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Quantitative analysis of arbutin and hydroquinone in strawberry tree (*Arbutus unedo* L., Ericaceae) leaves by gas chromatography-mass spectrometry

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The phenolic glycoside arbutin and its metabolite with uroantiseptic activity hydroquinone occur naturally in the leaves of various medicinal plants and spices. In this study, an extraction procedure coupled with gas chromatography-mass spectrometry (GC-MS) was developed to determine arbutin and hydroquinone content in strawberry tree (*Arbutus unedo* L., Ericaceae) leaves. The method showed good linearity (R^2>0.9987) in the tested concentration range (0.5-200 μg mL^{-1}), as well as good precision (RSD<5 %), analytical recovery (96.2-98.0 %), and sensitivity (limit of detection=0.009 and 0.004 μg mL^{-1} for arbutin and hydroquinone, respectively). The results obtained by the validated GC-MS method corresponded well to those obtained by high performance liquid chromatography (HPLC) method. The proposed method was then applied for determining arbutin and hydroquinone content in methanolic leaf extracts. The amount of arbutin in the leaves collected on the island of Koločep (6.82 mg g^{-1} dry weight) was found to be higher (t_{paired}=43.57, t_c=2.92) in comparison to the amount of arbutin in the leaves collected on the island of Mali Lošinj (2.75 mg g^{-1} dry weight). Hydroquinone was not detected in any of the samples. The analytical features of the proposed GC-MS method demonstrated that arbutin and hydroquinone could be determined alternatively by gas chromatography. Due to its wide concentration range, the method could also be suitable for arbutin and hydroquinone analysis in leaves of other plant families (Rosaceae, Lamiaceae, etc.).

KEY WORDS: *Ericaceae; food safety; GC-MS; HPLC; solvent extraction*

Arbutin is a hydroquinone-β-D-glucopyranoside present in the leaves of various plant species, especially in Ericaceae [*Arbutus unedo* L. (strawberry tree), *Arctostaphylos uva-ursi* (L.) Spreng. (bearberry), *Vaccinium vitis-idaea* L. (lingonberry)], while less often in some other species such as marjoram or *Origanum majorana* L. from the Lamiaceae family (1, 2). This phenolic glycoside is a secondary plant metabolite that forms as a plant's response against infectious diseases and environmental stress conditions (e.g. extremely low or high temperatures) (3, 4). Arbutin is used as an antiseptic for treating urinary infections as well as a skin whitening agent in cosmetics due to its inhibitory effect on tyrosinase activity (5, 6). Its bactericidal activity is probably brought by the hydroquinone released from arbutin by the action of β-glucosidase (3, 7). After ingestion, arbutin is absorbed from the gastrointestinal tract and hydrolysed by intestinal flora to form the aglycone hydroquinone (not to be confused with the naturally-occuring hydroquinone found in certain plants following release from arbutin upon plant β-glucosidase activity) (3, 6, 8). In herbal preparations used to fight pathogenic microorganisms, hydroquinone is recognized as an active substance at the site of action (urinary tract) and is crucial for therapeutic activity. Since hydroquinone appears to have hepatotoxic, nephrotoxic, and genotoxic potential and has been detected not only in glycolysated form as arbutin, but also in free form in the leaves of several plant families (9), its application in the treatment of human urinary infections should be controlled through food safety systems such as by monitoring the dietary intake of both hydroquinone and its glycoside derivative arbutin.

A. unedo, an evergreen wild shrub that grows in the Mediterranean region (10), contains many pharmacologically relevant polyphenols and one of its main phenolic compounds is arbutin (1, 11). *A. unedo* leaf infusions are known to have diuretic, astringent, and uroantiseptic properties and are used in folk medicine for treatment of hypertension, diabetes, and inflammation (12-15). As phenolic compounds have powerful antioxidative activity, the identification of individual compounds in leaves responsible for pharmacological activity has been the focus of interest in recent years.

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Several methods have been reported for arbutin and/or hydroquinone quantification in leaves, including high performance liquid chromatography (HPLC) with diode array detection (DAD) (2, 11, 16), UV detection (17, 18), mass spectrometry (MS) detection (19), as well as gas chromatography-mass spectrometry (GC-MS) (20), nuclear magnetic resonance (NMR) spectroscopy (1), and densitometry (21).

To the best of our knowledge, only one GC-MS method has been reported for arbutin quantification in plant leaf extracts (20) and no validated GC-MS method has been developed for the simultaneous quantification of arbutin and hydroquinone in leaves. The aims of this study were as follows: compare the effectiveness of different solvents for extracting arbutin and hydroquinone from *A. unedo* leaves, validate the GC-MS method for determining arbutin and hydroquinone in leaf extracts, and compare the results with those obtained by HPLC analysis. Finally, the proposed method was used to determine arbutin and hydroquinone content in *A. unedo* plant leaves growing at two different geographical locations.

MATERIALS AND METHODS

Chemicals and reagents

Standards of arbutin and hydroquinone were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol, dichloromethane, ethyl acetate, acetonitrile (all of HPLC grade) and formic acid (p.a.) were purchased from Merck (Darmstadt, Germany), while acetic acid (MS grade) was purchased from Sigma-Aldrich (Steinheim, Germany). N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1 % trimethylchlorosilane (TMCS) and N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) with 1 % TMCS were obtained from Restek (Bellefonte, PA, USA). Ultrapure water (MicroPure water purification system, 0.055 μS cm^{-1}, TKA; Thermo Fisher Scientific, Niederelbert, Germany) was used to prepare standard solutions for HPLC analysis.

Plant material

A. unedo leaves were collected in May 2013 on the Croatian islands of Mali Lošinj (GPS coordinates: 44°31′50″ N; 14°28′06″ E; 14 m a.s.l.) and Koločep (GPS coordinates: 42°40’34” N; 18°00’35” E; 34 m a.s.l.). The leaves of 25 randomly selected mature plants were harvested on a dry day and mixed to obtain randomly selected samples. The leaves were air-dried for twenty days in a well-ventilated room at 60 % air humidity and room temperature (22 °C), single-layered and protected from direct sunlight. The dried leaves were then milled in a laboratory mill and stored in plastic containers in a dark place at room temperature.

Preparation of sample extracts and standard solutions

Following literature data (4) for the evaluation of extraction efficiency, two extraction methods (sonication and vortexing) and two different volumes (5 and 15 mL) of four different solvents were tested: methanol, 80 % methanol, dichloromethane, and ethyl acetate. Three different sample weights (25, 50, and 200 mg) and two derivatisation reagents (BSTFA+1 % TMCS and MSTFA+1 % TMCS) were also considered. All of the experiments were carried out in triplicate with homogeneous plant material collected from the same site.

In the final optimised procedure, 25 mg of dried leaf sample was mixed with 5 mL of methanol in an ultrasonic bath for 30 minutes at room temperature. The mixture was vortexed after 15 minutes in order to additionally facilitate the extraction process. Extracts were filtered through a 0.45-μm Whatman filter paper and aliquots of 1 mL were used for further GC-MS analyses.

For the comparison of GC-MS and HPLC, 3 g of dried leaf sample was extracted with methanol using procedure described above. The obtained extracts were frozen and lyophilised in a vacuum freeze dryer Hetosic (Heto, Denmark). The lyophilisate was grounded in a laboratory mill and stored in a desiccator due to its hygroscopic characteristics. For GC-MS analysis, 25 mg of lyophilisate were mixed with 5 mL of methanol by vortexing for 10 min and an aliquot of 1 mL was used for further analysis.

An aliquot of 1 mL of extract (dried leaves and lyophilisate) was evaporated to dryness under a stream of nitrogen at room temperature. The dried extract was dissolved in 100 μL BSTFA with 1 % TMCS. The mixture was heated at 65 °C for 30 min, and 1 μL of sample cooled to room temperature was injected into the GC-MS system.

Each sample was analysed in triplicate and arbutin and hydroquinone content were expressed on dried leaf weight.

Stock standard solution (0.5 mg mL^{-1}) was prepared by dissolving arbutin and hydroquinone in methanol. The working standard solutions were obtained by using appropriate aliquots of stock standard solution diluted with methanol. Standard solutions were stored at –20 °C in a freezer.

One-mL aliquots of a set of working standard solutions ranging from 0.5 to 200 μg mL^{-1} for arbutin and hydroquinone were evaporated to dryness, derivatised, and analysed in the same way as leaf extracts.

GC-MS analysis

The analyses of the derivatised samples and standard solutions were carried out using a Trace 1300 gas chromatograph (Thermo Scientific, Milan, Italy) coupled to a ITQ 700 ion trap mass spectrometer (Thermo Scientific, Austin, TX, USA). The analytes were separated on a TG-5MS capillary column (30 m×0.25 mm ID, 0.25 μm film thickness) from Thermo Scientific (Runcorn, UK). Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. The

oven temperature programme was: 120 °C for 1 min, then increased to 280 °C at 20 °C min^{-1} and held for 3 min. Both injector and transfer line temperatures were set at 280 °C. Samples (1 µL) were injected in split mode with a split ratio of 10:1. The MS detector operated in electron impact (EI) ionisation mode. The ion source temperature was set at 220 °C. Quantification was done in selected ion monitoring (SIM) using *m/z* 254 for arbutin and *m/z* 239 for hydroquinone. External standard calibration was used: peak area of each target analyte was plotted against its concentration. The analytes were identified by matching the retention times and mass spectral data with the calibration standards.

Validation of GC-MS method

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated using a signal-to-noise ratio of 3 and 10, respectively. Precision was expressed as relative standard deviation (RSD) using six replicates of standard solution at concentrations of 20 and 100 µg mL^{-1}. The recovery of the method was evaluated by analysing samples of methanolic leaf extracts of known analyte concentrations (N=6) spiked with arbutin and hydroquinone at concentrations of 20 and 100 µg mL^{-1}.

HPLC-DAD analysis

Five mg of lyophilisate was dissolved in 1.5 mL of ultrapure water, and centrifuged at 13,000×*g* at room temperature. Clear supernatant was decanted, mixed with a standard solution of arbutin (80 µg mL^{-1}) in 1:1 (v/v) ratio, filtered through a syringe filter (13 mm, polytetrafluoroethylene (PTFE) membrane 0.45 µm; Supelco, Bellefonte, PA, USA) and 20 µL of extract was injected into the HPLC system.

An Agilent 1260 Infinity HPLC system was consisted of a 1260 Quat Pump (G1311B), 1260 ALS Autosampler (G1329B), 1260 TCC Column heater (G1316A), and DAD 1260 VL+ (G1315C) detector (Agilent Technologies, Santa Clara, CA, USA). Separations were performed using a Zorbax Eclipse Plus C18 column, 4.6×150 mm in size, with a 1.8 µm particle diameter (Agilent, Technologies, Santa Clara, CA, USA). The mobile phase was (A) 0.1 % formic acid and (B) methanol. The gradient program was as follows: 0–2 min 95 % A, 2–6 min 95 % A to 85 % A, 6-8 min 85 % A to 75 % A, 8-12 min 75 % A to 30 % A, 12-14 min 30 % A to 5 % A, 14-20 min 5 % A, 20–25 min 5 % A to 95 % A. The flow rate was 0.5 mL min^{-1}. Arbutin was detected and quantified at 290 nm. Quantification was performed using the calibration curve of the arbutin standard, concentration range 20-150 µg mL^{-1}.

Data analysis

Excalibur software (ver. 2.1, Thermo Scientific, San Jose, CA, USA) was used for data acquisition and chromatographic data analysis. Microsoft Office Excel 2007 was used for statistical calculations. Comparison of the amount of arbutin in the samples collected on two localities (the islands of Koločep and Mali Lošinj) was performed by one-tailed paired *t*-test. Linear regression analysis using the least squares method was used to evaluate the calibration curve of analytes. Data are expressed as mean±SD (standard deviation of average value).

RESULTS AND DISCUSSION

Optimisation of the extraction procedure

Organic solvent extraction is the most common method for extracting phenolics (22). In order to establish the most effective extraction of arbutin and hydroquinone in dried leaf material, variables such as type and volume of solvent and extraction method were optimised. Since hydroquinone was not detected in the leaf samples, a standard solution of hydroquinone in methanol was added to samples to contain 1 mg g^{-1} dried weight to optimise the extraction procedure. This concentration was chosen according to the literature data regarding hydroquinone content in the leaves of *Arctostaphylos uva-ursi*, *Vaccinium vitis-idaea*, and *Origanum majorana* (17, 18). Arbutin and hydroquinone were extracted from samples with 5 or 15 mL of methanol, 80 % methanol, ethyl acetate, and dichloromethane using

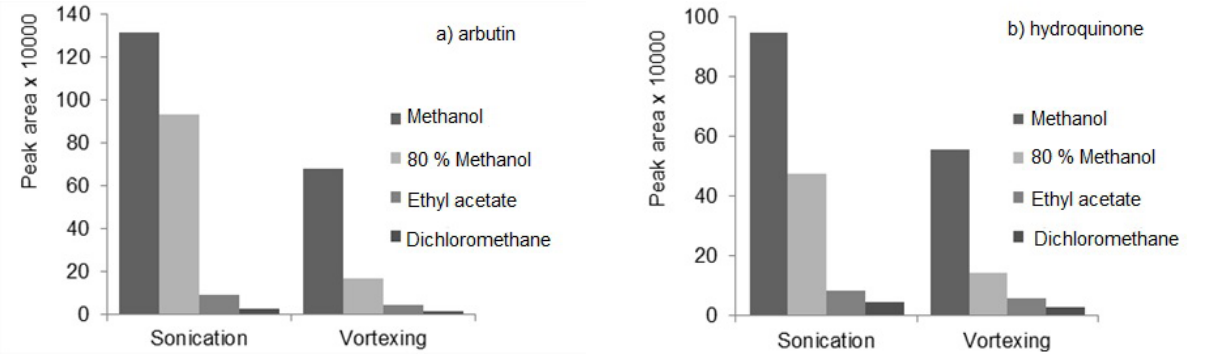

Figure 1 *Effect of solvent (methanol, 80 % methanol, ethyl acetate, and dichloromethane) and extraction type (sonication and vortexing) on the extraction efficiency of arbutin naturally present in* Arbutus unedo *leaf samples (2.80 mg g^{-1}) (A) and extraction efficiency of hydroquinone added to* A. unedo *leaf samples at 1 mg g^{-1} (B)*

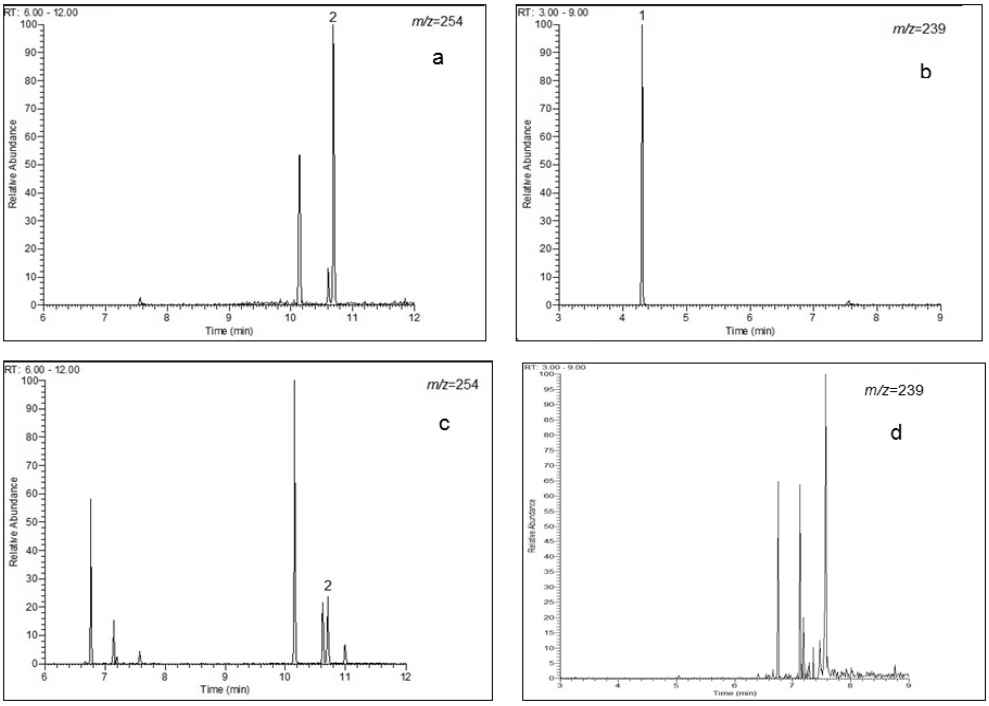

Figure 2 *Selected ion chromatograms for arbutin (*m/z*=254) and hydroquinone (*m/z*=239) standard solution (20 µg mL^{-1}) (a and b) and for a leaf extract sample obtained by the proposed GC-MS method (c and d).*
Peaks: 1=hydroquinone bis(trimethylsilyl ether); 2=arbutin penta(trimethylsilyl ether)

vortexing for 10 min or an ultrasonic bath for 30 min. Room temperature was used during extraction to prevent the degradation of the analytes due to the increased temperature (22). Figure 1 shows the extraction efficiency for different solvents and extraction types. Ultrasound-assisted extraction with methanol gave the highest recovery for both arbutin and hydroquinone. The volume of solvent did not affect the extraction efficiency, so 5 mL were used as the volume for all of the solvents in further procedures. As mentioned, sonication has been shown to be more effective than conventional vortexing. Most relevant studies performed arbutin and/or hydroquinone extraction from leaves using ultrasound assisted extraction (2, 17, 20). As for the amount of sample, 25 mg of sample showed better method precision, less baseline noise, and much better peak shape and resolution in the chromatogram compared to 50 or 200 mg. According to this experiment, the most suitable extraction procedure for arbutin and hydroquinone was ultrasound extraction with 5 mL of methanol for 30 min using 25 mg of dried leaf sample.

Optimisation of the derivatisation reaction

Due to the low volatility of the GC analysis target compounds, a derivatisation step using a silylation reagent was necessary to convert arbutin and hydroquinone to more volatile derivatives (4). BSTFA and MSTFA, both containing 1 % TMCS as the catalyst, were compared in this study. The temperature (60 °C) and reaction time (30 min) were chosen according to the preliminary experiment (data not shown) and recommendations from previous studies (4, 23). The type of derivatisation reagent did not affect the arbutin and hydroquinone peak shape and peak area. Since the BSTFA reaction produces corrosive by-products that can damage metal syringe needles and plungers as well as the capillary GC column, MSTFA+1 % TMCS was used as a derivatisation reagent in further analyses. The MSTFA+1 % TMCS volume sufficient to complete a derivatisation reaction was tested by dissolving dry extract (from leaf samples and arbutin and hydroquinone standard in methanol at 100 µg mL^{-1}) in 100 and 200 µL of

Table 1 *Analytical features of the proposed GC-MS method*

Analyte	LOD (µg mL^{-1})	LOQ (µg mL^{-1})	Precision[a] (RSD %)		Recovery[b] (%)	
			20 µg mL^{-1}	100 µg mL^{-1}	20 µg mL^{-1}	100 µg mL^{-1}
Arbutin	0.009	0.029	4.3	3.8	96.2	97.5
Hydroquinone	0.004	0.017	3.9	2.8	97.3	98.0

LOD–limit of detection; LOQ–limit of quantification
[a]*refers to standard solution of arbutin and hydroquinone at two concentrations (N=6)*
[b]*refers to the sample spiked with standard solution of arbutin and hydroquinone at two concentrations (N=6)*

derivatisation reagent. The results showed that a volume of 100 μL was sufficient to complete a derivatisation reaction. The optimised conditions for derivatisation were dissolving dry methanolic extract in 100 μL of MSTFA+1 % TMCS and heating the mixture at 60 °C for 30 min in a tightly capped glass tube.

Analytical features of the proposed GC-MS method

The proposed GC-MS conditions allowed a good separation of arbutin and hydroquinone with no interfering peaks. Figure 2 shows selected ion chromatograms for the standard solution of arbutin and hydroquinone (20 μg mL^{-1}) and for a sample containing 2.80 mg of arbutin per g of dried leaf weight. The calibration curves were linear (R^2>0.9987) over the tested concentration range (0.5-200 μg mL^{-1}). Table 1 shows the LOD, LOQ, and precision of the developed method as well as the analytical recovery of the studied compounds. The precision of the method (RSD<5 %) and analytical recovery (96.2-98.0 %) were comparable to the previously developed HPLC methods for arbutin and hydroquinone quantification in leaf extracts (17, 18) or arbutin quantification in medicinal plant extracts (24) as well as to the GC-MS method for arbutin quantification in leaf extracts (20). The LOD for arbutin (0.009 μg mL^{-1}) and for hydroquinone (0.004 μg mL^{-1}) obtained in this study showed an improvement in method sensitivity over the HPLC methods reported by Fecka and Turek (17) and Rychlinska and Nowak (18) (LODs>0.49 μg mL^{-1}) and the GC-MS method reported by Lamien-Meda et al. (20) (LOD for arbutin=0.13 μg mL^{-1}). An LOD for arbutin similar to the LOD achieved in our study was reported only for the HPLC method developed by Thongchai et al. (24) (LOD=0.005 μg mL^{-1}).

GC-MS and HPLC-DAD method comparison

For a comparison of the GC and HPLC methods, lyophilisate was analysed in triplicate and results were expressed as mg of arbutin per g of dried leaf. The GC result was 2.85 mg g^{-1}, while the HPLC analysis of arbutin content yielded 2.95 mg g^{-1}, which indicated that these results were in good agreement. The slightly lower level of arbutin obtained by the GC method could be explained by the loss of a certain amount of arbutin during the evaporation and derivatisation process; in HPLC analysis, the procedure is applied directly to the lyophilisate.

Sample analysis

The determination of arbutin and hydroquinone content in leaf samples is of great importance for estimating dosages of herbal products and evaluating the toxicity of hydroquinone to insure proper uroantiseptic activity. The proposed method was applied for determining arbutin and hydroquinone levels in *A. unedo* leaves from two geographical locations. *A. unedo* leaves collected on the island of Koločep showed statistically significant (t_{paired}=43.57, $t_{c(one\text{-}tailed)}$=2.92, $p \leq 2.63 \times 10^{-4}$) higher arbutin content (6.82±0.12 mg g^{-1}) compared to the leaves collected on the island of Mali Lošinj (2.75±0.06 mg g^{-1}). The differences in arbutin content may have been related to climate and soil characteristics of the sites where the plants grow. Our results for arbutin levels in *A. unedo* leaves are quite similar to those reported in other studies, ranging from 0.6 mg g^{-1} (1) to 12.4 mg g^{-1} (25). Hydroquinone was not detected in any of *A. unedo* leaf samples, which is in accordance with results reported by Pavlović et al. (16).

CONCLUSION

To the best of our knowledge, this is the first GC-MS method reported for the simultaneous analysis of arbutin and hydroquinone in leaf samples. In this study, methanol has been shown to be the most effective solvent for arbutin and hydroquinone extraction. The developed GC-MS method was shown to be sensitive, precise, and accurate. It is a good alternative for laboratories without liquid chromatographs and enables an unambiguous confirmation of the structure of the tested compounds. The analytical features of this method allow for arbutin and hydroquinone to be determined in a wide concentration range, which is why this method could also be used to analyse leaves of other plants used for therapeutic purposes.

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Conflicts of interest

The authors declare no conflict of interest.

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Kvantitativna analiza arbutina i hidrokinona u listovima obične planike (*Arbutus unedo* L., Ericaceae) plinskokromatografskom metodom uz detekciju masenim spektrometrom

Fenolni glikozid arbutin i hidrokinon, njegov metabolit s uroantiseptičkim djelovanjem, prirodni su sastojci lišća različitih biljaka koje se koriste u terapeutske svrhe te začinskih biljaka. U ovom radu optimiran je postupak ekstrakcije te je razvijena i validirana plinskokromatografska metoda uz detekciju masenim spektrometrom (GC-MS) za određivanje sadržaja arbutina i hidrokinona u lišću obične planike (*Arbutus unedo* L., Ericaceae). Metoda je pokazala dobru linearnost (R^2>0,9987) u ispitivanom koncentracijskom rasponu (0,5-200 μg mL^{-1}), kao i dobru preciznost (RSD<5 %), analitički povrat (96,2-98,0 %) i osjetljivost (granica detekcije=0,009 μg mL^{-1} za arbutin i 0,004 μg mL^{-1} za hidrokinon). Rezultati dobiveni validiranom GC-MS metodom u dobroj su suglasnosti s rezultatima dobivenim metodom tekućinske kromatografije visoke učinkovitosti (HPLC). Predložena metoda je primijenjena za određivanje sadržaja arbutina i hidrokinona u metanolnom ekstraktu lišća. Sadržaj arbutina određen u lišću obične planike prikupljenom na otoku Koločepu (6,82 mg g^{-1} suhe mase) bio je veći u usporedbi sa sadržajem arbutina u lišću prikupljenom na otoku Malom Lošinju (2,75 mg g^{-1} suhe mase). Hidrokinon nije detektiran ni u jednom uzorku. Analitičke značajke predložene GC-MS metode pokazale su da se arbutin i hidrokinon mogu alternativno određivati plinskom kromatografijom. S obzirom na široki koncentracijski raspon, metoda je pogodna za analizu arbutina i hidrokinona, ne samo u lišću obične planike, nego također i u lišću biljaka drugih porodica (Rosaceae, Lamiaceae, itd.).

KLJUČNE RIJEČI: *ekstrakcija otapalom; Ericaceae*; *GC-MS; HPLC; sigurnost hrane*