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ROOT SPECIFIC METHYLATED FLAVONES PROTECT OF *SCUTELLARIA BAICALENSIS*

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Plant specialized metabolites are small molecules known for their role in abiotic and biotic stress tolerance. Understanding of the individual functions of most of these metabolites remains unknown. A border of the root of annual plants is especially attractive to clarity how the plant roots withstand biotic and abiotic challenges. A main part of the metabolites in the root the plant *Scutellaria baicalensis* consists of the wide variety of methylated flavones. Eight most abundant of its, mono- and polymethylated, which present the beginning and end of the plant flavone biosynthesis pathway, respectively, were detected as phenoxide-ions over the root organs (bark, cambium, xylem and decayed core) by LC-MS. This inspection recovers their location within cambium and bark. The disposition of mono-methylated wogonin and oroxylin A with it's the putative potency to form the *o*-quinon anions (reductants) provide chemical protection of the root from reactive oxygen species. The tetra- and penta-methylated flavones arrange a passive hydrophobic physical barrier of the root bark. Environment threats necessitate the plant to produce the methylated flavones, which resistance mechanisms are embedded in the structures of their molecules.

Keywords: OMe flavones, catechol, o-qinone-anions; supper oxide anion; mass spectrometry.

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Introduction

During evolution, plants have acquired their capabilities to produce metabolites, which provide the adaptive mechanisms needed for survival in changing environment. Perennial plants accumulate metabolites in the roots ensuring their life cycle, including protection from environment threats [1–3]. As it often happens the metabolites in roots of those plants have medicinal properties. Because of what, such plants in the nature of East Asia were endangered [4]. The high demand for medicinal plants led to their large-scale cultivation, which could not but affect the metabolic picture. There is a huge body of papers on how to increase the production of some metabolites, bypassing the understanding of their role in the life of the plant itself. In Russian Federation, from Altai mounts until Pacific coast, such plants have still been preserved in a nature. These are like: *Astragalus membranaceus*, *Eleutherocóccus senticósus*, *Panax ginseng*, *Rhodiola rosea*, *Scutellaria baicalensis*. This fact makes it possible to record a natural pattern of plants metabolome and to find out what and how plant root metabolites in their wild habitat are resisting the challenges of the modern era of climate change.

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The root of the *S. baicalensis* has been widely used for a treatment of various ailments in the Asia-Pacific countries. The most well-known skullcap metabolites are chrysin **1**, nor-wogonin **2**, baicalein **3**, and two mono-methylated flavones: wogonin **4** and oroxylin A **5,** but the main biological activity is attributed to the last three. Methylated flavones make up of half of all extensive composition of polyphenolic substances in the plant root [5]. Such metabolite pattern is the direct signatures of spices phenotype and contains much information about a functional status of the plant [6, 7]. Knowledge of the role of methylated flavones in the life cycle of ~~t~~he plants remains sparse.

The composition of plant metabolites largely depends on a type of tissue and key environmental impacts: climate, soil microbiome and mineral pattern [8, 9]. Although a genomic have been well shed light on biosynthesis pathway of metabolites in the *S. baicalensis* [10, 11], however environmental factors influence the metabolic pattern at the end of this pathway. Facts about flavones in the root *S. baicalensis* was obtained mainly for the roots of cultivated plants adapted to their local ecosystems [5]. Information about the role flavones can only be obtained in indirect studies, for example, the distribution of flavones in the root by the time of vegetation or by organs [12]. A pioneer histochemical study of the roots of cultivated plants in the Asia-Pacific countries found glycosides of flavones **3** and **4** in xylem [13]. The last themselves were found in a peeling bark. A similar result was obtained by a scanning of the cross section of a root preparation by the direct method, which used the mass spectrometry imaging with protonated ions desorbed by a laser ablation [14]. A more thorough similar imaging provided a lot of details on localization of biosynthesis of metabolites using phenoxide-ions [15]. So, the baicalein **3** and wogonin **4** were found only predominantly along the perimeter of the root slice. The oroxylin A **5** that is an inherent constitute of the root was not mentioned in the reports. The study of isolated organs of plants with an extraction and LC-MS methods are remained arbitration approach for identifying of plant metabolites [16]. The uncertainty in a location of other methylated flavones of eight mostly abundant ones in the roots of the cultivated [5] and wild plant of Dauria [17], an ecogeographic region East of Baikal lake, which gave the name of species, caused this study for morphological distribution of the methylated flavones. The found distribution have been shed a light on their root protection role and how ones do this used chemical properties of catechol and pyrogallol moiety of the mains flavones: baicalein, wogonin and oroxylin A.

Experiment

Plant materials. The wild plant *S. baicalensis* were harvested in the vicinity of Orlovsky town, Trans-Baikal Territory. Then, the plant's roots have been dried in the shade and thoroughly checked to remove any foreign components.

Sampling. Four specimens were prepared from dried roots for this study: bark, cambium, xylem and core, rotten xylem along root axis represented by the mass of putrefactive residues (Fig. 1). Visual examination of transverse root fracture revealed a layered structure meaning the formation of the root xylem over the years separated by dark lines presumably of rests of dead cambium layer (Fig. 1a). The bark of dried roots was taken off manually exposing a dark brown surface (Fig. 1b, upper). The latter represents the remains of cambium. The bark of a live root is separable only together with a layer of the light xylem tissue of the last year of the plant's life (Fig. 1b, bottom). The cambium layer was scraped off along with a partly of the upper lay of xylem (Fig. 1c). The root insulted to a yellow xylem is a specimen of the root xylem (Fig. 1d). The core specimen is a dark yellow putrefied mass in funnel like hollow along the root (Fig. 1e, f). Procedure of the cultivation the hairy root culture has been described previously [17].

Extraction and analysis. About 100 mg grinded specimens and hairy root culture were stored with 1 mL ethanol (96%) for a couple days at ambient temperature then twice extracted at 50 °C for 2 h. Joined extracts were centrifuged 3 min at 15000 rpm. HPLC-MS analyses were performed at the Instrumental Centre of Biotechnology and Gene Engineering of Federal Scientific Center of the East Asia Terrestrial Biodiversity FEB RAS. The supernatant was injection into a column of an LC-UV-MS\MS instrument (Agilent Technologies 1260 Infinity analytical HPLC system and Bruker HCT ion-trap mass spectrometer). The ESI MS analyses were run in negative ion mode. Zorbax analytical reverse-phase C18 column (150 mm, 2.1 mm i.d., 3.5 μm particle size) was applied for separation. Separation was carried out using the following conditions: the column temperature was 40 °C, and the mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B). The following elution gradient with a flow rate of 0.2 mL/min was used: 0 min 20% B, 3 min 20% B, 25 min 80% B, 30 min 100% B, and then eluent B until 40 min. Spectroscopy of dissociative electron capture of baicalein **3** was performed on a laboratory setup under standard conditions [18]. Density functional theory calculations were performed with the Gaussian 09 set of programs to find the energies and localization properties of the virtual molecular orbitals of the **3**, **4**, **5** target molecules.

Results and Discussion

The selected ion monitoring of the phenoxide-ion $[M-H]^-$ [19] in ESI mass spectrometry was used for the evaluation of relative constitutes of methylated flavones in the root organs from bark to rotten core (Fig. 2). The monitoring carried out on eight mostly abundant methylated flavones. The profiles of ion chromatograms are collected with a time shift in three-dimensional form along the descending mass and the number of OMe groups, as shown in Fig. 2. The bark shows a noticeable content of final flavones **8** and **9** together main mono-methylated wogonin **4** and oroxylin A **5** (Fig. 2a). An abundance of flavone **8** and it glucuronoside **8**g in cambium is a specific feature of this organ (Fig. 2a, b). The xylem is represented by flavones **4**, **5**, **8** that resembles profile of root bark but with a less content of 6,7,8,6'-tri-OMe flavone **8** (Fig. 2c). The putrefactive remains of root core are practically presented by the target flavones **4** and **5** (Fig. 2d). It is noteworthy that last flavones are presented in approximately equal parts in all specimens, excluding xylem, in which **4** prevails twice.

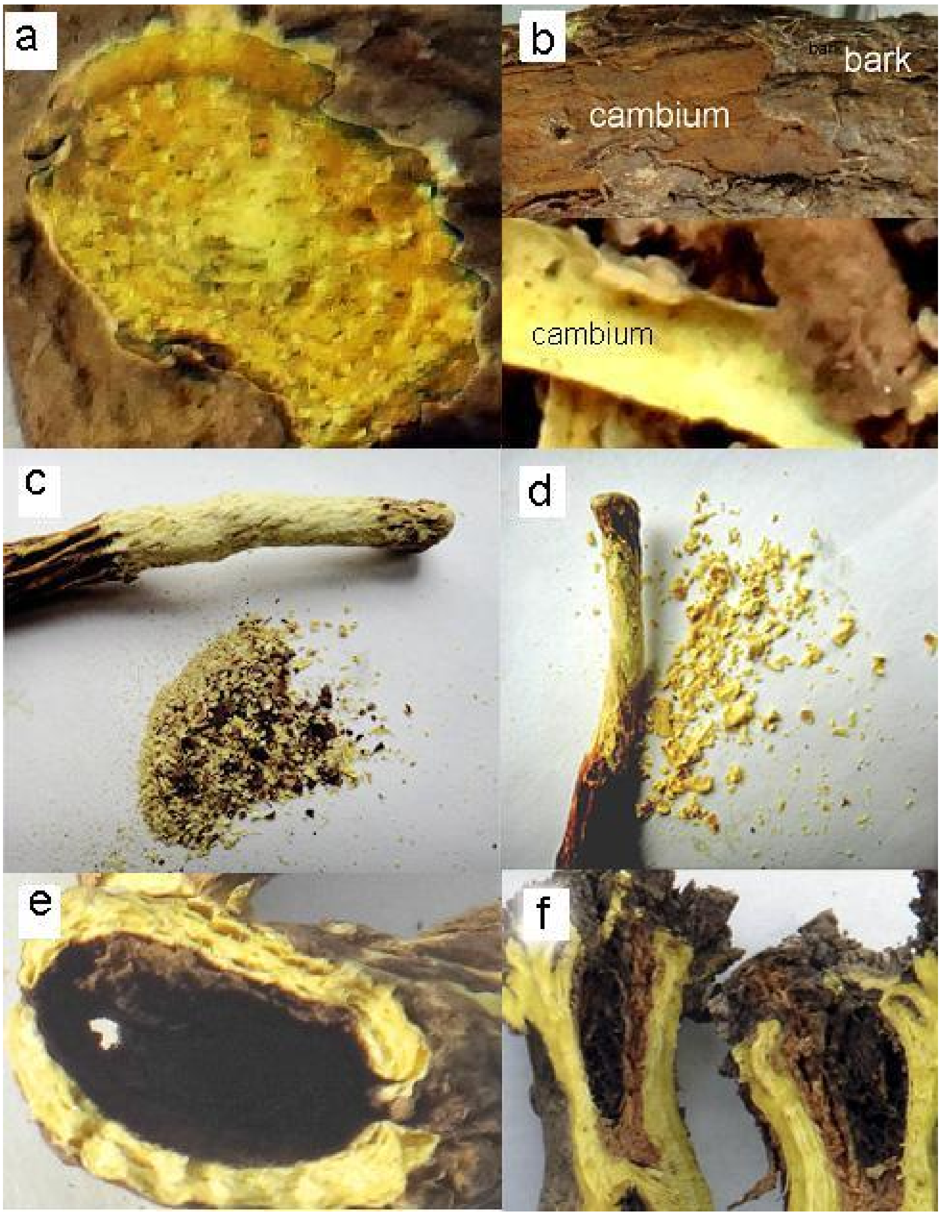

Fig. 1. Root specimens of the plant *Scutellaria baicalensis*: (a) the transverse root fracture revealed a layered structure meaning the formation of the root xylem over the years separated by dark lines; (b, upper) the bark of dried roots; (b, bottom) the bark of a live root with a layer of the light xylem tissue of the last year of the plant's life; (c) the cambium layer was scraped off along with a partly of the upper lay of xylem; (d) xylem; (e) the root tube hole and (f) it content, the mass of putrefactive residues of xylem.

The ionic profiles of free flavones, baicalein **3** and chrysin **1**, from which biosynthesis pathway splits on nor-wogonin **2** and baicalein**,** shown in inserts a-d in (Fig. 2)**.** The last both are precursors for wogonin **4** and oroxylin A **5**, respectively. Chrysin is actively synthesized in the direction of the cortex, the border of external threats, where synthesis **4** and **5** is mostly demand (Fig. 2b,2d). A low content of nor-wogonin **2,** which carries out the chemically active catechol moiety, can be regarded as increased need in it for synthesis of wogonin **4**. Abundance of final flavones in the root means high demand of baicalein **3** as a precursor its synthesis. The glucuronoside **8**g is a glycoside of particular interest due to an amphiphilicity of it molecular. A weak signal m/z 549 at 16 min for **8**g was measured only in the cambium specimen. The cambium forms a presumably hydrophobic layer between bark and xylem. The glycoside bond at C5 atom of the molecular **8**g almost completely dissociates in the ion source. As a result, the ion at m/z 373 of aglycone **8** is released at the 16th minute, while flavone **8** itself is eluted at 20.3 minutes. The hairy cells culture of the plant that grows in water broth produces more abundant glycoside **8**g. The fact allowed to run the collision dissociation of ion $[M\text{-}H]^-$ m/z 549 that have been provided the characteristic ms2 spectrum of fragments m/z 373 (15), 358 (70), 343 (15). An increasing synthesis **8**g by the cells indicates the purpose this glycoside for hydrophobic protection of the culture hairs or the plant root.

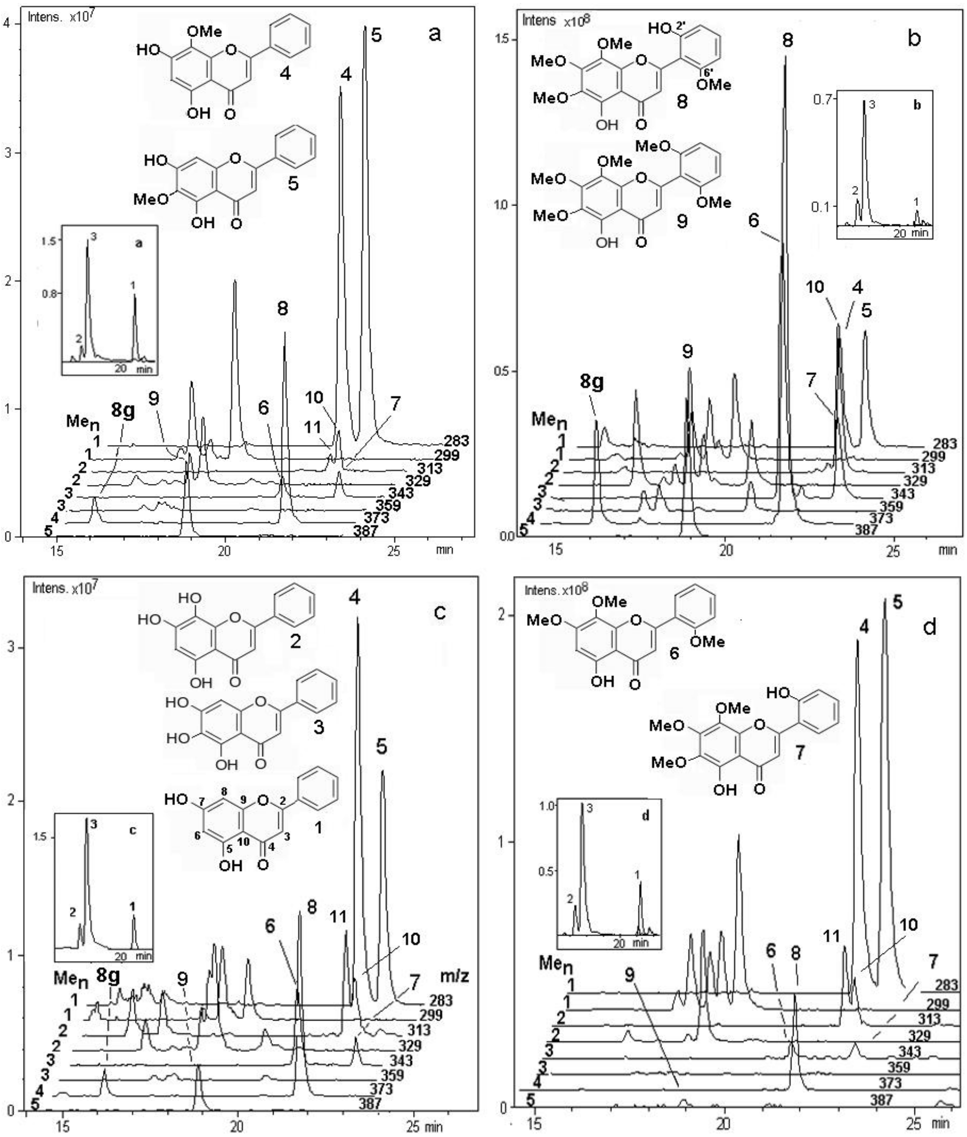

Fig. 2. Ion chromatograms for main methylated flavones of the root organs, including those beginnings of the biosynthesis pathway: (a) bark; (b) cambium. (c) xylem; (d) core (the rotten xylem along root axis represented by the mass of putrefactive residues). Annotation: (1) chrysin, (2) nor-wogonin, (3) baicalein, (4) wogonin, (5) oroxylin A, (6) 7,8,6'-tri-OMe-5-OH flavone, (7) 6,7,8-tri-OMe-2',5-di-OH flavone, (8) 6,7,8,6'-O-tetra-OMe-2'5-di-OH flavone, (9) 6,7,8,2',6'-O-penta-OMe-5-OH flavone, (10) 5,7-hydroxy-6,8 -di-methoxy flavone, (11) 5,8-hydroxy-6,7-di-methoxy flavone

The protective functions of the root flavones can be noticed in the comparison LC of the extracts of root xylem and hairy root culture. The xylem only consists of the glycosides **3**g, **4**g and **5**g of target flavones (Fig. 3a). Noteworthy, wogonin **4** and baicalein **3** have been actively produced by the culture cells already in first week (Fig. 3b). Meantime, the converting baicalein **3** to oroxylin A **5** by a methylation of 6-OH group does not occur to a noticeable extent in the culture cells. By the seventh week, a half of wogonin **4** was used up on glycosylation (Fig. 3c). Jointly sequential methylation of flavones **3** and **4** have been demonstrated the genetic program in the hairy root culture of this plant to synthesize polymethylated congeners **6-9** [17] but not performed it in degree as the plant root cambium does. Hydrophobic final flavones **8, 8**g and **9** should be attributed to the means of a physical barrier in the bark root [17]. It is assumed that an accumulation of glycosides **3**g and **5**g in the root serves as the stock for regeneration of a root damage that can be occurred during the growing season. Should note that all free flavones **2-9** practically absence in the root xylem (Fig. 3a). Their detection of negligible abundance should be attributed at the dead remains of the cambium from earlier life years of the plant, the dark rings on the root cross section (Fig. 1a). Climate and soil of the prairies Dauria caused the continuation of the biosynthesis pathway in the root of the plant *S. baicalensis* up to the final highly methylated flavones to protect presumably first and foremost of the cambium vital cells from environment threats.

The molecule of baicalein **3** carries out the pyrogallol moiety (6,7,8-tri-OH) which can be considerate as a combination of overlapping 5,6- and 6,7-catechol moieties. It seems unlikely that these both chemicals **2** and **3** have been accumulated in the xylem as free aglycones. The both should harm or rather to be deadly for the plant due to their high chemical activity. Those flavones most likely appear only in the intermediate stages during processes of methylation and subsequent glycosylation. They accumulate as glucuronides **2**g and **3**g in the root xylem, but the other part is converted to methylated derivatives **4** and **5**, respectively, by cambium cells to the side of periphery. With way the plant protects its own cells from the threat of autophagy causing by the free aglycones **2** and **3**. The pyrogallol moiety of baicalein **3** should be considered the initiator of a physical defense of the root body. When the root is damaged the 6,7-catechol moieties is freed from monosaccharide and converting to 6,7-ortho-quinone by inherent enzymes creating a necrotic tissue wall in the root body [20]. That may be why baicalin **3**g has be accumulated in significant quantities, since mechanical damage can have a much larger than point bio-invasion, the responsibility zone for nor-wogonin **2**. The other part of baicalein **3** that is produced towards the root periphery allegedly used as a precursor for the biosynthesis of the hydrophobic the molecules **8** and **9**. Observation of the flavones **4** and **5** in comparable amounts at the border of the root can indicate their different protecting roles at vicinity the bark.

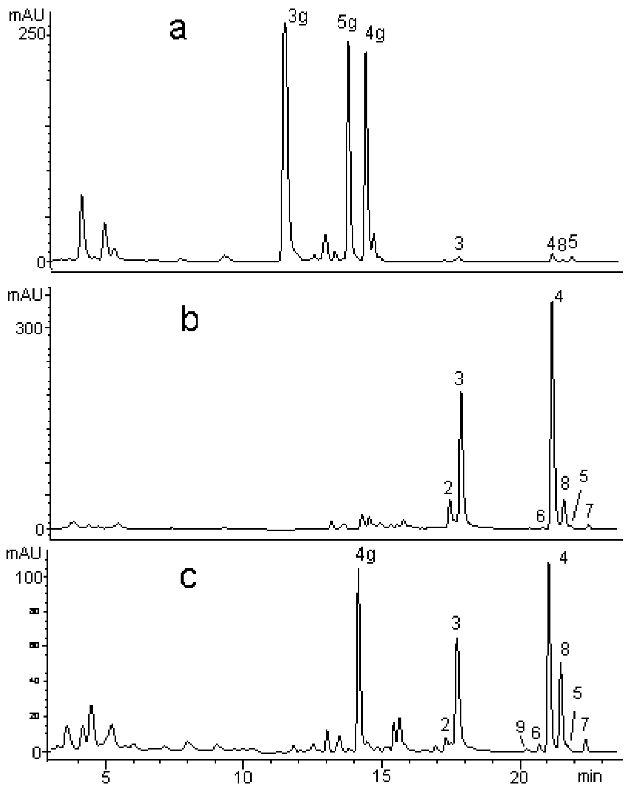

Fig. 3. Chromatograms of the extracts: (a) root xylem; (b) 7 and (c) 49 days of cultivation of the hairy roots culture of the *Scutellaria baicalensis*. Annotation: (2) nor-wogonin, (3) baicalein, (4) wogonin, (5) oroxylin A, (6) 7,8,6'-tri-OMe-5-OH flavone, (7) 6,7,8-tri-OMe-2,5-di-OH flavone, (8) 6,7,8, 6'-O-tetra-OMe-5,2'-di-OH flavone, (9). (3g, 4g, 5g) glucouronosides of baicalein 3, wogonin 4, oroxylin A 5, respectively

An environment stress at a plant is manifested as the action of a reactive oxygen species (ROS) first at all super oxide anion $O_2•^-$ [21, 22]. The last can be represented in a plant cell as carrier of the "leakage electrons" [23]. The energy of $O_2•^-$ is lower by 0.33 eV than ground state of the molecular O_2 [24] that is, it lies in the area of thermal energies. Meanwhile, the dissociative electron attachment (DEA) of gaseous thermal electrons by molecules of flavones and naphtoquinones that carry catechol moieties results the $[M-2H]^-$ ion and molecule H_2 [25, 26]. Last, having an attractive reputation in the clinic for having a regenerative potential [27], also plays the role of a signaling molecule in plant cells [28, 29]. At the same time, the energy level of the $[M-2H]^-$ ion of those natural antioxidants is 1.5-2.5 eV lower than the level of $O2•^-$ [24]. The fact suggested that the $[M-2H]^-$ ion (reductant) can restore the many of ROS. Indeed, DEA of 7,8-di-OH-5'-OMe isoflavone (retusin) produced the $[M-2H]^-$ ion (calculated the level energy -3.18 eV for water solvent conditions) [25] relative to ground state of the molecule. The value somehow explains order of magnitude greater antioxidant activity of retusin due to catechol moiety compares with known congeners [30]. The $[M-CH_3]^-$ ion from alone OMe group in the ring B (calc. value -2.19 eV for water solvent conditions) is another product-ion of DEA by retusin. These features allow to assume that wogonin **4,** in which 8-OH group of 7,8 catechol moiety of nor-wogonin **2**, methylated, interacts with the "leakage electrons" producing the reductant ($R_{7,8}^-$, calc. -2.31 eV) and molecule CH_4 in plant cells. Similar, oroxylin A **5** produces reductant ($R_{6,7}^-$, calc. -2.13 eV). The levels of the both reductants are quite low to be able to neutralize most of ROS [24]. Recently found the formation of methane in plants as well as hydrogen are constituted by plants biochemistry inherited from archaic cells [31,32]. The both H_2 and CH_4 play valuable roles in plant development and adaptation against environmental treats and changes.

To confirm the criterion of minimum energy of reductant to neutralize ROS, the study of formation gaseous reductants (ion m/z 268) in the collision experiments with glucuronosides of wogonin and oroxylin A in ESI mass spectrometer were performed (Fig. 4). The total ion current of glycosides **5**g and **4**g shares between ions $[M-H]^-$ m/z 459 and ions m/z 283 of its aglycones together with traces of their reductants m/z 268 (Fig. 4a). Noteworthy, the last has been formed from both flavones **4** and **5** already in the ion source at the threshold of ionization. The collision activation of the both glucuronosides **5**g and **4**g gave abundant phenoxide-ions m/z 283 for aglycones due to losses of glucuronlactones (Fig. 4b). The collision activation of such obtained the ions of aglycones provides a remarkable production of their reductants m/z 268 (Fig. 4c). A higher ratio ion m/z 268 to the parent ion m/z 283 for wogonin is consistent with its lower calculated energy than for this ion for oroxylin A. In favor of these scope on the reductant most of active phytochemicals of widely using spices (curcumin, capsaicin, gingerol, diosmetin, vanillin and so on) that have catechol moiety one of its OH groups protected by methylation [33, 34]. Thus, neutralizing of ROS with using the catechol carrying polyphenol intermediates, called reductants, is energetically justified.

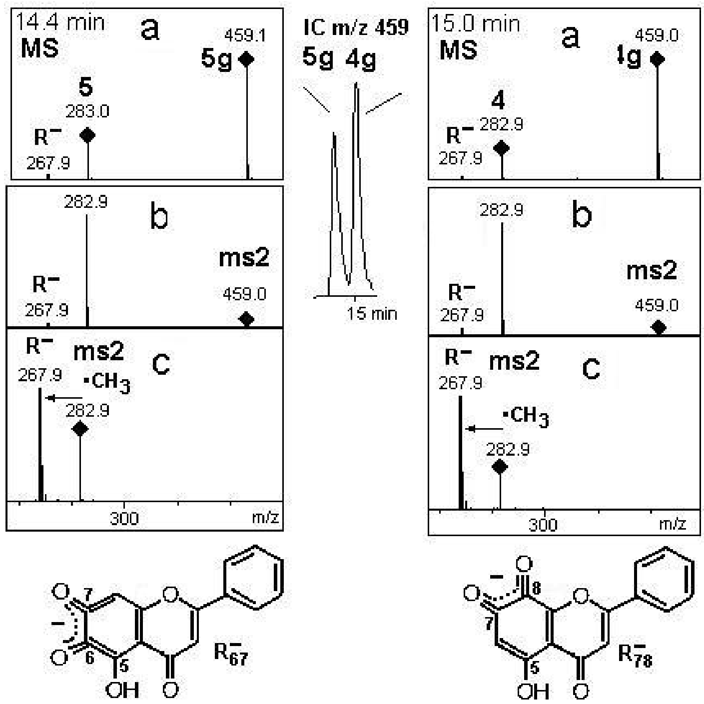

Fig. 4. Ion chromatogram of the glycosides oroxylin A **5**g, wogonin **4**g (center) and (a) their mass spectra which demonstrate the cleavage of aglycons as phenoxide-ions m/z 283 still in ion source. (b) Product ions of collision induced dissociation of ions $[M-H]^-$ for glycosides and (c) their aglycons. The last indicates a preference of the ion for wogonin **4** to form the reductant-ion m/z 268 relative one for oroxylin A **5**

Conclusion

In addition to the accumulated indirect evidences that polymethoxylated flavonoids can form part of plant protection against microbial pathogens, new data have been obtained involving the mono-methylated wogonin and oroxylin A as chemical agents. The both, due to the presence of chemically active di\tri-OH phenolic moiety, are transformed into ROS-reducing agent (reductant) under conditions of oxidative stress. Differences in the consists of the methylated flavones in organs of the plant root *S. baicalensis* shown their specific roles in a defense from environment threats. The modifications of root specific flavones by methylation within the root periphery have been used as a mechanism to modulate physical-chemical properties of cambium-cortex boundary. The ancient plant formed an effective chemical arsenal based on methylated flavones withstand to environmental threats. The report thus suggests the idea that plant-produced *o*-quinone-anion reagents and signaling molecules H_2, CH_4 within root-soil border might be an element of a survival strategy of the plant.

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