Application of high-temperature GC-MS for simultaneous identification and quantification of glycosidic forms of iridoids and flavonoids in plant samples

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Summary

High-temperature gas chromatography with mass spectrometry detection (HTGC-MS) was applied to separation and identification of iridoids (aucubin, catalpol), flavonoid aglycone (quercetin, apigenin, luteolin) and flavonoid glycosides (quercetin 3-*O*-α-D-arabinoside, quercetin 3-*O*-β-D-galactoside and quercetin 3-*O*-β-D-glucoside) in standard mixtures and 26 real natural matrices. Conditions of HTGC-MS were set in accordance with the best achieved separation of analytes and the highest value of signal in the used system. The developed two-step derivatization process allowed direct silanization of polar analytes in the plant material without the need of extraction. For the tested analytes, the calculated limits of detection were in the range of 2–35 mg·kg^{-1} and the values of retention indices ranged from 2858 for aucubin to 3859 for quercetin 3-*O*-α-D-arabinoside.

Keywords

iridoid; flavonoid; glycoside; high-temperature gas chromatography; mass spectrometry; plant

Flavonoids are a group of compounds that are extensively present in foodstuffs of plant origin. They belong to polyphenolic compounds known for their positive biological effects on the human organism. Their anti-inflammatory, antibacterial, antiviral, antiallergic, vasodilatory, antioxidant, antiulcer and antidiarrhoeal effects have been documented in many scientific papers [1–5]. Flavonoids are the products of secondary metabolic processes in plants [6]. In term of chemical structure they represent an extensive heterogeneous group of chemical compounds (chalcones, flavones, flavanols, flavandiols, anthocyanins, proanthocyanidines and aurones) [5, 7]. In plants, flavonoids occur as free aglycones or as glycosides with a bonded sugar component. This most frequently consists of carbohydrates: rhamnose, glucose, galactose, arabinose, xylose or rutinose [8]. To date more than 6000 types of flavonoids were identified [9]. Quercetin belongs to the best known flavonoids. In natural matrices it occurs most frequently in glycosidic forms [10–12].

Another important group of biologically active substances in plants are iridoids, which, due to their structure, belong to the group of monoterpenes. Their biological effects are similar to the polyphenolic substances, they protect the body from stress or mitigate its negative effects. Their neuroprotective [13], anticancer and antitumour [14], anti-inflammatory [15], antioxidative, antibacterial [16], antiviral [17] and anti-aging potency [18] is described in many scientific works. The

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synergistic effects between iridoids and polyphenolic compounds are well known, though their mechanism has not yet been explained and it is the subject of ongoing research [19, 20]. Therefore, determination of the content of these substances in plants, which are usually food components or are used for medicinal purposes, is relevant. Each plant matrix is a very complex mixture in terms of organic matter content, and it is important to use sufficiently effective and selective separation steps for their analysis.

In previous research works, determination of flavonoids and iridoids was performed by thin layer chromatography (TLC) [21, 22], while the currently preferred method is reverse-phase high-performance liquid chromatography (HPLC) using a diode array detector (DAD) or mass spectrometric (MS) detection [8, 11, 23]. To determine low concentrations or to improve the reliability of the analysis, HPLC coupled to MS/MS detection is used [23, 24]. Because isomeric forms of substances are usually present in the samples with the same MS or UV-Vis spectra, HPLC separation with a limited peak capacity when coupled to DAD, MS or MS/MS detection is insufficient. The use of gas chromatography for the determination of flavonoids and iridoids is also limited by the characteristics of the analytes and by the capabilities of the separation method. In the literature, derivatization processes that allow the determination of free forms of aglycons of flavonoids and iridoids by this method are published, while works published on the determination of aucubin and catalpol are the most frequent [25–27]. The use of gas chromatography (GC) for the determination of glycosidic forms of flavonoids after their derivatization was published only recently [28]. The volatility of analytes actually exacerbates with increasing molecular weight and thus reduces the GC separation usage possibility. We tried to solve this problem using high temperature gas chromatography.

High-temperature GC (HTGC) was developed and first published in 1980s. Although this analytical technique was initially not considered as sufficiently robust compared to HPLC or supercritical fluid chromatography, it has recently been successfully used for the analysis of hydrocarbons with the number of carbons in the molecule exceeding the value of 130 (simulated distillations), for the analysis of lipids, emulsifiers, detergents, polymeric additives, oligosaccharides, porphyrins and many other substances with a high boiling temperature [29–31]. In HTGC, for the extension of the boiling point distribution range of substances with low volatility, a column with a thin-film silicone stationary phase of 0.1 μm or less and with 0.53 mm internal diameter of column is used. In addition, the temperature of analysis reaches up to 450 °C. Under these conditions, the substances elute from the column by about 260–320 °C before their boiling temperature calculated for atmospheric pressure.

The aim of this work was to develop a new method for the determination of glycosidic forms of flavonoids and iridoids in plant materials using high-temperature gas chromatography.

MATERIALS AND METHODS

Chemicals and reagents

Acetonitrile and methanol were of HPLC grade from Merck (Darmstadt, Germany). Derivatization reagents *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), hexamethyl disilazane (HMDS) and trifluoroacetic acid (TFA) were from Supelco (Bellefonte, Pennsylvania, USA) and all were stored at 4 °C. Sucralose, aucubin, catalpol, apigenin, quercetin, luteolin, quercetin 3-*O*-β-D-galactoside, quercetin 3-*O*-β-D-glucoside and quercetin 3-*O*-α-D-arabinoside were from Sigma-Aldrich (St. Louis, Missouri, USA). The list of chemical structures of the studied flavonoids and iridoids is given in Fig. 1.

Instrumentation and apparatus

GC-quadrupole MS analyses were conducted on a 6890 GC and a 5973 MS system from Agilent Technologies (Santa Clara, California, USA). A DB-1 column (100% polydimethylsiloxane; Agilent Technologies) of dimensions 30 m × 0.53 mm internal diameter × 0.10 μm film thickness was used. The temperature program was from 120 °C, heated at 15 °C·min^{-1} to 350 °C and held for 1 min. Temperature of injector was set at 280 °C and 1 μl of the sample was injected in splitless mode (purge time 2 min) with a carrier gas (He) flow rate of 2.0 ml·min^{-1}. The transfer line was set at 320 °C and ion source at 230 °C, with electron ionization (EI) mode set at 70 eV, and mass range 45–800 Da in scan mode. Selective ion monitoring (SIM) mode was adopted for the quantitative analysis. The characteristic ions used for quantification of the respective silanized derivatives were *m/z* 308, 331, 361, 414, 471, 502, 559, 575 and 647. The transfer of analytes from the GC column to the MS system was performed using a silanized restrictor of 1 m × 0.1 mm. Data acquisition and processing were performed using MSD ChemStation software (Agilent).

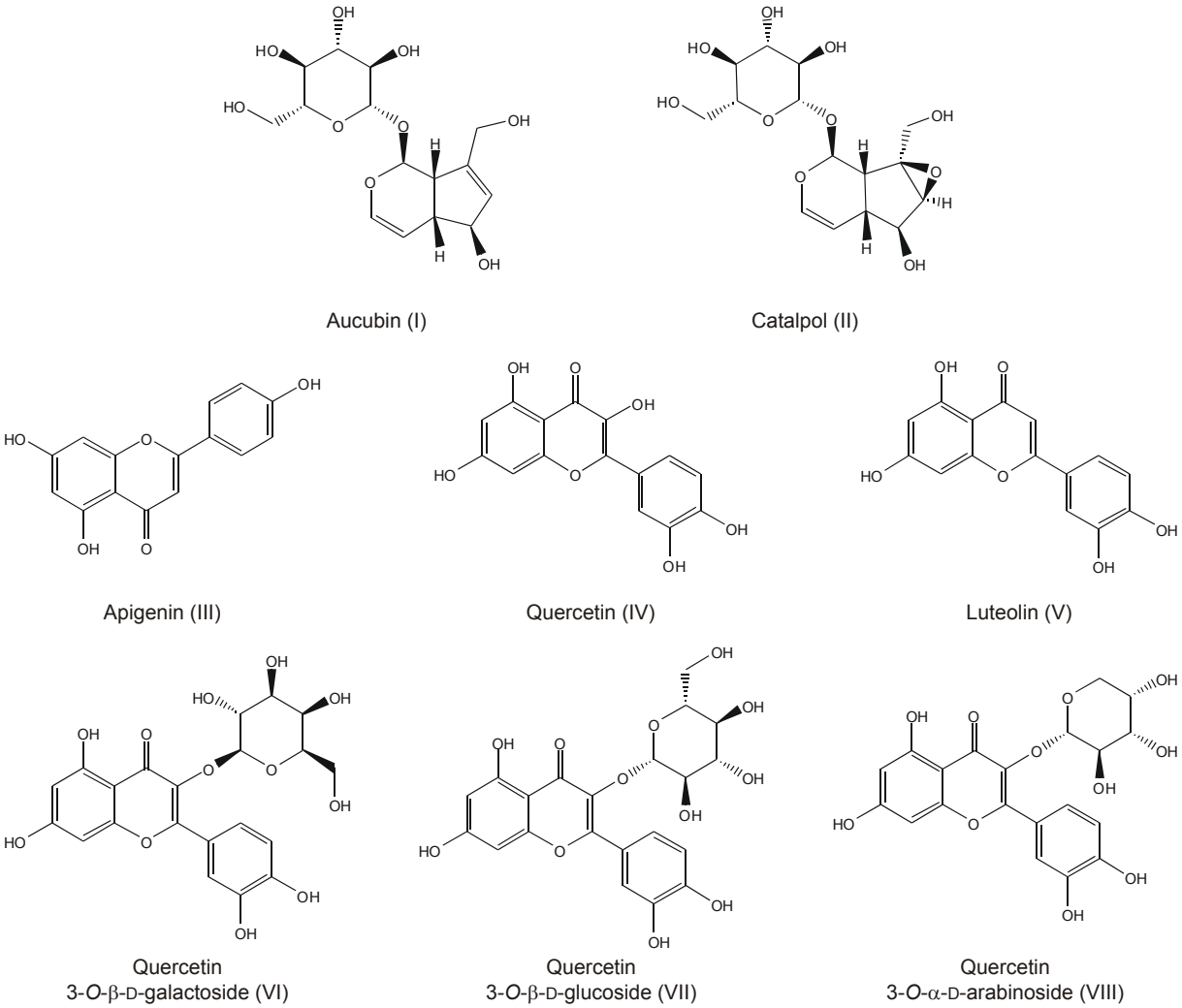

Fig. 1. Chemical structures of the studied iridiods (I, II) and flavonoids (III, IV, V, VI, VII, VIII).

Samples

For the analysis of iridoids and flavonoids, 26 plant materials of different origin (Asia, Africa, Central Europe) were used. All the samples were bought at a local market in Slovakia. For optimization and validation procedure of the analysis, the samples were modified as follows: 20 g of the sample was lyophilized and subsequently homogenized in an agate mortar. The material was stored at 8 °C while no degradation of the target compounds was observed within 2 weeks of preliminary experiments.

Derivatization procedure

Prior to HTGC-MS analysis, the analytes were derivatized by silylation reactions. Amounts of 50 mg of samples or 50 μl of standard solutions of analytes were weighed/injected into 2 ml vials. Then, 300 μl of internal standard acetonitrile solution (600 mg·l^{-1} sucralose) and 300 μl of silylating agent HMDS were added to the resulting solutions. To start the silylation reaction, 2 μl of TFA were added. The opened vials were inserted into a thermo-shaker heated to 40 °C and were shaken for 30 min at 15 Hz. Subsequently, 400 μl of derivatization agent BSTFA was added to the vials, vials were closed, placed back into the thermo-shaker heated for 80 °C and they were shaken for another 30 min at 20 Hz. Finally, the mixture was centrifuged, the supernatant was transferred to a new vial and 1 μl was injected for HTGC–MS analysis. This two-step method was used in order to achieve the simultaneous silylation of glycosidic and aglycone forms of the studied compounds. Without using the two-step silylation, e.g. by direct application of BSTFA as a silylation reagent, cleavage of carbohydrates occurs and thus makes the analysis of the glycosidic forms of flavonoids and iridoids by GC impossible.

Validation study

Validation was carried out by mixed standards and was assessed according to European SANCO guideline 12571/2013 [32]. The following parameters were evaluated during the validation of the analytic method: linearity, limit of detection (*LOD*) and limit of quantification (*LOQ*). Seven calibration levels of mixed standards in solvent were prepared to investigate the linearity. The method's *LOQ* was defined as the lowest spiked level that met the acceptability criteria regarding relative standard deviation (*RSD*) ≤20%. *LOD* and *LOQ* values were calculated based on the standard deviation of the response of the standard blank and the slope of calibration curve at signal-to-noise ratio of 3 and 10, respectively.

RESULTS AND DISCUSSION

Derivatization of iridoids and flavonoids

Trimethylsilyl derivatives are routinely employed in GC to increase the volatility and thermal stability of organic compounds containing active hydrogen. The combination of BSTFA and 1% trimethylchlorosilane is the preferred reagent mixture for trimethylsilylation of organic compounds with OH groups. However, this procedure cannot be used for derivatization of glycosidic forms of flavonoids and iridoids because the used reagents create aggressive environment leading to fission and decomposition of the carbohydrate part of molecule. Therefore, we developed a new two-step silanization process.

In the first step, partial silanization and simultaneous stabilization of derivatized molecules using HMDS as a silanization agent occurred. In the second step, reagent BSTFA addition to the mixture completed the silanization of active hydrogens without disintegration of the molecule.

The newly developed method allows silanization of substances directly in the tested plant material. In the derivatization process, silanization of the matrix (cellulose, starch, moisture, etc.) also occurred, which led to the formation of hexamethyl siloxane. This improved the solubility of silanized substances in the reaction medium. Moreover, the added value of the used method is attenuation of the matrix/target substance interactions (H-bond between non-derivatized compounds and matrix), which improves the yield of the method.

The efficiency of the derivatization procedure was investigated by analysing individual compounds by GC-MS system. All of the studied substances presented single products with no measurable amount of partially derivatized or underivatized products.

Optimization of chromatographic and detection conditions

All silanized compounds were separated on a column characterized by a high phase ratio of $\beta = 1325$. The selected type of column contributed to the reduction of retention times of the substances on the column, and thus resulted in their earlier elution at a lower temperature. We also achieved a shorter duration of the analysis of low-volatility substances. Furthermore, other parameters of chromatographic analysis have were tested: temperature gradient, flow of carrier gas, temperature of the injector and MS detector inlet temperature, in order to achieve complete separation of analytes. Selection of appropriate conditions also helped to avoid the formation of degradation products of analytes as well as their discrimination in the area of injector.

The separated compounds were quantified using MS detector in SIM mode. For each monitored substance, the most intensive fragmentation ions were chosen. Retention characteristics remained the decisive criterion for identification

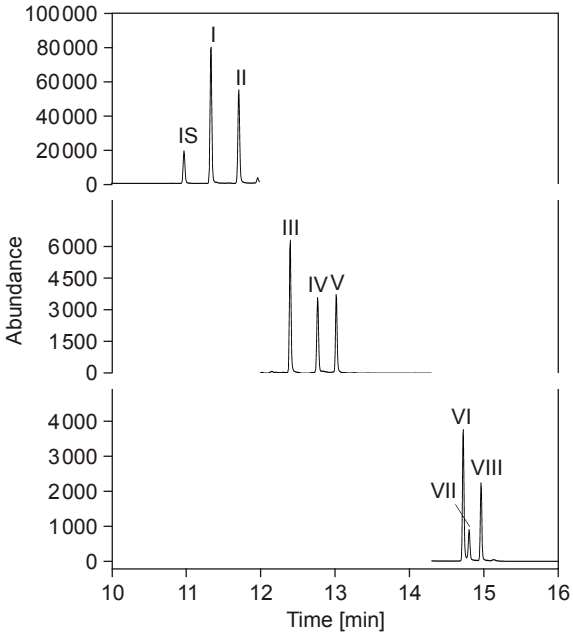

Fig. 2. Selected ion monitoring mass chromatograms of iridoids and flavonoids corresponding to a stock solution of 200 mg l^{-1} of each compound.

IS – sucralose, I – aucubin, II – catalpol, III – apigenin, IV – quercetin, V – luteolin, VI – Quercetin 3-*O*-β-D-galactoside, VII – Quercetin 3-*O*-β-D-glucoside, VIII – Quercetin 3-*O*-α-D-arabinoside.

Tab. 1. Retention and statistical characteristics of measured analytes.

Compound	Peak mark	*RI*	*m/z*	*a*	*b*	R^2	*LOD* [mg·kg^{-1}]	*LOQ* [mg·kg^{-1}]	*LDR* [mg·kg^{-1}]
Sucralose	IS	2772	308; 361	–	–	–	–	–	–
Aucubin	I	2858	361; 331	8702	–6517	0.988	2.0	6.2	2500
Catalpol	II	2949	361; 331	6981	–3574	0.988	2.5	7.8	2500
Apigenin	III	3129	471; 414	1093	–18077	0.974	21	65	3500
Quercetin	IV	3208	647; 575	3766	–788	0.983	1.8	5.6	2500
Luteolin	V	3291	559; 502	1731	–513	0.970	35	110	3500
Quercetin 3-*O*-β-D-galactoside	VI	3785	575; 647	923	–5494	0.998	11	34	3500
Quercetin 3-*O*-β-D-glucoside	VII	3809	575; 647	711	–3858	0.983	18	56	3500
Quercetin 3-*O*-α-D-arabinoside	VIII	3859	575; 647	752	–1375	0.994	9.0	28	3500

RI – retention index, *m/z* – selected ion, *a, b,* R^2 - linear regression data, *LOD* – limit of detection, *LOQ* – limit of quantification, *LDR* – linear dynamic range, encompasses from *LOQ* to the value in the table.

Tab. 2. Results of the analysis of iridoids and flavonoids by high-temperature GC-MS in real plant material samples.

Plant	Latin name	Part of herb	Content [mg·kg^{-1}]							
			I	II	III	IV	V	VI	VII	VIII
Allspice	*Pimenta dioica*	berries	ND	ND	ND	18	ND	ND	ND	ND
Basil	*Ocimum basilicum*	leaves	ND	ND	ND	88	ND	< 34	< 56	ND
Bay laurel	*Laurus nobilis*	leaves	77	12	ND	96	ND	103	316	94
Black pepper	*Piper nigrum*	cracked	ND	ND	ND	ND	ND	ND	ND	ND
Black pepper	*Piper nigrum*	ground	ND	ND	ND	ND	ND	ND	ND	ND
Brussels sprout	*Brassica oleracea*	leaves	22	< 7.8	395	13	ND	ND	ND	ND
Caraway	*Carum carvi*	seeds	ND	ND	ND	113	ND	117	77	ND
Celery	*Apium graveolens*	top	16	8	577	11	ND	ND	ND	ND
Chamomile	*Matricaria chamomilla*	flower	41	3670*	2350	142	924	60	ND	ND
Chives	*Allium schoenoprasum*	top	< 6.2	ND	< 65	45	ND	ND	63	ND
Cloves	*Syzygium aromaticum*	buds	< 6.2	ND	< 65	1770	< 110	37	321	< 28
Common nettle	*Urtica dioica*	all herb	ND	ND	ND	ND	ND	ND	ND	ND
Dill	*Anethum graveolens*	top	16	ND	155	9130*	< 110	229	194	156
Fennel	*Foeniculum vulgare*	seeds	ND	ND	< 65	1360	< 110	ND	ND	ND
Leek	*Allium porrum*	leaves	< 6.2	< 7.8	1220	28	< 110	ND	ND	ND
Littleleaf linden	*Tilia cordata*	flower	9	< 7.8	< 65	547	< 110	2650	< 56	30
Majoram	*Origanum majorana*	leaves	< 6.2	11	2010	60	5980*	43	< 56	ND
Oregano	*Origanum vulgare*	ground	ND	< 7.8	2890*	152	1970	ND	ND	ND
Parsley	*Petroselinum crispum*	root	20	8	200	ND	ND	ND	ND	ND
Parsley	*Petroselinum crispum*	top	7	< 7.8	2470	10	141	ND	ND	ND
Perforate St John's-wort	*Hypericum perforatum*	all herb	< 6.2	< 7.8	< 65	2320	< 110	6450*	2340	1090
Plantain	*Plantago lanceolata*	all herb	9700*	6850*	92	< 5.6	380	ND	ND	ND
Radish	*Raphanus sativus*	root	14	9	301	< 5.6	ND	ND	ND	ND
Rosemary	*Rosmarinus officinalis*	all herb	ND	24	926	76	6990*	ND	< 56	ND
Tomato	*Solanum lycopersicum*	fruit	10	< 7.8	647	7	ND	ND	ND	ND
Wild thyme	*Thymus serpyllum*	all herb	< 6.2	20	368	ND	6050*	ND	ND	ND

The data were calculated with the corresponding mass-calibration curve in the content units of milligrams per kilogram.
ND – not detected, * – value calculated from sample diluted 5 times.

of substances. The chromatogram of the analysed standard materials is shown in Fig. 2.

Fig. 2 clearly shows achievement of complete separation of all analytes. The greatest benefit of the developed method is the separation of quercetin 3-*O*-β-D-galactoside, quercetin 3-*O*-β-D-glucoside and quercetin 3-*O*-α-D-arabinoside. For their analysis, HPLC-MS was used so far, but it was facing a major problem of insufficient separation selectivity in terms of a poor resolution of analytes, and also a problem of detection units as analytes have similar fragmentation spectra. The retention indices (*RI*), selected ions *m/z* for MS detection in SIM mode, parameters of the calibration curves and the calculated *LOD* and *LOQ* values are listed in Tab. 1.

As presented in Tab. 1, nine standard substances were separated in the range of 1100 index units (i.u.), with an important difference of index units of two adjacent analytes that shows their mutual resolution. The lowest difference of index units had a value of 24 i.u. and regarded the substances quercetin 3-*O*-β-D-galactoside and quercetin 3-*O*-β-D-glucoside. The resolution of neighbouring substances in the selected mixture was higher than 1.5 and, therefore, all peaks were completely separated. To guarantee the correct identification of analytes in real samples based on identical *RI*, all *RI* values were determined with 1 i.u. accuracy. The values of slopes of the calibration curves for aucubin and catalpol were approximately one order of magnitude higher than the values of slopes for quercetin 3-*O*-β-D-galactoside, quercetin 3-*O*-β-D-glucoside and quercetin 3-*O*-α-D-arabinoside. This difference could be caused by the different efficiency of ionization of these compounds in the MS source and the low intensity of characteristic ions used for quantification purposes. *LOD* values were in the range from 2 $mg \cdot kg^{-1}$ for aucubin to 35 $mg \cdot kg^{-1}$ for luteolin. The *LOQ* values ranged from 6.2 $mg \cdot kg^{-1}$ for aucubin to 110 $mg \cdot kg^{-1}$ for luteolin.

Separation and identification of iridoids and flavonoids in real plant material samples

The determined contents of studied compounds in 26 samples obtained from a local market is listed in Tab. 2.

The chromatograms of perforate St John's-wort (*Hypericum perforatum)* sample under the optimal conditions are shown in Fig. 3.

To control the accuracy of analyte content determination, each sample was three times weighed in parallel, processed and analysed within one day. From the obtained data, the average content of individual substances in milligrams per kilogram was calculated. *RSD* values were in the range from 0.2% (for quercetin with the content of 2010 $mg \cdot kg^{-1}$ determined in marjoram *(Origanum majorana)*) to 19.8% (for quercetin 3-*O*-β-D-glucoside with the content 63 $mg \cdot kg^{-1}$ determined in the sample of chives (*Allium schoenoprasum)*). Low variation of peak area values for concentrations higher than 3-time *LOQ* indicated a good robustness of the tested method.

The highest content of iridoids was detected in plantain (*Plantago lanceolata*) with 9700 $mg \cdot kg^{-1}$ for aucubin and 6850 $mg \cdot kg^{-1}$ for catalpol. A significant content of iridoids was observed in chamomile (*Matricaria chamomilla),* where 3670 $mg \cdot kg^{-1}$ of catalpol was detected.

The highest content of apigenin, with a value of 2890 $mg \cdot kg^{-1}$, was determined in oregano, the highest content of quercetin was determined in dill *(Anethum graveolens)*, and rosemary (*Rosmarinus officinalis)* contained the highest amounts of luteolin with a value of 9690 $mg \cdot kg^{-1}$.

The content of glycosidic forms of flavonoids was scarcely assessed in scientific works. In our work, we evaluated the contents of quercetin 3-*O*-β-D-galactoside, quercetin 3-*O*-β-D-glucoside and quercetin 3-*O*-α-D-arabinoside in selected

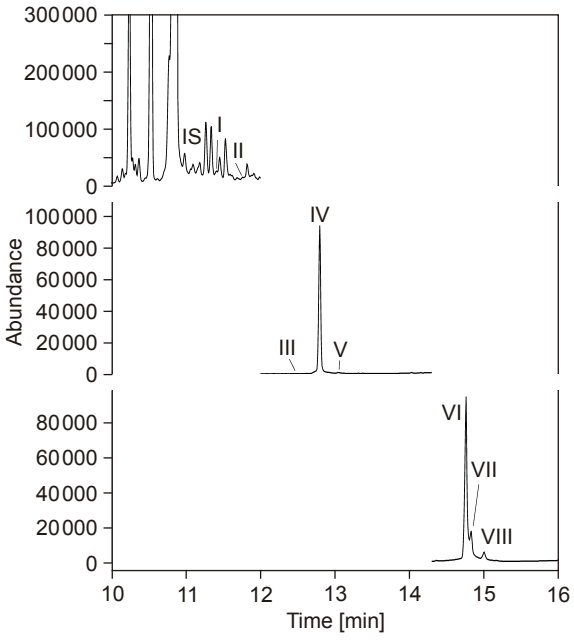

Fig. 3. Selected ion monitoring mass chromatograms corresponding to a real perforate St John's-wort (*Hypericum perforatum)* sample.

IS – sucralose, I – aucubin, II – catalpol, III – apigenin, IV – quercetin, V – luteolin, VI – Quercetin 3-*O*-β-D-galactoside, VII – Quercetin 3-*O*-β-D-glucoside, VIII – Quercetin 3-*O*-α-D-arabinoside.

plant matrices. The highest content of these compounds was observed in perforate St John's-wort, with 6450 mg·kg^{-1} quercetin 3-*O*-β-D-galactoside. A higher content of quercetin 3-*O*-β-D-galactoside (2650 mg·kg^{-1}) was also found in littleleaf linden (*Tilia cordata*). In most samples, however, quercetin 3-*O*-β-D-galactoside, quercetin 3-*O*-β-D-glucoside and quercetin 3-*O*-α-D-arabinoside were below *LOD*.

CONCLUSION

This work presents, for the first time, the use of HTGC-MS for the analysis of iridoids, glycosylated flavonoids and their aglycones in plant materials. Generally, it deals with the analysis of low-volatility polar compounds, containing 3–8 hydroxy groups in their structure. The method development included also a new two-step derivatization process based on the use of appropriately selected silylating agents. The developed separation method, together with the process of sample treatment, can serve as an alternative tool for the analysis of other polar and low-volatility chemicals present in spice plants and herbal products. The main advantages of this method are its high effectivity and selectivity of separation that facilitate reliable qualitative and quantitative analysis of glycosidic flavonoids.

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