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Rapid HPLC method for scopoletine determination in *Weigela* leaves based on one-step sample preparation by extractive freezing-out

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The new rapid method was proposed for scopoletine (7-hydroxy-6-methoxy-2*H*-1-benzopyran-2-one) determination in *Weigela* leaves. It is based on the one-step sample preparation *via* extractive freezing-out (EF) with subsequent HPLC analysis. The preparation time does not exceed 1 h, and the determination limit is 0.5 mg of scopoletine per 100 g of plant mass.

The study of natural organic compounds in plants remains of significant interest due to many reasons, including the identification of effective markers of plant adaptation to extreme environmental conditions (drought, heat, frost, cold, soil salinization, *etc.*).[1] It is also important to search for promising biologically active substances (BAS) useful in cosmetics and medicine.[2] However, the solvation of such problems requires modern methods of extraction and physical-chemical analysis.

Although much of attention is paid to the development of systems for physical-chemical identification of organic compounds,[3–5] methods for the isolation of target components and preliminary preparation of biological samples for the stage of instrumental investigation are usually limited to liquid and solid-phase extractions.[6,7] Some other technologies of extraction from the plant mass are being developed and actively used. In particular, supercritical fluid extraction (SFE-technology) utilizes carbon dioxide in its supercritical state,[8] however its efficiency for extracting polar organic compounds is not enough.[9] It is also known that subcritical water (T = 100–250 °C, p ~12.5 MPa) can be used as the extractant of plant BAS.[10] In all these cases, such technologies are not appropriate for low-budget laboratories due to the need for special equipment associated with high pressure and careful adherence to the process temperature, and significant material costs.

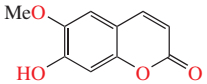
Previously, we developed a new approach to the technology of extraction of organic substances from water-containing samples, including biological ones, which is based on the formation of phase boundary in the initial homogeneous system.[11] It used the extractive freezing-out (EF),[11] wherein the target compounds are extracted under conditions of crystallization of the aqueous part of sample during the cooling after addition of a soluble (or partially soluble) non-freezing solvent, which is isolated into a separate liquid phase during the process. The combination of EF with centrifugation (EFC) allowed us to significantly increase the extraction efficiency for target substances by increasing their partition coefficients between the extractant liquid phase and ice solid phase.[12,13] It is possible to achieve 25–30 fold enrichment degree for analytes in the resulting extract by the one step extraction.[14] To date, the theoretical foundations of this method have been established thus providing the explanation of obtained regularities and prediction of effectiveness of such extraction systems.[13,15,16]

The acquired experience of using EFC in analytical practice allowed us to note some important features and advantages over other extractive methods.[12–17] The enrichment degree and efficiency of extraction of organic compounds from water (*i.e.*, the especially polar solvent) exceed those for the traditional liquid extraction. It is possible to use water-soluble extractants without any additional chemical sample modification, *e.g.*, salting out. Prepared extracts do not contain water and dispersed particles even in the case of acetonitrile and are compatible with the reversed-phase HPLC. The method makes it possible to extract substances from highly contaminated, dispersed samples directly and without any additional operations, such as filtering. It is also cheaper than liquid and solid-phase extraction, including QuEChERS (the abbreviated from 'Quick, Easy, Cheap, Effective, Rugged, Safe' name of kits for the sample preparation, including solid-phase sorbent), due to a significantly reduced number of operations, chemical utensils, and extractants. It is crucially indispensable for studies of thermolabile organic compounds, as well as favorable for improving working conditions and safety since it significantly reduces the volatility of toxic solvents and extractable substances. The selectivity of extraction is controlled by the variation of extractant, pH, and EFC conditions.

Thus, the present work was aimed at the development of express method based on EFC for the determination of one compound, scopoletine, in plant material.

The research object was leaves of three sorts of *Weigela* and *Wagnera Thunb.* possessing a different adaptability degree for the natural region: 'Augusta' (unstable), 'Eva Ratke' (medium), and 'Arlequin' (stable). They were selected due to the presence of triterpenoids[18] and coumarin derivatives in the leaves and stems of this plant.[19] Pharmacological properties of coumarins, in particular, allow us to expect their possible applications in the treatment of some diseases.

Scopoletine (7-hydroxy-6-methoxy-2*H*-1-benzopyran-2-one) is representative of natural coumarins. It was found in the roots of *Scopolia carniolica Jacq*. It is also present in various parts of some



Scopoletine

higher plants.[20] Moreover, its concentration may be increased in virus infected plants.

Known methods for scopoletine determination in plant raw materials are based on liquid extraction by alcohols and other solvents.[20,21] Thin-layer, gas and high-performance liquid chromatography are most often applied for its identification and quantitative assessment.[20–23] In those cases, the extract is separated from the plant mass and, if necessary, evaporated to remove water before the step of instrumental analysis.

We have previously identified the endogenous organic substances in ethanol extracts of *Weigela* leaves using GC-MS (Table 1). As one can see from these data, phytol and ethylglucopyranoside were present in substantially larger amounts among the identified compounds in the extracts. In addition, the vegetation significantly increases the relative content of scopoletine and squalene in the extracts. It should be noted that it was the summer period characterized by droughts and lack of moisture in the plant leaves.

The step of extraction and concentration of organic substances from plant objects is usually quite time-consuming and multi-stage process requiring a special equipment. The method of liquid extraction traditionally contains a number of serious disadvantages (*e.g.*, multistage) and limitations due to the formation of stable dispersions. A common feature is bringing pre-prepared immiscible phases into contact: aqueous solution or water-containing object (moisture content in plant objects reaches 70–80%) and extractant (insoluble organic liquid, sorbent, gas).

Expansion of the number of methods for the isolation of valuable natural organic substances, including polar and soluble, from water-containing media remains an important task for biochemistry. The main criteria include increasing the extraction efficiency, reducing the thermal and chemical act on the sample, reducing the number of analysis stages, and satisfactory expenses. In many aspects, this corresponds to our new approach,[11,12] which combines extraction with freezing-out.

The experimental procedure is a result of optimization of EFC conditions for obtaining the extract of *Weigela* leaves.[†] The conditions for the quantitative determination of scopoletine were additionally optimized taking into account its UV-VIS spectrum (Figure 1), which is in a good agreement with the reported data.[‡,24]

Table 1 Identified components in *Weigela* and *Wagneri* ethanol extracts and their relative content for different vegetation periods (2015–2017).[a]

Compound	Relative content (%)[b]	
	April	July
Ethyl α-D-glucopyranoside[c]	43±2	47±4
Ethyl palmitate	6±1	4±1
Ethyl 9,12,15-octadecatrienoate	14±3	6±2
Phytol	29±3	28±3
Scopoletine	0	4±1
Squalene	0	7±2
γ-Sitosterol + β-Sitosterol	8±2	2±1
Vitamin E	0	2±0.5

[a] A Focus SSL/DSQ II GC-MS instrument (Thermo Fisher Scientific) operating in the detection mode of 30–600 Da (positive ion) at ionization energy of 70 eV and equipped with a TR-5MS column (30 m×0.25 mm, ID = 0.25 μm) was used. [b] The averaged component content for all the *Weigela* sorts was established by the method of internal normalization using GC-MS in the TIC-mode. [c] Its presence was additionally confirmed by the derivatization into the acetyl derivative.

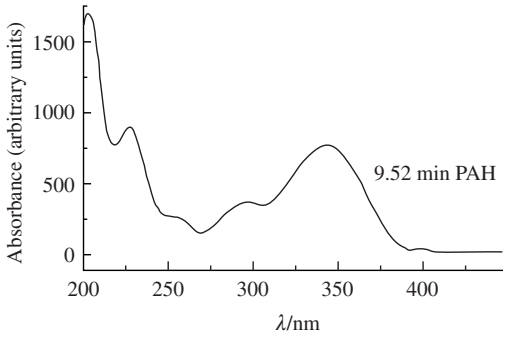


Figure 1 UV-VIS absorption spectrum of scopoletine solution in acetonitrile (340 μg ml^{-1}).

Figures 2 and 3 show HPLC chromatograms for a standard solution of scopoletine and *Weigela* leaf extract, respectively. Their comparison indicates satisfactory parameters of the chromatographic separation of extracted components.

The scopoletine content was calculated according to the method of absolute calibration performed on a series of standard solutions. The calibration graph of the area dependence of scopoletine chromatographic maximum on its concentration in the solution was described by function $y = 31394x + 414643$ with the correlation coefficient $R^2 = 0.99$.

It has been previously found in model experiments that scopoletine is completely extracted from the aqueous solutions into MeCN during the EFC procedure. Such a high efficiency of extraction from water into MeCN during the EFC process has already been established for a number of organic compounds.[12,13,16] The relative error ($n = 8$, $P = 0.95$) of scopoletine content in *Weigela* leaves did not exceed 20% in the concentration range from 1 to 50 mg per 100 g. The determination limit was 0.5 mg per 100 g.

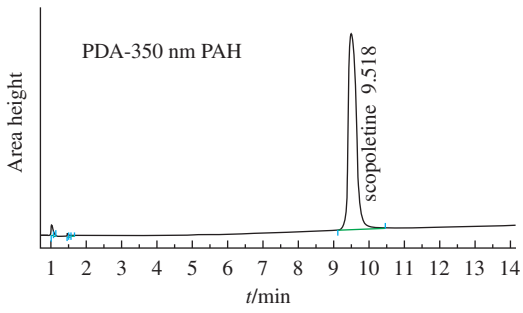


Figure 2 HPLC chromatogram of scopoletine solution in MeCN (340 μg ml^{-1}).

[†] Crushed leaves (2 g) were grounded in a ceramic cup with quartz sand (0.2 g). The prepared mixture was quantitatively transferred into glass vials (Chromacol, capacity of 12 ml), and the water–acetonitrile mixture (5 ml, 1 : 1, v/v) as an extractant was added. The vials closed with stoppers were placed on a shaker operating in the mode of constant mechanical mixing for 15 min. The samples obtained after the control weighing (the mass of vials with the sample should not differ by more than 0.4 g) were then placed in the rotor (4000 rpm/1650g) of experimental installation[17] for carrying out the EFC for 25 min at $T = -27 \pm 1$ °C. The acetonitrile extracts released into a separate liquid fraction were decanted from the surface of frozen solid aqueous phase into other glass vials. Scopoletine content in the extracts was determined by HPLC-DAD (diode array detector).

[‡] An Accela-600 HPLC-system equipped with a diode array detector (Thermo Fisher Scientific) operating at 350 nm and a Kromasil C18 column (5 μm×120 mm, ID of 2.1 mm, Lumex) was used. The gradient eluent mode was 5% isopropanol in phosphoric acid (0.125%) during 0 to 3 min, then the concentration of isopropanol was increased to 25%. The eluent flow was 400 μl min^{-1}, and the sample volume was 5 μl.

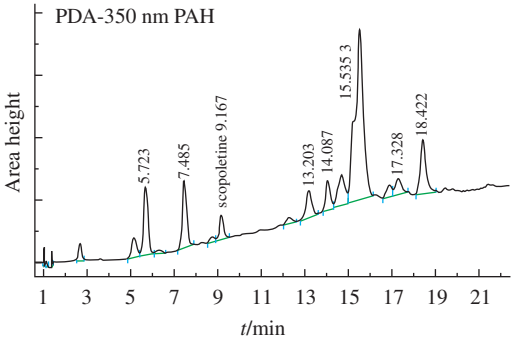


Figure 3 HPLC chromatogram of EFC leaf extract of *Weigela* and *Wagnera Thunb.* in MeCN.

In addition, it was revealed by GC-MS that the acetonitrile extracts obtained by the EFC-method do not contain ethyl α-D-glucopyranoside in contrast to an extraction with liquid EtOH. It remains in the frozen aqueous part of the sample during the EFC procedure. Therefore, the selectivity of scopoletine extraction was already improved at the step of isolation of the target components from the plant mass. We have previously observed a similar effect.[25] The EFC method improved significantly the conditions of ruscogenin isolation from the roots of *Ruscus ponticus* separated at this step from the fructose, glucose and sucrose.

Moreover, the isolation stage of scopoletine from the leaf extract takes no more than 30 min and does not require any filtering or drying (water solubility in acetonitrile at –27 °C is less than 4%). As a result, it reduces essentially the overall analysis time.

Figure 4 shows the results of scopoletine determination in fresh *Weigela* leaves. The highest scopoletine content was found in the leaves of the *Weigela* sort 'Arlequin'. These results do not contradict the information on its content in other plants, such as *Physalis alkekengi* containing about 83 mg of scopoletine per 100 g of plant mass.[26]

In conclusion, a new express method for the scopoletine determination in plants was proposed and verified using *Weigela* leaves as the example. The sample preparation step is based on the extractive freezing-out of target component in combination with the centrifugation from the plant mass. This EFC method increases the extraction selectivity towards the scopoletine allocation. The quantitative assessment of its content was carried out using reversed-phase HPLC-DAD.

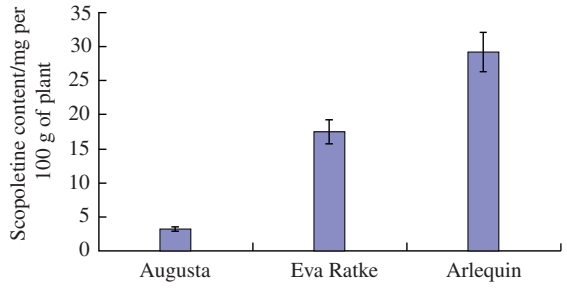


Figure 4 Scopoletine content in *Weigela* leaves harvested in 2015–2017 (natural leave moisture).

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