



# Authentication of two eighteenth century juniper-containing drug remains by HPLC–MS/MS and GC–MS

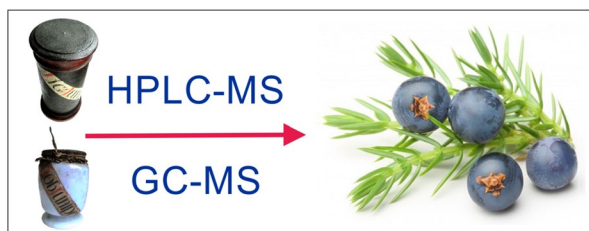
Karel Nesměrák<sup>1</sup> · Tomáš Lener<sup>1</sup> · Anton Korban<sup>1</sup> · Martin Štícha<sup>2</sup>

Received: 12 May 2023 / Accepted: 14 June 2023 / Published online: 12 July 2023  
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## Abstract

Two historical remains of juniper-containing drugs, dating from the eighteenth century, were analyzed using two separation techniques with mass detection (HPLC–MS/MS and GC–MS). As reference material, replicates of one of these analyzed historical remains, juniper preserve, were prepared according to period recipes. Although the HPLC–MS/MS method was suitable for authenticating the origin of a drug prepared from juniper berries (i.e., juniper preserve), it proved unsuitable for a drug containing only juniper wood. In contrast, GC–MS was able to demonstrate that this drug did indeed contain juniper wood. Thus both studied samples were verified to be juniper-containing drugs. A remarkable stability of some glycosides was observed in the samples analyzed. The presence of viridiflorin was demonstrated for the first time in the juniper plant, and a detailed ESI<sup>+</sup>-MS<sup>n</sup> fragmentation of this substance was proposed.

## Graphical abstract



**Keywords** Chemotaxonomy · GC–MS · HPLC–MS · *Juniperus communis* · Tandem mass spectrometry · Viridiflorin

## Introduction

Common juniper (*Juniperus communis* L.), or a related species, is a plant that has been used by mankind since time immemorial. The plant enchanted our ancestors with its distinctive scent and has been empirically found to have interesting medicinal, preservative, and disinfectant properties. Juniper, especially its berries, has been used as a spice, as a

drug in medicine, as well as for religious purposes (as part of incenses). Its millennia-long use is evidenced by archeological findings from the Bronze Age, in which juniper has been archaeobotanically demonstrated, based on seed morphology [1]. Babylonian medical sources describe the alleged psychotropic effects of juniper-containing mixtures for medicinal and religious purposes [2]. In ancient Egypt, wide medicinal use of juniper is mentioned in the famous Ebers Papyrus [3, 4], but its cosmetic use is also documented [5, 6]. The importance of juniper to the ancient world is further evidenced by the numerous references to it in the Bible [7]. A summary of ancient knowledge of the medicinal and other uses of juniper was captured by Pliny the Elder (23/27–79 AD) in his remarkable *Naturalis historia* [8]. Pliny mentions the usefulness of juniper berries for abdominal and chest pains and notes their diuretic properties. As a prophylaxis

✉ Karel Nesměrák  
nesmerak@natur.cuni.cz

<sup>1</sup> Department of Analytical Chemistry, Faculty of Science, Charles University, Prague, Czech Republic

<sup>2</sup> Mass Spectrometry Laboratory, Section of Chemistry, Faculty of Science, Charles University, Prague, Czech Republic

against snake bites, he recommends anointing the body with crushed juniper berries. Pliny’s slightly younger contemporary Pedanius Dioscorides (c. 40–90 AD) in his treatise *Περὶ ὕλης ἰατρικῆς* (On Medical Material)—which was mandatory for centuries to come—states that drinking juniper decoction has diuretic effects, is good for the stomach, and is effective in chest diseases, coughs, flatulence, colic, and malignant ulcers [9]. In the following centuries, juniper was widely used for these effects. In 1679, a German physician Benjamin Scharff (1651–1702) even published a comprehensive treatise on juniper and its uses: *Συν τῶ θεῶ Ἀρκευθολογία seu juniperi descriptio curiosa* [10]. To this day, juniper is a medicinal plant included in many pharmacopeias [11].

As part of our long-term focus on authenticating and studying the composition of historical remains of drugs [12], we had the unique opportunity to analyze two juniper-containing drugs from the eighteenth century. Their source is a peculiarly preserved equipment from the pharmacy of the Capuchin monastery in Hradčany, Prague, now in the collections of the National Museum in Prague, which preserves Baroque medical preparations in their original containers that have not been opened since the late eighteenth century [13]. From this source, we have already analyzed the historical remains of the following preparations: chicory extract [14], senna extract [15], opium-containing preparations [16, 17], ipecacuanha root [18], and baroque ointments [19].

In this paper, we present an analysis of two historical remains of juniper-containing drugs (Fig. 1): (i) “*Lignum juniperi*”, that is, juniper wood (from the appearance of the sample it is probably mixed with the bark of this tree), and (ii) “*Rob juniperi*”, that is, a preserve made from the juice of juniper berries, usually sweetened with sugar or honey, and concentrated by evaporation to a syrupy consistency. According to period literature [20, 21], juniper wood was used in decoctions as a sudorific (a drug that induces sweating), a diuretic, and an anti-catarthal remedy. The indications for the juniper preserve were similar. Furthermore, the period literature indicated that “*a nonnullis rusticorum panacea & theriaca vocatur*” (by some of the plebeians, it is called a panacea and theriac) [20, 21].

To authenticate these two historical remains of juniper-containing drugs, we used a chemotaxonomic approach consisting of confirming the presence (or absence) of substances characteristic of the plant, in our case juniper. To the best of our knowledge, this analysis of a more than 200-year-old sample of juniper-containing drugs has not yet been reported in the literature. Several typical substances from the group of mono-, di-, and sesquiterpenes [22–24], phenolic compounds [25, 26], flavonoids [27, 28], and coumarins [29] have been described in juniper. Since the plant material was heated in the preparation of the juniper preserve, we first replicated the preparation of this medical preparation



**Fig. 1** **a** The baroque pharmaceutical jar designed “*Lig. Junip.*”, which was the source of the analyzed sample of juniper wood (National Museum, inv. no. H2-4842), **b** the appearance of the representative sample of the content of the jar, **c** The baroque pharmaceutical jar designed “*Rob Juniper.*”, which was the source of the analyzed sample of the juniper preserve (National Museum, inv. no. H2-4957), **d** the appearance of the representative sample of the content of the jar

according to the recipes of that time to obtain suitable reference material (the method of replicating is a relatively recent approach in studies from the history of science [30, 31]). In the second part of the work, we used HPLC–MS/MS and GC–MS for screening the substances typical of juniper to confirm (or refute) the authenticity of the studied samples. Finally, we present a detailed analysis of the high-resolution mass spectrum of isoflavone viridiflorin, the occurrence of which has not been described in juniper and, at the same time, has only been slightly and incompletely described in the literature.

## Results and discussion

### Replication of the preparation of “*Rob juniperi*” according to the period recipes

To obtain suitable reference material for the analysis of H2-4957 *Rob juniperi* sample, a modern replication of this juniper-containing drug was first performed from fresh juniper berries.

In the aforementioned Capuchin pharmacy, the drugs were prepared according to the *Dispensatorium pharmaceuticum Austriaco-Vinnense* of 1729 [20]. According to this pharmacopeia (Fig. 2a), *Rob juniperi* was prepared by mixing six pounds (about 2,520 g) of fresh juniper berries with half a pound of powdered white sugar (about 210 g; so, the ratio of berries to sugar is 12:1). The mixture was boiled down to the consistency of preserve, while heating gently. During replication, however, it was found that the amount of water contained in the berries was insufficient to prepare the preserve. Therefore, it was necessary to add water repeatedly and mash the berries to obtain a product that is at least similar to preserve. However, there were still residues of berries in the final replicate of preserve. The replicate (Fig. 2b) is hereafter referred to as R1.

Therefore, a second experiment was carried out in which the recipe of the *Pharmacopoeia Austriaca* of 1855, which



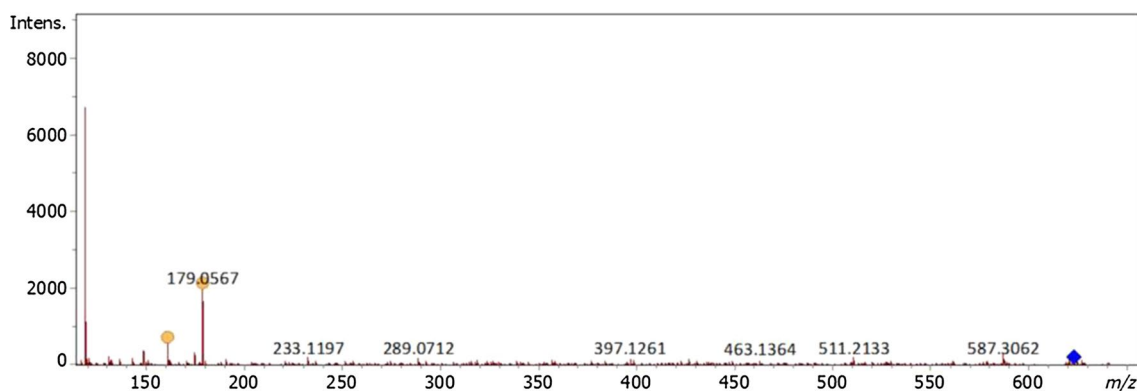
**Fig. 2** a The original Latin recipe for *Rob juniperi* according to *Dispensatorium pharmaceuticum Austriaco-Viennense* form 1729 [20], b the appearance of a replicate R1 of juniper preserve prepared by the eighteenth century recipe, c the appearance of a replicate R2 of juniper preserve prepared by the nineteenth century recipe

is more than a century younger, was replicated [32]. According to this recipe, the mashed juniper berries were boiled with water in a ratio of about 1:4 and then strained through a cotton cloth. The resulting liquid was evaporated to the consistency of honey and sugar was added in a 3:1 ratio, and the mixture was heated until the sugar was dissolved. The replicate (Fig. 3c) is hereafter referred to as R2.

### Screening for markers of juniper by HPLC–MS/MS

HPLC with high-resolution tandem mass spectrometry was chosen as the first method for the analysis of both analyzed samples. For measurements, the method according to Innocenti et al. [27], was chosen and subsequently modified in the gradient program. The stationary phase was an XBridge® BEH C18 column (150×3 mm, 2.5 µm), tempered at 30 °C. The mobile phase was binary, where component A was 0.1% aqueous formic acid and component B was 100% acetonitrile. The mobile-phase flow rate was 0.4 cm<sup>3</sup> min<sup>-1</sup>. Multi-step gradient elution with a total analysis time of 50 min was used as follows: 0–15 min, constantly 75% A; 15–25 min, linearly decreasing 75–65% A; 25–35 min, linearly decreasing 65–50% A; 35–40 min, linearly decreasing 50–0% A; 40–47 min, constant 0% A; 47–48 min, linearly increasing 0–75% A; 48–50 min, constantly 75% A. The extraction of the compounds from the samples for HPLC–MS/MS was carried out using a 7:3 (v/v) mixture of ethanol and water, the pH of which was adjusted to 2.0 using formic acid.

In the analysis of the historical remains H2-4957 *Rob juniperi*, replicates R1 and R2 as well as current fresh and dried juniper berries were used as reference materials, as a wide range of possible compounds could be expected, most of which are not commercially available as standards. In addition, the use of replicates R1 and R2 simulated the possible degradation of substances during the preparation of the juniper preserve. Analysis of these samples identified 67 substances in the ESI negative mode, some of which were present in only one sample. Of these, it was possible



**Fig. 3** ESI<sup>-</sup>–MS<sup>2</sup> spectrum of the product ions of the viridiflorin heptoxide precursor [M–H]<sup>-</sup> ion  $m/z=623.1960$  (marked by the diamond)

to characterize 37 substances based on a comparison with the literature and simultaneously by high-resolution tandem mass spectra analysis. Table 1 summarizes 24 compounds that were detected in at least two of the samples studied or in the historical sample.

No sucrose or other simple sugar was detected in the analyzed historical remains H2-4957 *Rob juniperi* (in contrast to both replicates). We can only assume that sucrose was either forgotten to be added by a period apothecary or was destroyed by microbial decomposition over the centuries. Evidence that the historical remains H2-4957 *Rob juniperi* is a juniper-containing drug was provided by the finding of a total of six substances characteristic of juniper, both in the historical remains and in the reference materials, namely quercetin 3-*O*- $\beta$ -D-xylopyranoside, apigenin 7-*O*- $\beta$ -D-glucoside, kaempferol 3-*O*- $\beta$ -D-xylopyranoside, quercetin, quercetin, and viridiflorin heptoxide. Moreover, the substance identified as viridiflorin heptoxide has not yet been described in the literature as characteristic of

juniper. The stability of these compounds is also notable, although four of them are glycosides (similar stability of glycosides was observed in historical remains of senna [15]). A total of six juniper characteristic compounds (procyanidin B<sub>2</sub>, apigenin, amentoflavone, isoflavone, imbricatolic acid, and isocupressic acid) were detected only in modern reference materials. They are likely to have been degraded over time.

In the analysis of the historical remains H2-4842 *Lignum juniperi*, fresh juniper wood (in two variants: peeled and with bark) was used as reference material because, as mentioned above, the historical remains are probably juniper wood mixed with its bark. Analysis of these samples identified only eight substances in the negative ESI mode, of which only four of them were present in both the historical remains and the reference material (Table 2). Moreover, none of these four substances is specific enough to provide unequivocal evidence that the historical sample H2-4842 *Lignum juniperi* is juniper wood.

**Table 1** Chemical substances found by the HPLC–MS/MS in the analyzed historical remains H2-4957 *Rob juniperi* (HR), replicates of this preparation R1 and R2, and current fresh (BF) or dry (BD) juniper berries (retention time in HPLC, identity and CASRN of the

compound, formula, experimental  $m/z$  of  $[M-H]^-$  ion, presence of the substance in the analyzed sample, reference used for confirmation of the substance)

$t_r$ /min	Compound, CASRN	Formula	$m/z$ $[M-H]^-$	Presence in sample					References
				HR	R1	R2	BF	BD	
1.7	Citric acid (77-92-9)	C <sub>6</sub> H <sub>8</sub> O <sub>7</sub>	191.0563		✓	✓	✓	✓	
1.7	Sucrose (57-50-1)	C <sub>12</sub> H <sub>22</sub> O <sub>11</sub>	341.1086		✓	✓			
2.2	Procyanidin B <sub>2</sub> (29106-49-8)	C <sub>30</sub> H <sub>26</sub> O <sub>12</sub>	577.1360		✓	✓	✓		[24, 25]
2.3	Catechin (154-23-4)	C <sub>15</sub> H <sub>14</sub> O <sub>6</sub>	289.0719		✓	✓	✓		[25, 26]
2.4	Rutin (153-18-4)	C <sub>27</sub> H <sub>30</sub> O <sub>16</sub>	609.1477				✓	✓	
2.6	Quercetin 3- <i>O</i> - $\beta$ -D-glucopyranoside (482-35-9)	C <sub>21</sub> H <sub>20</sub> O <sub>12</sub>	463.0899		✓	✓	✓	✓	[28]
2.9	Diethyl fumarate (623-91-6)	C <sub>8</sub> H <sub>12</sub> O <sub>4</sub>	171.067	✓					
3.5	Quercetin 3- <i>O</i> - $\beta$ -D-xylopyranoside (549-32-6)	C <sub>20</sub> H <sub>18</sub> O <sub>11</sub>	433.0773	✓	✓	✓	✓	✓	[33]
4.0	Apigenin 7- <i>O</i> - $\beta$ -D-glucoside (578-74-5)	C <sub>21</sub> H <sub>20</sub> O <sub>10</sub>	431.0988	✓		✓	✓	✓	[27, 28]
5.1	Kaempferol 3- <i>O</i> - $\beta$ -D-xylopyranoside (61117-16-6)	C <sub>20</sub> H <sub>18</sub> O <sub>10</sub>	417.0000	✓	✓	✓	✓	✓	[26]
5.3	Quercetin (117-39-5)	C <sub>15</sub> H <sub>10</sub> O <sub>7</sub>	301.2172	✓	✓	✓	✓	✓	[26, 27]
5.5	Quercetin (522-12-3)	C <sub>21</sub> H <sub>20</sub> O <sub>11</sub>	447.0922	✓	✓	✓	✓	✓	[25]
6.0	Unresolved	C <sub>20</sub> H <sub>18</sub> O <sub>9</sub>	401.0878		✓	✓	✓	✓	
6.8	Luteolin (491-70-3)	C <sub>15</sub> H <sub>10</sub> O <sub>6</sub>	285.0897		✓	✓		✓	[27, 29]
12.7	Viridiflorin heptoxide <sup>a</sup>	C <sub>29</sub> H <sub>36</sub> O <sub>15</sub>	623.1960	✓		✓	✓	✓	– <sup>a</sup>
16.1	Diosmetin (520-34-3)	C <sub>16</sub> H <sub>12</sub> O <sub>6</sub>	299.2009				✓	✓	[34]
16.4	Apigenin (520-36-5)	C <sub>15</sub> H <sub>10</sub> O <sub>5</sub>	269.0442		✓	✓	✓	✓	[27–29]
17.2	Negundin B (844692-51-9)	C <sub>20</sub> H <sub>22</sub> O <sub>6</sub>	357.1343			✓	✓	✓	[35]
21.9	Amentoflavone (1617-53-4)	C <sub>30</sub> H <sub>18</sub> O <sub>10</sub>	537.0782		✓	✓	✓	✓	[25, 27]
30.3	Unresolved	C <sub>21</sub> H <sub>22</sub> O <sub>7</sub>	385.1296				✓	✓	
33.7	Sequoiiflavone (21763-71-3)	C <sub>31</sub> H <sub>20</sub> O <sub>10</sub>	551.0974		✓	✓	✓	✓	[36]
38.6	Unresolved	C <sub>19</sub> H <sub>28</sub> O <sub>3</sub>	303.1974		✓	✓		✓	
39.1	Imbricatolic acid (6832-60-6)	C <sub>20</sub> H <sub>34</sub> O <sub>3</sub>	321.3434		✓	✓		✓	[37, 38]
40.3	Isocupressic acid (1909-91-7)	C <sub>20</sub> H <sub>32</sub> O <sub>3</sub>	319.2148		✓	✓	✓	✓	[29, 37]

<sup>a</sup>For a detailed of MS/MS, see section “High-resolution mass spectrometry of viridiflorin”



**Table 2** Chemical substances found by the HPLC–MS/MS in the analyzed historical remains H2-4942 *Lignum juniperi* (HL), fresh juniper wood peeled (WP) and fresh juniper wood with bark (WB) (retention time in HPLC, identity and CASRN of the compound, for-mula, experimental  $m/z$  of  $[M-H]^-$  ion, presence of the substance in the analyzed sample, reference used for confirmation of the substance)

$t_r$ /min	Compound, CASRN	Formula	$m/z$ $[M-H]^-$	Presence in sample			References
				HL	WP	WB	
2.8	Unresolved	$C_{25}H_{32}O_{10}$	491.1916	✓	✓	✓	
5.2	Unresolved	$C_{26}H_{32}O_{11}$	519.1878	✓	✓	✓	
37.7	[6]-Gingerol (23513-14-6)	$C_{17}H_{26}O_4$	293.1763	✓	✓	✓	[39]
39.1	Imbricatic acid (6823-60-6)	$C_{20}H_{34}O_3$	321.3434	✓		✓	[37, 38]

### Screening for markers of juniper by GC–MS

Because HPLC–MS/MS analysis did not provide sufficient chemotaxonomic evidence for the historical remains H2-4842 *Lignum juniperi*, we used GC–MS for this evidence. To capture volatile organic compounds, we used the headspace solid-phase micro-extraction method, which has been used in the literature for the analysis of this class of compounds from juniper [40]. Divinylbenzene-carboxen-polydimethylsiloxane (DVB/CAR/PDMS) fiber was used while optimizing the extraction time (3, 5, and 10 min). An extraction time of 5 min was chosen as a sufficient extraction time (considering the intensity/extraction time ratio). Due to the lack of volatility of some analytes, the extraction was performed both at laboratory temperature 25 °C and after previous heating for 5 min at 90 °C. Separation of volatile organic compounds after desorption from the fiber was performed by a modified method according to Adams [22]. Of the substances found, the characteristic substances of juniper were identified based on agreement with the literature [41–45]. The substances found in at least two samples analyzed are summarized in Table 3. A total of 12 substances were found in historical remains H2-4842 *Lignum juniperi* and modern reference material. Therefore, it can be concluded that the historical remains are indeed juniper wood, from which volatile organic substances can still be extracted after more than 200 years.

The developed headspace solid-phase micro-extraction GC–MS method was also applied to a second historical remains H2-4957 *Rob juniperi*, as well as fresh and dried juniper berries. A wider range of volatile organic compounds was obtained than in the case of the juniper wood analysis. A total of 27 compounds were identified in at least two samples analyzed; these are summarized in Table 4. Of these, 25 were present in both the historical remains H2-4957 *Rob juniperi* and the reference materials. Interestingly, a total of four substances were found only in the historical remains H2-4957 *Rob juniperi*. In the case of the substances ylangene,  $\alpha$ -muurolene, and  $\alpha$ -calacorene, these are terpenoids found in the essential oil of juniper in very

low concentrations [46]. In contrast to fresh or dried current reference material, these substances are concentrated in the historical remains as a result of the preparation of the juniper preserve; therefore, it was possible to detect them. In the case of  $\gamma$ -selinene, this substance is typical of juniper needles [42], and its finding in the historical remains H2-4957 *Rob juniperi* suggests that juniper berries were contaminated with needles during preparation by period apothecary. These findings support the conclusion of the authenticity of these historical remains as a juniper-containing drug made on the basis of HPLC–MS/MS measurements.

### High-resolution mass spectrometry of viridiflorin

For the identification of compounds in HPLC–MS/MS screening, high-resolution tandem mass spectrometry was used, and measured spectra were confirmed by comparison with the literature. An exceptional substance found in the analysis of a historical H2-4957 *Rob juniperi* (and reference material used) was determined to be viridiflorin heptoxide, eluting at 12.7 min. It is an isoflavone, first isolated from the plant *Tephrosia viridiflora* [48]. It was later described also in plants *Ficus carica* [49] and *Millettia brandisiana* [50]. Here, viridiflorin was detected for the first time in *Juniperus communis*.

Because the viridiflorin mass spectrum is not described in detail in the literature nor has the fragmentation mechanism yet been published, we focused on a detailed study of its spectrum. In the ESI negative mode (Fig. 3), the molecular ion  $[M-H]^-$  with  $m/z=623.1960$  (formula  $C_{29}H_{36}O_{15}$ , mass 624.59 Da) is found at a retention time of 12.7 min. On the other hand, in the ESI positive mode (Fig. 4a), the  $[M+H]^+$  ion is found at the same time with  $m/z=399.1433$  (formula  $C_{22}H_{22}O_7$ , mass 398.41 Da). The difference between the two fragmentations gives the formula  $C_7H_{14}O_8$  (mass 226.181 Da), which probably corresponds to a sugar unit from the heptoxide class. Thus, in the positive ESI mode, the substance already dissociates in the ionic source under the cleavage of  $C_7H_{14}O_8$ . Moreover, the ESI<sup>+</sup> spectrum is much richer in fragments than the spectrum in negative mode, so

**Table 3** Chemical substances found by the GC–MS in the analyzed historical remains H2-4942 *Lignum juniperi* (HL), fresh juniper wood peeled (WP) and fresh juniper wood with bark (WB) (retention timein GC, identity and CASRN of the compound, formula, experimental  $m/z$  of molecular ion, presence of the substance in the analyzed sample, reference used for confirmation of the substance)

$t_r$ /min	Compound, CASRN	Formula	$m/z$	Presence in sample						References
				Extraction I. <sup>a</sup>			Extraction II. <sup>b</sup>			
				HL	WP	WB	HL	WP	WB	
7.2	$\alpha$ -Pinene (80-56-8)	C <sub>10</sub> H <sub>16</sub>	136.2	✓	✓	✓	✓	✓	✓	[41–44]
7.4	(±)-Camphene (79-92-5)	C <sub>10</sub> H <sub>16</sub>	136.2			✓		✓	✓	[42, 43]
7.7	$\beta$ -Pinene (127-91-3)	C <sub>10</sub> H <sub>16</sub>	136.2			✓			✓	[42, 43]
7.8	$\beta$ -Myrcene (123-35-3)	C <sub>10</sub> H <sub>16</sub>	136.2			✓				[42–44]
8.0	3-Carene (13466-78-9)	C <sub>10</sub> H <sub>16</sub>	136.2			✓		✓	✓	[42, 43]
8.2	D-Limonene (5989-27-5)	C <sub>10</sub> H <sub>16</sub>	136.2	✓	✓	✓		✓		[42–44]
9.7	Terpinen-4-ol (562-74-3)	C <sub>10</sub> H <sub>18</sub> O	154.3		✓	✓		✓	✓	[42, 43]
11.0	$\alpha$ -Copaene (3856-25-5)	C <sub>15</sub> H <sub>24</sub>	204.4	✓	✓	✓	✓	✓	✓	[41–44]
11.4	$\alpha$ -Cedrene (469-61-4)	C <sub>15</sub> H <sub>24</sub>	204.4		✓		✓			[41]
11.5	$\beta$ -Cedrene (546-28-1)	C <sub>15</sub> H <sub>24</sub>	204.4	✓		✓		✓		[41]
11.6	<i>cis</i> -Thujopsene (32435-95-3)	C <sub>15</sub> H <sub>24</sub>	204.4	✓	✓		✓	✓	✓	[42, 44]
11.7	$\gamma$ -Muurolole (30021-74-0)	C <sub>15</sub> H <sub>24</sub>	204.4		✓	✓		✓	✓	[42]
12.0	$\beta$ -Cadinene (523-47-7)	C <sub>15</sub> H <sub>24</sub>	204.4	✓	✓	✓		✓	✓	[41, 42, 44]
12.0	(+)-Cuparene (16982-00-6)	C <sub>15</sub> H <sub>24</sub>	202.3	✓	✓		✓	✓		[41, 42]
12.1	(–)-Calamenene (483-77-2)	C <sub>15</sub> H <sub>24</sub>	202.3	✓	✓	✓	✓	✓		[41]
12.9	$\alpha$ -Cedrol (77-53-2)	C <sub>15</sub> H <sub>26</sub> O	222.4	✓			✓		✓	[41]
13.1	Cadalene (483-78-3)	C <sub>15</sub> H <sub>18</sub>	198.3	✓	✓	✓	✓	✓	✓	[41, 42]
13.8	(–)-Isolongifolol acetate (18367-70-9)	C <sub>17</sub> H <sub>28</sub> O <sub>2</sub>	264.4	✓			✓			[45]

<sup>a</sup>Extracted from sealed vial at temperature 25 °C for 5 min<sup>b</sup>The sealed vial containing the sample was tempered at 90 °C for 5 min, followed by extraction for 5 min

we have also proposed a scheme for the possible fragmentation of viridiflorin under this ionization (Fig. 4b). The validity of this suggestion is supported by the fact that some of the fragments found are analogous to the mass spectra of other flavonoids and isoflavonoids [51, 52].

## Conclusions

Using HPLC–MS/MS, it was possible to confirm the authenticity of historical remains of juniper preserve from the eighteenth century, in which a number of substances typical of juniper have been preserved for more than 200 years since its preparation. Moreover, the stability of some of the glycosides has been observed over two centuries. The authenticity of the second historical remains, juniper wood, was demonstrated by SPME–GC–MS on the basis of the detection of typical volatile organic compounds. This method also proved to be useful for the authentication of juniper preserve. In addition, the occurrence of isoflavone viridiflorin in *Juniper communis* is described for the first time and a detailed study of its mass spectrum, including the design of a fragmentation mechanism, is presented.

## Experimental

### Analyzed samples, reference material

Historical remains of both juniper-containing drugs were taken in the autumn 2022 from the original baroque apothecary jars preserved in the collections of the National Museum in Prague. Sample H2-4842 was taken from a wooden pharmaceutical jar (height 14.0 cm, diameter 9.0 cm; Fig. 1a), which is labeled “*Lig: Junip:*”. The sample consisted of small pieces of yellowish to orange-colored wood, with some pieces showing bits of grayish bark on the edges. Sample H2-4957 was taken from a milk glass pharmaceutical jar (height 10.0 cm, diameter 7.0 cm; Fig. 1c), covered with an original leather lid and labeled “*Rob Juniper:*”. After carefully removing the cap, a sample was taken with a porcelain spoon. The sample had a lumpy texture, dark brown, sometimes even black in color, formed by long drying of originally pasty coatings.

The current fresh and dried juniper berries were used as reference material. Fresh juniper berries were collected in December 2022 in the western part of the Czech Republic and stored in a freezer. The dried juniper berries were purchased from a spice store. In addition, current

**Table 4** Chemical substances found by the GC–MS in the analyzed historical remains H2-4957 *Rob juniperi* (HR) and current fresh (BF) or dry (BD) juniper berries (retention time in GC, identity and CASRN of the compound, formula, experimental  $m/z$  of molecular ion, presence of the substance in the analyzed sample, reference used for confirmation of the substance)

$t_R$ /min	Compound, CASRN	Formula	$m/z$	Presence in sample				References
				HR <sup>a</sup>	HR <sup>b</sup>	BF	BD	
7.1	(±)- $\alpha$ -Thujene (2867-05-2)	C <sub>10</sub> H <sub>16</sub>	136.2	√	√	√		[41]
7.2	$\alpha$ -Pinene (80-56-8)	C <sub>10</sub> H <sub>16</sub>	136.2	√	√	√	√	[41–44]
7.4	(±)-Camphene (79-92-5)	C <sub>10</sub> H <sub>16</sub>	136.2		√	√		[42, 43]
7.5	Thuja-2,4(10)-diene (36262-09-6)	C <sub>10</sub> H <sub>14</sub>	134.2		√		√	[22]
7.7	Sabinene (3387-41-5)	C <sub>10</sub> H <sub>16</sub>	136.2		√	√		[41]
7.7	$\beta$ -Pinene (127-91-3)	C <sub>10</sub> H <sub>16</sub>	136.2		√	√	√	[42, 43]
7.8	$\beta$ -Myrcene (123-35-3)	C <sub>10</sub> H <sub>16</sub>	136.2			√	√	[42–44]
8.2	D-Limonene (5989-27-5)	C <sub>10</sub> H <sub>16</sub>	136.2	√	√	√	√	[42–44]
8.2	o-Cymene (527-84-4)	C <sub>10</sub> H <sub>14</sub>	134.2	√		√	√	[22]
8.2	$\beta$ -Phellandrene (555-10-2)	C <sub>10</sub> H <sub>16</sub>	136.2			√	√	[42]
8.3	1,8-Cineole (470-82-6)	C <sub>10</sub> H <sub>18</sub> O	154.3	√			√	[41]
8.4	$\gamma$ -Terpinene (99-85-4)	C <sub>10</sub> H <sub>16</sub>	136.2		√	√		[42]
9.0	Linalool (78-70-6)	C <sub>10</sub> H <sub>18</sub> O	154.3	√		√		[41]
9.7	Terpinen-4-ol (562-74-3)	C <sub>10</sub> H <sub>18</sub> O	154.3	√		√		[42, 43]
10.5	Bornyl acetate (76-49-3)	C <sub>12</sub> H <sub>20</sub> O <sub>2</sub>	196.3		√	√	√	[41]
10.8	$\alpha$ -Cubebene (17699-14-8)	C <sub>15</sub> H <sub>24</sub>	204.4		√	√	√	[41]
11.0	Ylangene (14912-44-8)	C <sub>15</sub> H <sub>24</sub>	204.4	√	√			[41]
11.0	$\alpha$ -Copaene (3856-25-5)	C <sub>15</sub> H <sub>24</sub>	204.4	√	√	√	√	[41–44]
11.2	(–)- $\beta$ -Elemene (515-13-9)	C <sub>15</sub> H <sub>24</sub>	204.4	√	√	√	√	[41]
11.7	(1Z,4Z,7Z)-1,5,9,9-Tetramethyl-1,4,7-Cycloundecatriene (400822-79-9)	C <sub>15</sub> H <sub>24</sub>	204.4		√	√	√	[47]
11.7	$\gamma$ -Muuroleone (30021-74-0)	C <sub>15</sub> H <sub>24</sub>	204.4		√			[42]
12.0	$\beta$ -Cadinene (523-47-7)	C <sub>15</sub> H <sub>24</sub>	204.4	√		√	√	[41, 42, 44]
11.8	$\alpha$ -Muuroleone (10208-80-7)	C <sub>15</sub> H <sub>24</sub>	204.4	√	√			[41]
11.9	$\beta$ -Selinene (17066-67-0)	C <sub>15</sub> H <sub>24</sub>	204.4		√	√	√	[41]
12.2	$\gamma$ -Selinene (515-17-3)	C <sub>15</sub> H <sub>24</sub>	204.4	√	√			[41]
12.2	Selina-3,7(11)-diene (6813-21-4)	C <sub>15</sub> H <sub>24</sub>	204.4		√		√	[41]
12.2	$\alpha$ -Calacorene (21391-99-1)	C <sub>15</sub> H <sub>20</sub>	200.3	√	√			[41]

<sup>a</sup>Extracted from sealed vial at temperature 25 °C for 5 min<sup>b</sup>The sealed vial containing the sample was tempered at 90 °C for 5 min, followed by extraction for 5 min

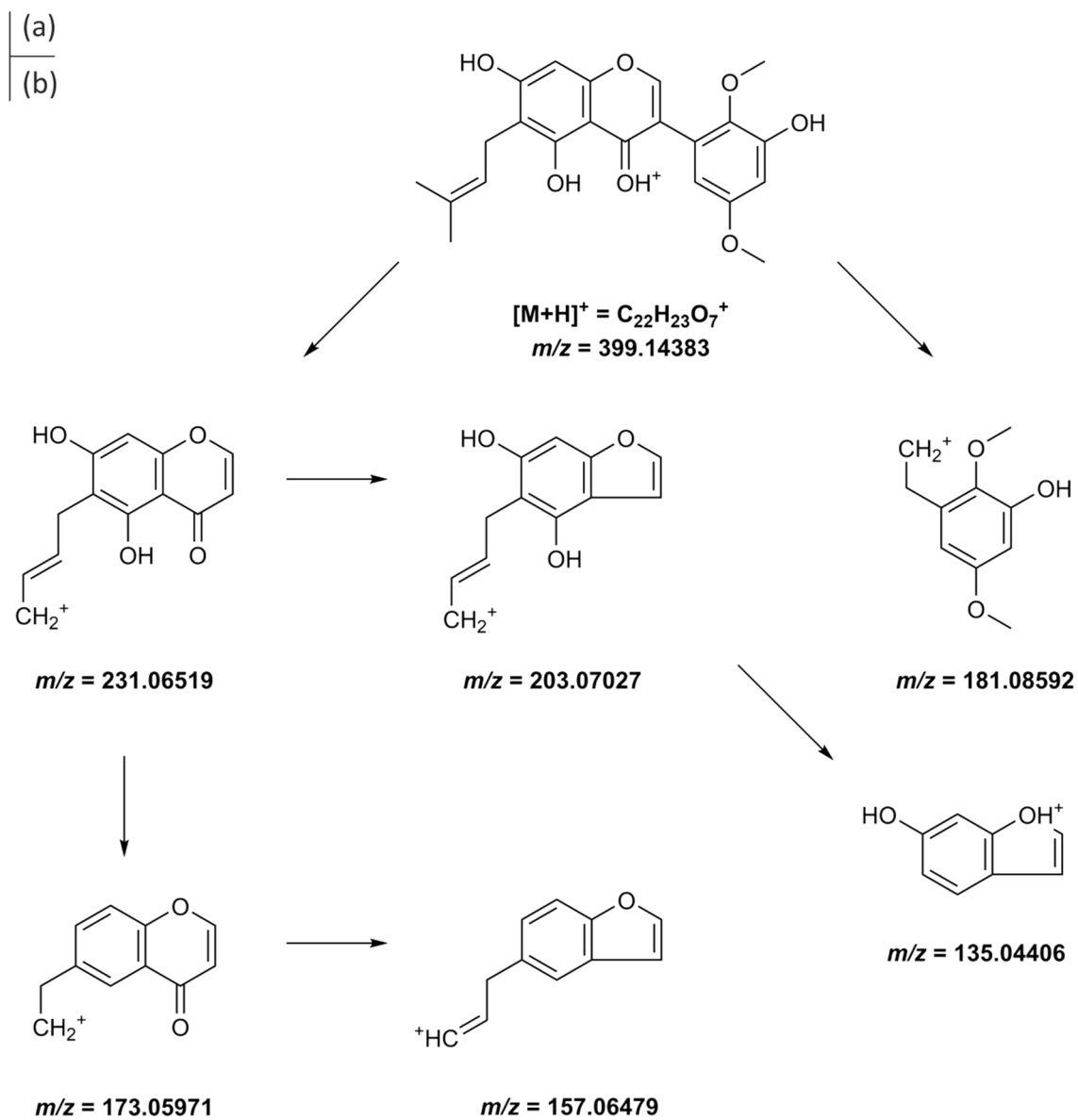
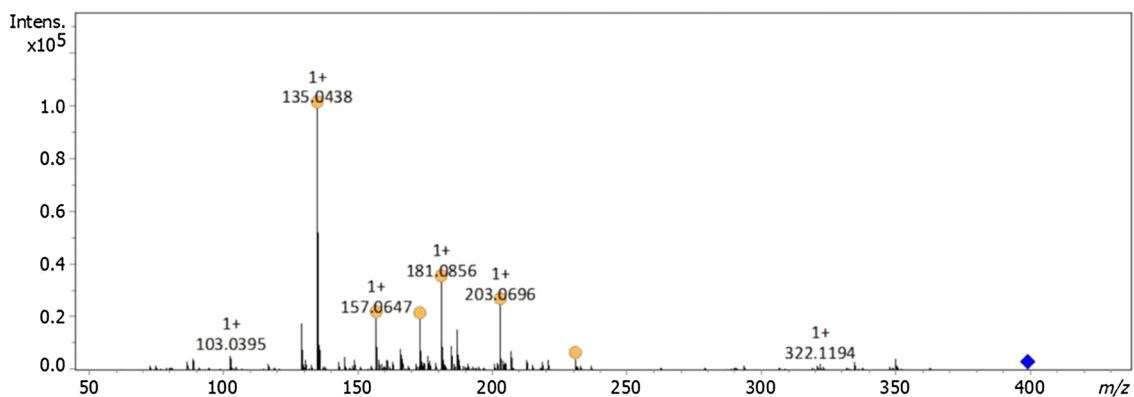
fresh juniper wood obtained from the same plant that was the source of fresh berries was used. The current juniper wood was used in two variants: peeled and barked. Wood samples were kept in a refrigerator before analysis.

## Chemicals

The chemicals used were: acetonitrile (for HPLC–MS; Macron Fine Chemicals, USA), ethanol 96% (p.a.; Penta, Czech Republic), formic acid 98% (p.a.; Lach-Ner, Czech Republic), sucrose (p.a., Lach-Ner, Czech Republic). Deionized water with a specific conductivity < 0.05  $\mu\text{S cm}^{-1}$ , obtained using a Milli-Q instrument (Millipore, USA), was used.

## Replication experiments

The preparation of the R1 replicate was based on the literature [20]. Exactly about 3 g of fresh juniper berries (previously crushed in a porcelain mortar) was weighed. The weighted berries were transferred to a porcelain dish, 0.25 g of sucrose was added, and the mixture was placed in a water bath. After 5 min in the water bath, when the berries had softened, they were again crushed with a porcelain pestle and the mixture was left in the water bath for another 5 min. As the mixture was observed to dry out, enough water was added to submerge the berries. The mixture was stirred with a glass rod, and when almost all the water had evaporated, the procedure was repeated a total of three times. The preparation was stopped after 32 min



**Fig. 4** **a** ESI<sup>+</sup>-MS<sup>2</sup> spectrum of the product ions of the viridiflorin precursor  $[M+H]^+$  ion  $m/z=399.1433$  (marked by the diamond), **b** proposed ESI<sup>+</sup>-MS<sup>2</sup> fragmentation of viridiflorin (the  $m/z$  values are calculated)



of heating; the mixture was left to cool and then kept in the refrigerator.

The preparation of the R2 replicate was based on the literature [32]. Exactly about 3 g of fresh juniper berries (previously crushed in a porcelain mortar) were weighed. The weighted berries were transferred to a porcelain dish, 25 cm<sup>3</sup> of water was added, and the mixture was heated for 15 min on a water bath while stirring occasionally with a glass rod. The mixture was then strained through a cotton cloth. The brown liquid obtained was placed in a porcelain dish, and heated for 13 min in a water bath. The liquid was thus thickened to the consistency of honey. The resulting paste was weighed and 350 mg of sucrose was added and dissolved in the paste in a water bath stirring for 2 min. The obtained replicate was kept in a refrigerator.

### HPLC–MS/MS procedures, instrumentation

To extract the sample for HPLC–MS/MS analysis, exactly about 100 mg of the sample (homogenized in an agate mortar) was weighed in an Eppendorf tube. A volume of 1.5 cm<sup>3</sup> of extraction agent (a mixture of ethanol and water 7:3 (v/v), pH adjusted to 2.00 with formic acid) was added to the tube. Subsequent extraction was carried out on a vortex at 1000 rpm for 30 min. The extract was then filtered using a 0.2 µm PVDF centrifuge filter. In the case of the extraction of the juniper wood samples, after the addition of the extraction agent, the Eppendorf tube was placed in an ultrasonic bath for 2.5 h and then kept in a refrigerator for 7 days. Then the wood extracts were filtered using a 0.2 µm PVDF centrifuge filter.

For HPLC–MS/MS, an Agilent 1290 Infinity II LC System with a binary pump model was used. An XBridge® BEH C18 column (150×3 mm, 2.5 µm), tempered at 30 °C, was used. The binary mobile phase of 0.1% aqueous formic acid (solvent A) and acetonitrile (solvent B) was used with a flow rate of 0.4 cm<sup>3</sup> min<sup>-1</sup>. The gradient elution program is described in section “Screening for markers of juniper by HPLC–MS/MS.” The volume of injected sample was 5 mm<sup>3</sup>.

High-resolution tandem mass spectrometry detection was performed on a Bruker QqTOF compact instrument controlled by Compass of Control 4.0 software (Bruker Daltonics, Germany). Compass DataAnalysis 4.4 (Build 200.55.2969) software (Bruker Daltonics, Germany) was used for data processing. ESI-MS<sup>2</sup> data were collected in both positive and negative ion modes; the scans ranged from  $m/z$  50 to  $m/z$  1000. The drying gas temperature was 220 °C and its flow rate was 3.0 dm<sup>3</sup> min<sup>-1</sup>. The cone voltage was 2800 V. The measured mass spectra were analyzed using Compass CompoundCrawler 3.0 software (Bruker,

Germany) and compared with the literature and databases PubChem [53], ChemSpider [54], and ChEBI [55].

### GC–MS procedures, instrumentation

For GC–MS analysis, approximately 50 mg of the homogenized sample was weighed into a 4 cm<sup>3</sup> glass vial sealed with a septum. Headspace solid-phase micro-extraction was performed on a 50/30 µm DVB/CAR/PDMS fiber held by a manual fiber holder (both Supelco, USA). Two extraction methods were used: (i) extraction at 25 °C for 5 min, and (ii) the sealed vial containing the sample was first tempered at 90 °C for 5 min, followed by extraction for 5 min.

The GC–MS measurements were performed on a GCMS-QP2010 Ultra instrument connected to the quadrupole (both Shimadzu). Separation was achieved by Rxi-1301 Sil MS column (60 m×0.25 mm, 0.25 µm; Restek). Helium with linear velocity of 35.0 cm s<sup>-1</sup> served as mobile phase. The injection was performed in splitless mode during with time of 1 min at temperature of injector 250 °C (to prevent saturation of the MS detector, a 1:50 split was used when extracts from fresh juniper berries were analyzed). The column temperature program was as follows: initial isotherm 50 °C for 2 min, heating to 290 °C at 20 °C min<sup>-1</sup> with a final isotherm for 1 min. Mass detection parameters: electron ionization 70 eV, ion source temperature 200 °C, interface temperature 200 °C,  $m/z$  were registered in scan mode in a range 29–300. GCMS Postrun Analysis software was used to process and evaluate the chromatograms and MS spectra. The measured spectra were compared with NIST/EPA/NIH 02 Mass Spectral Library [56], and only spectra with similarity > 92% were taken into account.

**Acknowledgements** The financial support by the project Cooperation Chemistry of Charles University is gratefully acknowledged.

**Funding** Open access publishing supported by the National Technical Library in Prague.

**Data availability** The experimental data supporting the findings of this study are available from the corresponding author, K.N., upon reasonable request.

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