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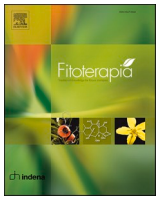

Research paper

Analysis of bitter compounds in traditional preparations of *Gentiana purpurea* L

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ABSTRACT

Roots of *Gentiana purpurea* are known to have an intense bitter taste due to its high content of secoiridoids. In folk medicine roots have commonly been prepared as water decoctions, soaked in ethanol, or boiled with milk, wine, or beer. The aim of this study was to explore how various historical preparation methods influence yields of major bitter compounds in *G. purpurea*. HPLC-DAD analysis revealed that maceration with 40% and 70% ethanol, boiling with acetic acid (3% and 6%), vinegar and raw milk gave the highest extraction yields of gentiopicrin. Erythrocentaurin was detected when the roots were added to cold water before boiling, possibly because of enzymatic degradation. In contrast, erythrocentaurin was not detected in preparations where roots were added to boiling water, or when they were extracted with acetic acid or alcohol. The results stress the significance of traditional preparation methods to optimize yield of bioactive compounds.

1. Introduction

Preparation method is often overlooked in assessments of historical herbal medicine, but extraction with water versus milk or wine, and cold versus hot, all impact efficacy and yield [1,2]. In the preparation of herbal drugs, the most used solvents are water or aqueous ethanol. The preparations are typically prepared with boiling water, as an infusion or maceration. In ethanol extraction, the plant material is usually soaked for several hours or days. However, older sources describe a myriad of other liquids for preparation of traditional medicine, including milk, vinegar, wine, or beer [3]. In both 1st century Greek texts attributed to Dioscorides and in the 10th century Anglo-Saxon Bald's Leechbooks, the use of breastmilk in combination with plant ingredients is recommended for preparation of extracts with medicinal properties [1]. Older texts including the Ebers Papyrus (15th century BCE) list herbal medicines made with water, beer, milk, wine, and honey [4].

In Scandinavia herbal medicine traditions are influenced by both environment, prevalent common ailments as well as borrowed knowledge from elsewhere. The Scandinavian medicinal flora is species rich, but several species are more commonly used than others and over longer time periods, these include *Angelica archangelica* L., *Juniperus communis* L., *Hypericum perforatum* L., and *Gentiana purpurea* L. [5]. The latter, *G. purpurea* (Gentianaceae), is a perennial plant with dark purple corollas. The roots have an intense bitter taste which is due to its high content of secoiridoids [6–8]. The medicinal use of *G. purpurea* roots has a long tradition in Norway. Both humans and animals were treated with different kinds of root preparations. The medical doctor and medical historian Ingjald Reichborn-Kjennerud (1856–1949) describes that the roots were used against the Black Death [9]. It was regarded as a universal remedy, and its use included the treatment of colic, vomiting, throat infection, tuberculosis, chest diseases, bronchitis, edema, diarrhea, and jaundice, and it was used as an appetizer to improve digestion [3,9]. It was also used externally; a tincture was applied on the nipples to discourage from breastfeeding, on sore cow's teats, and on joints affected by gout. The roots were also sold in pharmacies, and doctors commonly prescribed them for stomach diseases. They were preferably collected in late autumn, and the roots were used either fresh or cut in pieces and dried. Different preparation modes are described. Most commonly, the roots were soaked in alcohol to make a tincture or boiled in milk or water. Boiling in beer or cream is also mentioned, as is the preparation in

Abbreviations: DC, decoction cold extractant; DW, decoction warm extractant; HPLC-DAD, high performance liquid chromatography-diode array detector; I, infusion; LOD, limit of detection; LOQ, limit of quantification; M, maceration.

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vinegar or wine. It was also chewed as crude drug [3,9–11].

Gentiopicrin (synonym gentiopicroside) is the major secoiridoid (49.7–67.7 mg/g), while amarogentin (2.7–3.6 mg/g) is regarded as the major bitter principle in *G. purpurea* roots [6,8]. Erythrocentaurin is reported as a degradation product from gentiopicrin [12–14] and formed during extraction of *G. purpurea* roots with boiling water [8]. Additionally, the secoiridoids swertiamarin and sweroside are present in the roots [8]. The bitter secoiridoids are regarded as the active ingredients in medicinal preparations of gentian roots and are bitter taste receptor agonists. Amarogentin is reported to be 5000 times more bitter than gentiopicrin [6] and regarded as the most bitter molecule of natural origin [15,16]. Bitter compounds are agonists of the bitter receptors (TAS2R), which were initially found in the taste buds of the mouth and thought to be an evolutionary mechanism to avoid ingestion of poisonous compounds [17]. Today 25 TAS2R are known in humans [18], and they are present in a number of organs, such as respiratory, gastrointestinal and urinary tracts, heart, brain, and pancreas. Their physiological functions are under investigations, and they are proposed to be involved in the regulation of appetite and digestion, modulating gut hormone release, and relaxation of smooth muscle relaxation [17–19]. Stimulation of bitter receptors in the airways is supposed to decrease airway obstruction and proposed as a drug target for treating asthma and other obstructive lung diseases [20].

No scientific studies have so far investigated the influence of traditional methods of extraction and preparation on the yield of the bitter compounds in the *G. purpurea* root preparations. Because of the variation in lipophilicity, pH, temperature, and ethanol content of the liquids used for the traditional preparation of medicines, one can assume that the different preparation methods will have an impact on the yields of these metabolites. We aimed to evaluate the relevance of the historical methods of extraction and preparation in a modern phytochemical framework. This was tested through the following research questions: 1) How different solvent compositions mimicking traditional preparation methods of *G. purpurea* affect the yield of analytes from the root; and 2) how different preparative methods such as boiling, infusion and maceration affects the yields.

2. Material and methods

2.1. Plant material

Gentiana purpurea L. (Norwegian *søterot*) (Gentianaceae) was collected in Vang i Valdres, Norway (coordinates 60°59′29.5"N, 8°37′41.7″E), 1060 masl, the 12th of August 2021. The roots were cleaned, cut into small pieces, and spread out for air-drying immediately after the collection to avoid fermentation. The dried roots were ground into a powder with a knife mill (Brabender, Duisburg, Germany; 4 mm sieve). A voucher specimen is deposited at the Department of Pharmacy, University of Oslo; reference number RL-20210812-gp-r.

2.2. Chemicals and reagents

The analytical standards gentiopicrin, sweroside (≥ 99% purity), and harpagoside (≥ 98% purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Amarogentin and erythrocentaurin were isolated from *G. purpurea* [8], and the purity determined by HPLC as >98% (supporting information, **Fig. S1**). HPLC-grade acetonitrile was purchased from VWR, deionized water was obtained with a Millipak (0.22 μm filter) purification system from Milli-Q (Molsheim, France). Oude Geuze Boon sour beer, Bodin brown ale and Domaine Tariquet white wine were bought from the Norwegian monopoly for wine and spirits. Tuborg pilsner beer, Tine fat free milk (0.1% fat), Tine whole fat milk (3.8% fat). Tine cream (18% fat), and Heinz apple cider vinegar was bought from a local grocery. pH was measured using Ph-indicator strips (MColorpHast, Darmstadt, Germany). Raw milk (4% fat) was provided by Bygdø Kongsgård (Oslo, Norway).

2.3. Extraction and sample preparation

Powdered roots of *G. purpurea* (40 mg, < 4 mm particle size) was extracted with 20 mL extraction solvent, either by adding the plant material to room temperature (RT) solvent and brought to boil, by adding to boiling solvent, or by extraction with solvent at RT. Type of solvents and extraction time are shown in Table 1. The table also includes pH of each preparation. The extracts were transferred to 20 mL volumetric flasks and filled up to the final volume with distilled water. After extraction with milk, an internal standard of 10.0–25.0 μg/mL harpagoside was added, and the extract was diluted to 50 mL with cold acetonitrile. The extracts were then centrifuged at 4000 RPM for 10 min, and precipitated proteins and plant material were removed from the extract. Excess acetonitrile was removed on a miVac centrifugal concentrator (Genevac Ltd., Ipswich, United Kingdom), and the extract was diluted with water to a final volume of 20 mL. The sample solutions were filtered through a 0.45 μm PTFE filter before HPLC analysis.

2.4. Quantitative analysis with HPLC-DAD

HPLC-DAD analysis was performed on a LaChrom Elite HPLC system (VWR-Hitachi, Tokyo, Japan) equipped with an L-2455 diode array detector, and a Kinetex C18 100 A (150 × 4.6 mm) column (Phenomenex, Torrance, CA, USA) was used for separation. Elution was performed using a gradient of mobile phase A (H_2O) and B (acetonitrile) with the following time schedule: 5% B, 0–3 min; 5–25% B, 3–20 min; 25–90% B, 20–45 min; 90% B, 45–50 min. The flow rate was 1 mL/min, injection volume 10 μL, temperature 25 °C, and absorbance was recorded at 225, 246 and 275 nm. Quantification was based on individual standard curves for each analyte. Gentiopicrin, sweroside, erythrocentaurin and amarogentin were accurately weighed, dissolved in methanol and diluted to appropriate concentrations (Table 2). Standard curves were based on three parallels with eight different concentrations, and UV maximum for each standard employed: gentiopicrin 275 nm, sweroside 246 nm, erythrocentaurin and amarogentin 225 nm. The sample solutions were filtered through a PTFE syringe filter (0.45 μm). The results are expressed as μg/mL and are the averages of three independent extractions and analysed in triplicate. Method validation was performed according to Harris and Lucy, 2020 [21] for linearity, precision, range, selectivity, and sensitivity.

2.5. Statistics

Statistical analysis was conducted by using the GraphPad Prism 9 software (GraphPad). Analysis was done by ordinary one-way ANOVA test and Tukey's multiple comparison test. The extracts were divided in four groups for ANOVA test based on relevance of comparison: Water decoctions and infusions; ethanol and acetic acid containing extracts and water; complex extractants containing wine, beer, and apple cider vinegar; complex extractants containing dairy products. Values are expressed as mean ± SD and refers to three independent extractions analysed in triplicate.

3. Results

3.1. Method validation

The method was suitable for quantification of analytes. Correlation between concentration and all standards were linear within applied range, with correlation coefficients over 0.99. The limit of detection and quantification were suitable for concentrations found in extracts, indicating sufficient sensitivity (Table 2). The method also showed selectivity, and all analytes were separated at baseline at their respective absorbance maximums. No peaks with the same retention time as gentiopicrin, sweroside, erythrocentaurin and amarogentin at relevant wavelengths were observed in the HPLC analysis of the extractants. For

Table 1
Preparation methods of extracts and pH measured in each extract.

Extractant	Decoction[a]			Infusion[b]		Maceration[c]	pH
	30 min	60 min	120 min	3 min	5 min	24 h	
Water	DW	DC	DC	I	I		5
Ethanol							
- 5%	DC						6
- 10.5%	DC						6
- 40%						M	5
- 70%						M	5
Acetic acid							
- 3%	DC						2–3
- 6%	DC						2
Pilsner, 4.5% alcohol	DC						4–5
Sour beer, 7% alcohol	DC						3–4
Brown ale, 8% alcohol	DC						4–5
White wine, 10.5% alcohol	DC						3
Apple cider vinegar, 5% acetic acid	DC						2–3
Fat free milk	DC						6–7
Whole milk, 3.8% fat	DC						6–7
Cream, 18% fat	DC						6–7
Raw milk	DC						6–7

[a] Powdered roots were added to boiling water and boiled for 30 min (DW), or added to cold water, brought to boil and boiled for 30 min, 60, or 120 min (DC).
[b] Powdered roots were added to boiling water and infused for 3 or 5 min (I).
[c] Powdered roots were added to 40 or 70% ethanol and macerated for 24 h at room temperature (M).

Table 2
Calibration curve, LOD and LOQ for *Gentiana purpurea* bitter compounds **1**–**4**.

Standard	Calibration curve	R^2	Concentration range (μg/mL)[a]	LOD (μg/mL)[b]	LOQ (μg/mL)[c]
Gentiopicrin (**1**)	y = 62,377.7× + 10,806.7	0.9999	1–200	0.31	0.94
Sweroside (**2**)	y = 75,695.3× + 79,627.7	0.9996	1–200	2.3	6.9
Erythrocentaurin (**3**)	y = 313,025.5× + 314,114.3	0.9989	0.5–100	2.2	6.5
Amarogentin (**4**)	y = 92,161.8× + 28,187.3	0.9999	0.5–100	0.29	0.88

[a] Based on 8 different concentrations.
[b] LOD (limit of detection), 3.3 × standard deviation of the y-intercepts of regression line/ slope of the regression line (σ/S).
[c] LOQ (limit of quantification), 10 × standard deviation of the y-intercepts of regression line/ slope of the regression line (σ/S).

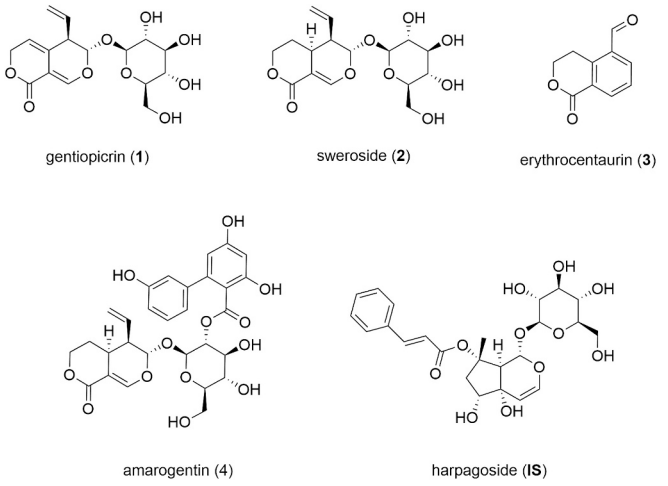

Fig. 1. Chemical structures of compounds analysed in *G. purpurea* preparations. Harpagoside was used as internal standard (is) in the HPLC-DAD analysis of milk preparations.

milk, exclusion of peaks with the same retention time as harpagoside was also verified. Precision was determined through inter- and intra-day variations in analysis of water extracts. The relative standard deviation of inter-day variations ranged from 1.0 to 1.5% for gentiopicrin, sweroside and erythrocentaurin, and 3.7% for amarogentin. Inter-day variation showed a relative standard deviation of 0.9–1.4% for gentiopicrin, sweroside and amarogentin, and 4.0% for erythrocentaurin, over 3 days.

3.2. Extraction results

The concentration of gentiopicrin, sweroside, amarogentin, and erythrocentaurin (see Fig. 1 for structures) in traditionally prepared *G. purpurea* root preparations was determined by HPLC-DAD. (See Fig. 2) Firstly, the roots were prepared with water as the extraction solvent using different boiling times and temperature where the water was added to the roots (Table 1), the results are shown in Table 3. There is a significant difference in gentiopicrin concentration dependent on both preparation mode and length of extraction. Infusion for 5 min gave the highest yield of gentiopicrin (89.9 ± 6.0 μg/mL), while hot extraction for 30 min gave the highest yields of sweroside (28.1 ± 1.0 μg/mL) and amarogentin (5.0 ± 0.1 μg/mL). There were only small differences in the extraction yield of gentiopicrin with infusions (3 and 5 min) and decoction for 30 min where roots were added to boiling water. However, the yields were significantly lower when gentian roots were added to cold water and brought to boil before extraction for 30–120 min. During these extractions, detectable amounts of erythrocentaurin were found with highest amounts after 30 min. Furthermore, the yield of erythrocentaurin decreased with boiling time.

There were little differences in the yields of sweroside, though decoction (30 min) showed a significantly higher yield (28.1 ± 1.0 μg/mL) than decoction (120 min) (20.1 ± 1.5 μg/mL), infusion (3 min) (22.0 ± 1.4 μg/mL) and decoction (30 min, boiling) (20.8 ± 3.3 μg/mL). For amarogentin again decoction (30 min) showed significantly higher yield (5.0 ± 0.1 μg/mL) than infusion (3 min) (3.5 ± 0.3 μg/mL) and decoction (30 min, boiling, but plant material added to cold water) (3.3 ± 0.4 μg/mL). In addition, the yield of amarogentin significantly decreased from decoction (30 min) to decoction (60 min) (3.0 ± 0.9 μg/mL) and to decoction (120 min) (1.7 ± 0.6 μg/mL).

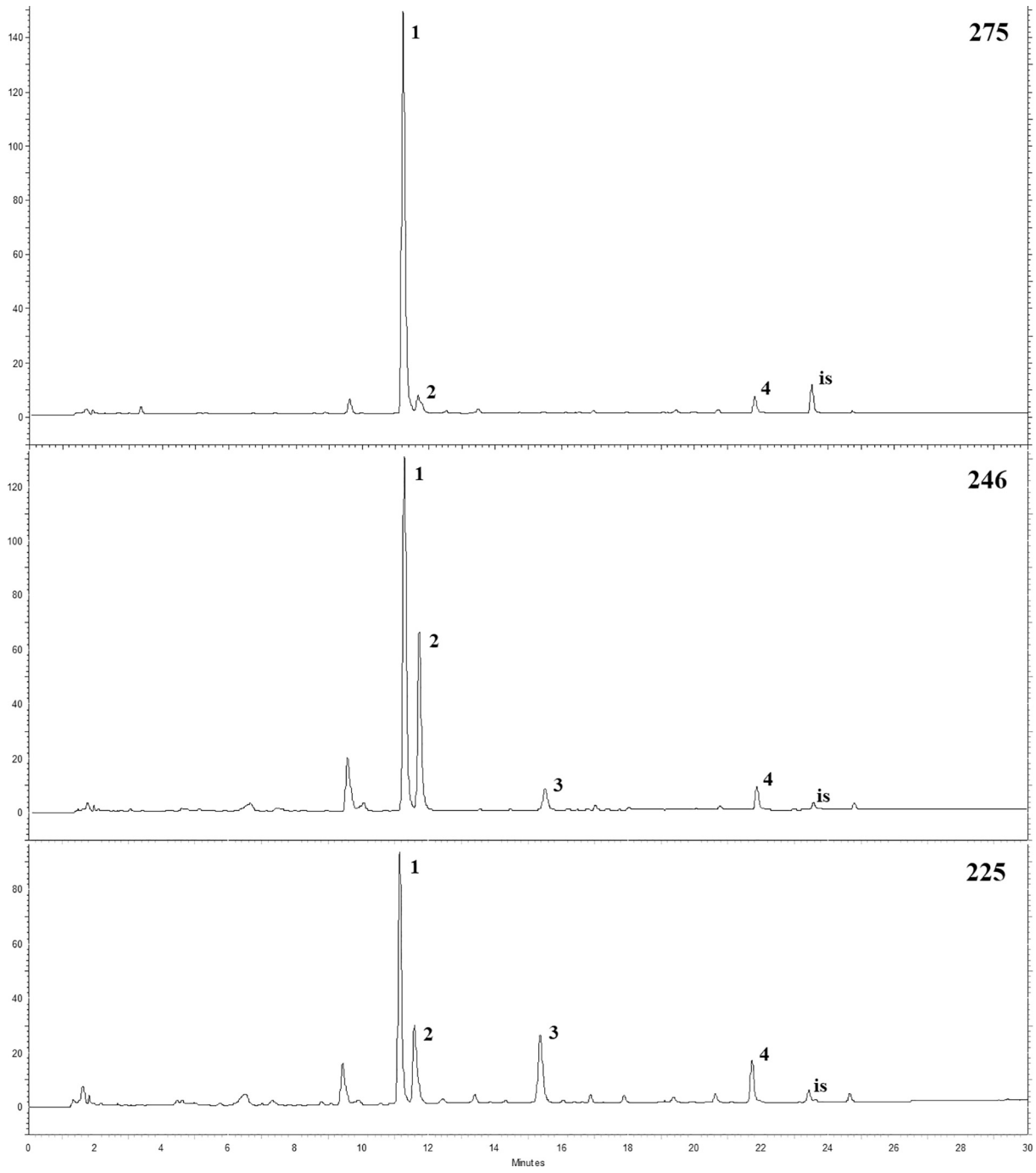

Fig. 2. HPLC-DAD chromatograms (225, 246 and 275 nm) of *G. purpurea* water extract with harpagoside (is) added as an internal standard. Analysed compounds are gentiopicrin (**1**), sweroside (**2**), erythrocentaurin (**3**), and amarogentin (**4**). Chemical structures are shown in Fig. 1.

Table 3
Concentration of gentiopicrin, sweroside, erythrocentaurin, and amarogentin in *Gentiana purpurea* preparations made with hot water.

Extraction method	**Gentiopicrin** (μg/mL ± SD)	**Sweroside** (μg/mL ± SD)	**Erythrocentaurin** (μg/mL ± SD)	**Amarogentin** (μg/mL ± SD)
Decoction (30 min) (DW)	83.7 ± 5.3 $^{a\ b\ c}$	20.8 ± 3.3 ^{a}	< LOD	3.3 ± 0.4 ^{a}
Decoction (30 min) (DC)	76.4 ± 1.0 ^{b}	28.1 ± 1.0 ^{b}	4.1 ± 0.8 ^{a}	5.0 ± 0.1 ^{b}
Decoction (60 min) (DC)	72.6 ± 6.5 ^{c}	23.3 ± 1.1 $^{a\ b\ c}$	2.5 ± 0.8 $^{a\ b}$	3.0 ± 0.9 ^{ac}
Decoction (120 min) (DC)	57.4 ± 4.0 ^{d}	20.1 ± 1.5 $^{a\ c}$	1.9 ± 0.3 ^{b}	1.7 ± 0.6 ^{c}
Infusion (3 min) (I)	86.5 ± 1.3 $^{a\ b}$	22.0 ± 1.4 ^{a}	< LOD	3.5 ± 0.3 ^{a}
Infusion (5 min) (I)	89.9 ± 6.0 ^{a}	25.1 ± 2.0 $^{a\ b}$	< LOD	4.2 ± 0.4 $^{a\ b}$

Different superscript letters in the same column indicate significant differences obtained by one-way ANOVA test and Tukey's multiple comparison test ($P < 0.05$). Plant material was added to boiling water and boiled for 30 min (DW) or infused for 3 or 5 min (I), or the plant material was added to cold water, brought to boil, and boiled for 30, 60 or 90 min (DC). ($n = 3$).

Maceration with 70% ethanol gave the overall highest yields of gentiopicrin (117.9 ± 5.7 μg/mL), and this was significantly higher than with boiling water where cold water was added to the roots, but not significantly higher than maceration with 40% ethanol or decoction with 3% acetic acid (Table 4). Decoctions with 5% and 13% ethanol gave significant lower gentiopicrin yields, but only with 5% ethanol detectable amounts of erythrocentaurin was observed. Only small differences were observed for sweroside and amarogentin when different ethanol or acetic acid concentrations were employed during the extractions.

Lastly, the extraction yields were quantified in preparations made with boiling beer, wine, vinegar and milk (Tables 5–6). These treatments also resulted in small differences in the yields of sweroside and amarogentin. Extraction with apple cider vinegar (5% acid) gave a significant higher yield of gentiopicrin compared to decoction with water ($p < 0.0001$), but there were no significant differences in the extraction yields for apple cider vinegar and 3 and 6% acetic acid.

The gentiopicrin yield seemed to increase with increasing concentration of ethanol in the liquids. Extraction with sour beer (7% ethanol) and brown ale (8% ethanol) gave significant higher yields than pilsner beer (4.5% ethanol) ($p < 0.05$ and 0.01, respectively). Extraction with 10.5% white wine (101.1 ± 4.3 μg/mL) gave approximately the same yield as with brown ale (103.9 ± 1.3 μg/mL). Compared to extraction with ethanol having the same alcohol concentrations, there were no significant differences between alcohol containing drinks or aqueous ethanol.

For extraction with milk and dairy products (Table 6), gentiopicrin yield was dependent on fat content. The yield of fat free milk (79.1 ± 3.5 μg/mL) was comparable with 30 min water extraction. Milk with 3.8% and cream with 18% fat gave a significantly higher yield of gentiopicrin (105.1 ± 7.0 and 106.0 ± 3.1 μg/mL, respectively) compared to fat free milk ($p < 0.0001$). These yields were also comparable with 40% EtOH and 6% acetic acid. Unhomogenized, unpasteurized raw milk gave the highest yield of gentiopicrin (116.5 ± 7.7 μg/mL), though not significantly different compared to 3.8 and 18% fat content. In addition, raw milk gave the highest recorded extraction yield of sweroside (37.3 ± 1.5 μg/mL).

4. Discussion

The yields of gentiopicrin and erythrocentaurin were highly linked to both extraction method and extraction time for aqueous extractions. The highest yield of gentiopicrin was achieved when boiling water was added directly to powdered root, whilst the concentration of erythrocentaurin was undetectable. On the contrary, when root material was added to cold water and brought to boil the yield of gentiopicrin was markedly lower, whilst erythrocentaurin was higher. Furthermore, the extractions by maceration in 40% and 70% EtOH and decoction with vinegar showed among the highest yield of gentiopicrin, with undetectable amounts of erythrocentaurin (Fig. 3). As the variables in these extractions are linked to reduction of enzymatic activity, the conversion of gentiopicrin to erythrocentaurin can be caused by enzymatic degradation by β -glucosidase and possibly also other enzymes. β-Glucosidase is found in all living organisms, including plants [22]. It is stable at 40 °C and a pH of 5.2–6.4 and shows increased activity and decreasing stability with increasing temperature [23]. Maximum activity is observed at 60 °C and pH of 4.4–5.2 and decreases drastically as the temperature approaches 80 °C. In extracts with pH < 4, such as vinegar, acetic acid, sour beer, and white wine (Table 1), the low pH will contribute to reduced enzyme activity. Similarly with dairy products, pH was 6–7 which is above the range for maximum activity. Gentiopicrin contains a β-linked glucose moiety, and its linkage to the aglycone is probably hydrolysed by β-glucosidase during the heating in water. The mechanism behind the further conversion into erythrocentaurin may involve other enzymes as well. Previous studies have shown that β-glucosidase can transform gentiopicrin into erythrocentaurin [24]. Similar transformations have also been observed with mixtures of intestinal bacteria [12,25]. The secoridoid swertiamarin, which is also present in *G. purpurea* roots [8], can also be transformed into erythrocentaurin by intestinal bacteria [26]. The addition of EtOH seems to protect against degradation into erythrocentaurin. Extraction with 5% and 10.5% EtOH showed erythrocentaurin yields of 0.9 ± 0.1 μg/mL and detectable, though unquantifiable, amounts respectively, suggesting that the enzyme activity decreases with increasing EtOH concentration. Furthermore, there were no significant difference in gentiopicrin yields for 40% EtOH, 70% EtOH, 3% acetic acid and 6% acetic acid, indicating that enzymatic activity is crucial for gentiopicrin degradation during extraction.

The yields of sweroside and amarogentin were not correlated to erythrocentaurin yield, as for gentiopicrin. This is evident from the 30 min extraction in water brought to the boil with powdered drug, which showed a high yield of both erythrocentaurin (4.1 ± 0.8 μg/mL) and sweroside (28.1 ± 1.0 μg/mL), and a low yield of gentiopicrin (76.4 ± 1.0 μg/mL). Sweroside has been shown to be enzymatically degraded by β-glucosidase into an unknown metabolite, different from erythrocentaurin [27]. For amarogentin there were no conclusive patterns to the yield based on extractant, though boiling in ethanol concentrations over 5% and acetic acid seemed to give slightly increased yields. Amarogentin is reported to be more resistant to enzymatic degradation, possibly due to the diphenyl group [27].

Sour beer, brown ale, and white wine all had comparable yields of gentiopicrin despite their differing ethanol concentration and pH of 7% and 3, 8% and 4–5, 10.5% and 3, respectively. They were however higher than the extractions with pure 10.5% ethanol, with brown ale and white wine being significantly higher. As mentioned, erythrocentaurin was detectable in 10.5% ethanolic extraction, but this was not the case for sour beer, brown ale, and white wine. It therefore seems that these latter, more complex, extractants are more effective at reducing enzymatic activity during the boiling step of extraction. Despite this apparent synergistic effect, 5% apple cider vinegar with a pH around 2.5 gave higher yields than any of the complex extractions, though the difference was significant only compared to sour beer.

Fat content was important for the gentiopicrin yield during extractions with milk. With fat free milk, the yield was comparable to pure water and 5% ethanolic extractants. With the addition of fat, as seen with the 3.8% milk, the yield was significantly higher and comparable to

Table 4
Concentration of gentiopicrin, sweroside, erythrocentaurin, and amarogentin in *Gentiana purpurea* preparations made with water, ethanol or acetic acid.

Extractant	Gentiopicrin (μg/mL ± SD)	Sweroside (μg/mL ± SD)	Erythrocentaurin (μg/mL ± SD)	Amarogentin (μg/mL ± SD)
Water (DC)	76.4 ± 1.0 ^{a}	28.1 ± 1.0 ^{a}	4.1 ± 0.8 ^{a}	5.0 ± 0.1 $^{a\ c\ d}$
5% EtOH (DC)	83.2 ± 2.6 $^{a\ b}$	24.4 ± 1.6 ^{a}	0.9 ± 0.1 ^{b}	4.3 ± 0.1 $^{b\ c\ d}$
10.5% EtOH (DC)	89.2 ± 3.4 ^{b}	24.5 ± 0.9 ^{a}	< LOQ	5.9 ± 0.5 $^{c\ d}$
40% EtOH (M)	109.7 ± 2.3 ^{c}	29.1 ± 6.8 ^{a}	< LOD	4.4 ± 0.1 ^{d}
70% EtOH (M)	117.9 ± 5.7 ^{c}	27.0 ± 3.8 ^{a}	< LOD	5.2 ± 0.7 $^{a\ b\ c\ d}$
3% Acetic acid (DC)	114.0 ± 4.5 ^{c}	25.4 ± 1.2 ^{a}	< LOD	5.8 ± 0.7 $^{a\ c}$
6% Acetic acid (DC)	106.2 ± 7.5 ^{c}	24.9 ± 0.5 ^{a}	< LOD	5.4 ± 0.2 $^{a\ b\ c\ d}$

Different superscript letters in the same column indicate significant differences obtained by one-way ANOVA test and Tukey's multiple comparison test ($P < 0.05$). Plant material was added to cold extractant, brought to boil, and boiled for 30 min (DC), or the plant material was macerated at room temperature for 24 h (M). ($n = 3$).

Table 5
Concentration of gentiopicrin, sweroside, erythrocentaurin, and amarogentin in *Gentiana purpurea* preparations made with beer, wine, or vinegar.

Extractant	Gentiopicrin (μg/mL ± SD)	Sweroside (μg/mL ± SD)	Erythrocentaurin (μg/mL ± SD)	Amarogentin (μg/mL ± SD)
Pilsner beer	81.6 ± 5.8 [a]	22.8 ± 1.8 [a]	< LOQ	5.0 ± 0.4 [a]
Sour beer	97.7 ± 6.6 [b]	26.5 ± 3.8 [a b]	< LOD	5.7 ± 0.7 [a b]
Brown ale	103.9 ± 1.3 [b c]	25.7 ± 2.6 [a b]	< LOD	7.0 ± 1.0 [b]
White wine	101.1 ± 4.3 [b c]	32.0 ± 5.1 [b]	< LOD	5.6 ± 0.4 [a b]
Apple cider vinegar	113.4 ± 7.8 [c]	25.7 ± 0.5 [a b]	< LOD	5.7 ± 0.4 [a b]

Different superscript letters in the same column indicate significant differences obtained by one-way ANOVA test and Tukey's multiple comparison test ($P < 0.05$). Plant material was added to cold extractant, brought to boil, and boiled for 30 min. ($n = 3$).

Table 6
Concentration of gentiopicrin, sweroside, erythrocentaurin, and amarogentin in *Gentiana purpurea* preparations made with dairy products.

Extractant	Gentiopicrin (μg/mL ± SD)	Sweroside (μg/mL ± SD)	Erythrocentaurin (μg/mL ± SD)	Amarogentin (μg/mL ± SD)
Fat free milk	79.1 ± 3.5 [a]	21.9 ± 1.8 [a]	1.0 ± 0.1 [a]	5.0 ± 1.1 [a]
Milk 3.8% fat	105.1 ± 7.0 [b]	28.2 ± 3.5 [b]	1.1 ± 0.3 [a]	6.0 ± 0.1 [a]
Cream 18% fat	106.0 ± 3.1 [b]	29.4 ± 0.4 [b]	1.8 ± 0.9 [a]	4.8 ± 0.9 [a]
Raw milk	116.5 ± 7.7 [b]	37.3 ± 1.5 [c]	2.0 ± 0.8 [a]	6.9 ± 1.0 [a]

Different superscript letters in the same column indicate significant differences obtained by one-way ANOVA test and Tukey's multiple comparison test ($P < 0.05$). Plant material was added to cold extractant, brought to boil, and boiled for 30 min. (n = 3).

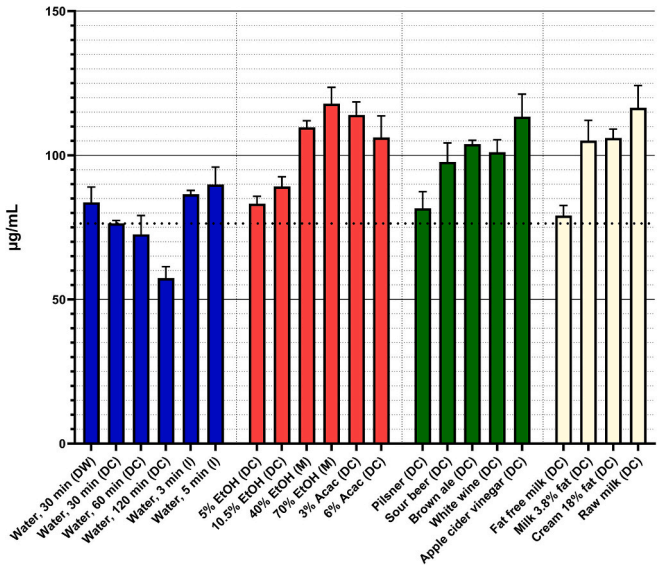

Fig. 3. Gentiopicrin yield (μg/mL) from all extractions ($n = 3$). Extractions were performed by adding the plant material to boiling water (DW), by adding the plant material to cold water before boiling (DC), by infusion (I), or by maceration (M). See Table 1 for details about the extraction methods.

the yields from 6% acetic acid and 40% EtOH maceration. Furthermore, the yield did not increase when the fat percentage was raised to 18% during extraction with cream and stayed consistent with the yield from 3.8% fat. This indicates that increasing the fat percentage far beyond 3.8% is not beneficial for gentiopicrin yield. Interestingly, raw milk provided the highest yield of gentiopicrin, comparable with 70% EtOH maceration, though not significantly higher than the other fat milk extractions. In addition, these extractions also provided the overall highest yield of sweroside and one of the highest yields of amarogentin.

The main differences between raw milk and processed milk are the former being unpasteurized and unhomogenized. Raw milk has significantly bigger fat globules than homogenized milk [28]. This seems to be beneficial for the extraction yield of gentiopicrin and especially the more lipophilic amarogentin. Extractions with milk appeared to provide some protection against degradation of gentiopicrin, as the yields of erythrocentaurin were lower than pure water and comparable to 5% EtOH. Milk fat droplets are encased in an outer membrane of polar phospholipids and associated proteins, such as immunoglobulins and highly glycosylated glycoproteins [29]. These emulsifying compounds may enhance the solubility of gentiopicrin, sweroside and amarogentin, all of which exhibit limited to low water solubility. Furthermore, the outer membrane protects its contents from enzymatic degradation, which could explain the reduced yield of erythrocentaurin [28,29]. There were no significant differences in the amount of degradation to erythrocentaurin among the milk extractions.

Results obtained with this study is in accordance with our recent publication (Zhang et al. 2023, [8]), where we showed that gentiopicrin is the dominating secondary metabolite in *G. purpurea* roots collected in Norway, and that erythrocentaurin was formed during extraction with boiling water when the roots were added to cold water before heating. With the present study, we have evidence for a biotransformation of gentiopicrin into erythrocentaurin when the roots are added to cold water before heating and suggest that an enzymatic reaction is involved, possibly also influenced by native bacteria. We have also identified amarogentin as a major bitter principle in *G. purpurea* roots. Amarogentin is a secoiridoid glycoside with an additional biphenyl moiety that is found to have great impact on the bitterness value [16]. Amaroswerin and amaropanin are related bitter compounds containing the biphenyl substituent and previously reported from *G. purpurea* collected in Switzerland [6,30]. They were found in less amounts compared to amarogentin and were not quantified in the present study.

A limitation with the present study was the relatively low drug-solvent ratio of 2 mg/mL that was used. Other quantitative studies on *Gentiana* species have often used concentrations of 20–25 mg/mL [31,32], although with the purpose to quantify secoiridoids and other constituents in the plant material, some of them present in low concentrations. We could quantify the major secoiridoids gentiopicrin, sweroside, and amarogentin in all preparations, but only for gentiopicrin we were able to observe significant differences between the different preparation methods. If a larger drug-solvent ratio was used, we would obtain a higher yield of all analytes and consequently more pronounced differences between the different extraction methods would be expected. Additionally, given the small sample size of only 40 mg powder per preparation and a particle size below 4 mm, variations in powder size between each preparation may have led to uneven distribution of particles between each preparation, which could potentially influence on the extraction yield.

5. Conclusions

This study shows that the traditional preparation methods for

G. purpurea root gave high yields of its bitter compounds, compounds that are regarded as important for the medicinal effects. Significantly higher yields of the major compound gentiopicrin were observed with raw milk, vinegar and 70% EtOH as extraction solvent compared to water. This study highlights the specific efficacy of extraction using historical solvents and stresses the relevance of investigating both the ingredients as well as preparation methods in ethnopharmacological studies of traditional medicine. The efficacy of historical extraction solvents in the preparation of traditional medicine has been a subject of considerable interest and exploration. Throughout history, various solvents such as water, ethanol, and oil have been employed to extract bioactive compounds from medicinal plants, aiming to harness their therapeutic properties. These solvents have proven effective in isolating a diverse range of phytochemicals, including alkaloids, flavonoids, and terpenoids, which may contribute to the pharmacological activity of traditional medicines. While water-based extractions are often preferred for their safety and simplicity, ethanol and oil extractions are valued for their ability to capture a broader spectrum of compounds, including those with limited water solubility. The choice of solvent can significantly impact the composition and potency of the final herbal preparation, reflecting the complex interplay between the solvent's physicochemical properties and the plant's biochemistry. As we continue to investigate historical remedies with modern scientific methods, we will continue to shed light on the understanding of historical extraction solvents for optimized preparation of traditional medicines.

CRediT authorship contribution statement

Håvard Hoel: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Hugo J. de Boer:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Conceptualization. **Anneleen Kool:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Helle Wangensteen:** Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2024.105932.

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