh leaves contained 378 mg GAE/100 g FW. The TPC of the flower extracts ranged from 1.9 to 2.8 mg GAE/g FW, which agrees with the data presented by Rop et al. [29] and Garzón and Wrolstad [3], where the TPC values of the flower extract were 3.3 mg GAE/g FW and 406 mg GAE/100 g FW, respectively. On the contrary, Garzón et al. [6] reported two to three times higher results of freeze-dried samples of red, orange, and yellow flower extracts (909, 688, and 538 mg GAE/100 g FW, respectively) than in our study. This mismatch could be caused

**Fig. 4** Correlation of total phenolic content determined by spectroscopic method (GAE) with the sum of phenolic compounds determined by HPLC/MS/MS method. The correlation coefficient together with coefficient of determination is shown



by the use of different plant species, extraction solvent, or/and freeze-drying of samples.

The effect of sample pre-treatment on TPC was evident in the samples of seeds, stems, and leaves. Fresh seeds and leaves provided lower values compared to their frozen or dried forms.

In general, spectrophotometric and chromatographic methods gave comparable results of total amount of PPs with a relatively high correlation coefficient,  $r=0.8865$  (Fig. 4). The exceptions are again seed extracts, which are somewhat out of direct correlation, but the difference is not as obvious as in the case of correlation with the ABTS method (Figure S3a). As already mentioned, seeds probably contain phenolic compounds that were beyond the scope of this study; however, they possess high antioxidant properties.

Furthermore, the TPC results were also consistent with the TEAC determined by the ABTS method with a correlation coefficient of  $r=0.8888$  (Figure S3b), indicating a good correlation of both spectrophotometric methods.

### Total anthocyanin content

The TAC was determined using the pH-differential method using two buffers with different pH (pH 1 and pH 4.5). The amount of sample extract added to the buffers was optimised by gradually increasing its volume and monitoring the corresponding increase in absorbance. According to the literature [3, 6], pelargonidine-3-sophoroside, delphinidin-3-dihexoside, and cyanidin-3-sophoroside are the main anthocyanins present in nasturtium flowers; however, their individual concentrations depend on the colour of the petals. Whereas, pelargonidine-3-sophoroside is the major anthocyanin in orange flowers, delphinidin-3-dihexoside predominates in red flowers and their concentration is even similar in yellow flowers [3, 6]. For this reason, the standard of pelargonidin-3-glucoside (pg-3-glu) was selected for the determination of TAC as in the original method described by Giusti and Wrolstad [24], instead of cyanidin-3-glucoside (cy-3-glu) used later by Garzón et al. [6].

Additionally, the absorption spectra of the orange and yellow extracts were measured to confirm the choice of the correct wavelength (Figure S1). While the absorption maximum of orange flower extract was 504 nm, which corresponds to absorption maximum of pelargonidin, the absorption maximum of yellow flower extracts was shifted to the longer wavelength (514 nm) due to the contribution of delphinidin [24].

The mean TAC values of all measured samples are shown in Fig. 3c. The anthocyanins content is negligible in all parts of the plant except the flowers. The highest

anthocyanin content was found in orange flowers (about 1.18 mg pg-3-glu/g FW), which is in good agreement with Garzón et al. [6], despite the fact that the TAC was calculated as cy-3-glu equivalent in that work. In the case of yellow flowers, the TAC value was significantly lower (about 0.27 mg pg-3-glu/g FW), which is consistent with the results of Garzón et al. [6], who found 0.32 mg cy-3-glu/g FW using the HPLC technique, however, nondetectable amount of cy-3-glu equivalent was reported using pH-differential method.

### Conclusion

A total of 30 biologically active compounds were determined using RP-HPLC with mass spectrometric detection. All compounds were separated within 7.5 min using a rapid gradient elution of acetonitrile in acidified water and a Kinetex Polar column packed with core-shell particles. The individual MRM transition was optimised for selective and sensitive quantification using mass spectrometry in MRM mode. In addition, a comprehensive stepwise optimisation was required for compounds without commercially available standards.

The distribution of target compounds in extracts prepared from individual parts of the *T. majus* sample was investigated. Furthermore, the effect of sample pre-treatment (drying or freezing) before the storage of the plant on the content of target compounds was monitored.

It was found that the content of the compounds strongly depends on both the plant part and the sample pre-treatment. The highest and lowest concentrations of PPs were determined in leaves and seeds, respectively. Furthermore, the leaves also contained the greatest amount of GTL, while its content in the stem, seeds, and flowers was much lower and reached comparable values. Regarding the sample pre-treatment, it was confirmed that freezing sharply reduced the GTL content; however, the content of PPs remained similar to that of fresh one. In contrast, drying did not considerably affect the content of biologically active compounds. Their concentration was comparable or slightly lower than that of fresh samples. Therefore, compared to freezing, drying can be considered a definitely better method for storing the plant.

Furthermore, antioxidant capacities together with total phenolic and anthocyanin contents were determined using spectrophotometric methods. The highest antioxidant capacity and TPC were found in the leaves. On the contrary, these parameters were the lowest in stem. Anthocyanins occurred mainly in orange flowers. The spectrophotometric results were consistent with those obtained by the HPLC/MS/MS method, which was proven by the high correlation coefficient.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00217-022-04126-4>.

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**Author contributions** LČ: conceptualization, methodology, formal analysis, writing–review and editing, supervision. JK: data curation, writing–original draft. TŠ: investigation, data curation.

## Declarations

**Conflict of interest** The authors have no competing interests to declare that are relevant to the content of this article.

**Compliance with ethics requirement** This study does not involve any human or animal testing.

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