

## Article

# Optimized Centrifugal Partition Chromatography (CPC) Protocol for Isolation of Urease Inhibitors: Magnoflorine and Berberine from *Berberis vulgaris* Extracts

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**Abstract:** In recent years, an increasing interest in phytotherapy has been observed. Parallel to the research on the total extracts of plant material, numerous studies on the activity of single molecules derived from plants are being conducted to address their mechanisms of action and determine active doses and eventual interactions. Despite this phenomenon, the isolation of individual compounds is a bottleneck due to its difficulty and cost. This work presents the results of a careful optimization of magnoflorine and berberine (isoquinoline alkaloids) recovery from a commonly distributed shrub, *Berberis vulgaris*, growing in Poland and Georgia, using CPC. Both compounds are known for their numerous medicinal properties, which makes the isolation methodology an important area of research. Additionally, CPC has the ability to isolate high-quality compounds in large quantities, which makes it an effective and easy-to-commercialize method. For a successful separation, the biphasic solvent system composed of hexane, butanol, ethanol, and water in a ratio (3:12:4:16 v/v/v/v) was used in the ascending mode, together with the flow rate of 8 mL/min and rotation speed of 1600 rpm. The method was selective for both compounds, and it delivered good results for both root and stem extracts from the plant. The qualitative composition of alkaloids in the studied extracts determined by HPLC-ESI-QTOF-MS/MS confirmed the presence of berberine, magnoflorine, jatrorrhizine, and palmatine alkaloids from the group of isoquinolines. The isolates, magnoflorine and berberine, were subjected to the *Helicobacter pylori* growth inhibition assay and urease inhibition test to assess whether, next to the previously proved anticancer properties, these compounds are characterized by *H. pylori* inhibition. MGN was found to exhibit inhibitory potential against urease (IC<sub>50</sub> = 25 mg/L).

**Keywords:** magnoflorine; berberine; alkaloids; centrifugal partition chromatography; *Berberis vulgaris*; natural substance; extraction



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## 1. Introduction

The deepening of knowledge on the chemistry and activity of plant medicines and their standardization has brought them renewed interest. Recent years have brought an increase in the number of publications on phytochemical research and related pharmacological studies, which have been a major manifestation of this approach. Numerous side effects of chemical drugs constantly trigger interest in natural herbal remedies that often bear similar pharmacological potential with a better pharmacokinetic profile. Within three years, 2021–2023, 3481

publications on alkaloids—the natural compounds discussed in this study—have been listed in the Pubmed database. Among them, isoquinoline alkaloids (IQAs) are known for their diverse medicinal properties. Some of them are successfully used in medicine, e.g., in cancer or dementia treatment; however, the scientific literature is still providing new information on their applications.

*Berberis vulgaris* is one of the species from the largest genus of woody plants, genus *Berberis*, belonging to the family Berberidaceae, with approximately 500 species worldwide. *B. vulgaris* is a plant whose natural range covers areas of Eurasia and some regions of North America. In Europe, it can be found from the Scandinavian Peninsula to the Balkan Peninsula, and also in Asia, reaching from the Middle East to Siberia. In Europe, common barberry naturally occurs in the wild [1].

In traditional medicine, various parts of *B. vulgaris*, such as the fruit, bark, root, and stem, have been used to treat various ailments. The shrub contains IQAs, flavonoids, phenolic acids, vitamin C, organic acids, and many other compounds. Alkaloids from the plant constitute a key group of secondary metabolites that play an important role in the pharmacological profile of the plant. Among them, protoberberine alkaloids such as berberine, palmatine, and jatrorrhizine, and aporphine alkaloids such as magnoflorine (MGN), were identified [2]. The latter group of alkaloids is the second most commonly distributed subgroup of IQAs in plants after benzyloquinoline alkaloids. One of the leading compounds from this group is magnoflorine. It proved to be widely synthesized among the representatives of Papaveraceae, Berberidaceae [3], Annonaceae [4], Rutaceae [5], Euphorbiaceae [6], Menispermaceae [7], and others.

MGN has a number of medicinal properties. It is used in diabetology [8] as an anti-inflammatory and antioxidant [9,10] component. Moreover, MGN exhibits antiviral [11], antibacterial [12], and antifungal [13] properties. Recent years have delivered results on its anticancer properties and potential application in the treatment of breast cancer [14] and gastric cancer [15,16]. Nevertheless, the mechanism of action and the influence of MGN on the progression of cancer, which is of particular interest to the authors, have not yet been fully understood.

When discussing the composition of barberry shrubs, it is necessary to mention berberine—an isoquinoline alkaloid from the protoberberines' subgroup that can be perceived as a chemotaxonomical marker of the Berberidaceae botanical family. Berberine belongs to the most studied compounds of natural origin. Traditionally, it has been used as a cholagogenic, anthelmintic, antioxidant, and hepatoprotective compound; however, more recent studies confirm its antidepressant, cholesterol-diminishing, antihypertensive, anti-diabetic, immunomodulatory, and anti-inflammatory properties [17]. In the latest scientific literature, there is an increasing number of scientific reports on the application of berberine in the prevention and treatment of cancers including breast, colon, lung, and cervical, as well as glioblastoma and leukemia, *in vitro* [18,19].

Literature screening on the biological properties of MGN shows us that there are still some gaps to be filled concerning the studies on this alkaloid. In this work, the authors present a method for isolating MGN from *Berberis vulgaris*, both collected in Poland and Georgia, using a hydrostatic countercurrent chromatography technique, CPC. Thanks to the application of this method, high-purity alkaloids from the selected plant material were obtained together with tailored protocols, promising their preparative recovery on a commercial scale directly from a crude extract.

The isolated alkaloids, MGN and berberine, were tested for their properties against *Helicobacter pylori* bacteria. The infected habitat in humans is the mucus layer covering the epithelial containers. The infection is usually asymptomatic and may include non-ulcer dyspepsia, peptic ulcers, and atrophic decomposition of the secondary mucosa—atrophy. The treatment of *H. pylori* infections is of particular importance as the strain is listed as a human carcinogen. Numerous studies show that the populations with a high incidence of cancer are also very susceptible to *H. pylori* infections [20], which are progressing with

triggered inflammatory conditions inducing parietal lesions and hypochloridia. In the final stage, a multi-stage process of carcinogenesis may occur [21].

During *H. pylori* infection, macrophages, crucial components of innate immunity, are recruited to the gastric mucosa. Macrophages exhibit plasticity and flexibility, with two polarization outcomes: M1 (proinflammatory) and M2 (anti-inflammatory and tissue-repair). The balance between M1 and M2 polarization and their mediators plays a role in the immune response. The activation of M2 macrophages induced by Th2 cytokines, especially interleukin-4 (IL-4) through the signal transducers and activators of the transcription 6 (STAT6) pathway, has gained attention in *H. pylori* infection research [22].

So far, berberine has shown marked efficacy in the inflammation and cancer treatment of the digestive system. The alkaloid was reported to effectively treat *H. pylori*-induced CAG with low side effects and better resistance than common antibiotics. Berberine promotes the polarization of M2 macrophages during *H. pylori* infection, whereas its anti-inflammatory properties are associated with the inhibition of M1-polarized macrophages. Berberine activates the IL-4-STAT6 signaling pathway, a crucial pathway in M2 macrophage activation and anti-inflammatory responses [23]. As both alkaloids were proven to show anticancer properties in the treatment of gastric cancer, the goal of the study was to test the *H. pylori* inhibitory potential on the two isolates to study the potential of MGN in relation to berberine.

## 2. Materials and Methods

### 2.1. Plant Material and Extractions

The plant material used in the research was the root of *Berberis vulgaris* purchased in a local herbal shop (Proherbis Jaroslaw Wolanski) in Poland, and the stem of *Berberis vulgaris* collected in Tbilisi, Georgia, in 2019. The cut stem of *Berberis vulgaris* was transported to Poland in its entirety, where it was cut and dried at 35 °C by the authors of the publication and used for the study. Its authenticity was confirmed by Dr. Anna Bozhadze and Prof. Wirginia Kukula-Koch, the co-authors of this manuscript. The samples of plant material are stored by the authors in the Department of Pharmacognosy with Medicinal Plants Garden of the Medical University of Lublin.

Both the stem and root samples were extracted under the following conditions. Portions of 10 g of powdered plant material from each plant were extracted with methanol using a pressurized liquid extractor (also known as accelerated solvent extractor—ASE) (ASE 100, Dionex, Sunnyvale, CA, USA) in the following conditions: static time: 10 min, number of cycles: 4, working pressure: 96 bar, temperature: 90 °C, purge time: 120 s, and purge volume: 60%. Extraction under these conditions was repeated in 4 cycles, and then extracts from individual plant organs were combined and evaporated. Plant extracts were evaporated to dryness in a rotary evaporator at 45 °C. The dried residue was weighed and used for alkaloid recovery. The obtained dried residues were refrigerated at 4 °C until further tests. Reagents used during extraction—methanol was of analytical purity and was purchased from Avantor Performance Materials (Gliwice, Poland). This particular extracting solvent was selected based on the former experiments of the authors, confirming the strongest extraction capacity of methanol in *Berberis siberica* samples [24].

### 2.2. Fractionation of the Extract by CPC

#### 2.2.1. The Selection of the Biphasic Solvent Composition for the Fractionation of *Berberis vulgaris* Extracts

The selection of a proper biphasic solvent system composition that will be selective enough in the fractionation of a crude extract by CPC chromatographs is a critical step. To recover alkaloids from *Berberis vulgaris* methanolic extract, a series of tests were conducted using different solvent systems to select the most suitable composition. The tested solvent mixtures included different ratios of hexane, buthanol, ethanol, and water in the ranges from 2 to 18 volume parts for every solvent pair, so as to create 20 parts in total for each of the two groups of solvents: n-hexane with buthanol and ethanol with water. Then, the volume of ethanol and water was left in 20 parts, and further modifications to the ratio of n-hexane and

buthanol were introduced. Then, 20 mg of the extract was added to each of the best mixtures of solvents. The two best mixtures in terms of the settling times and proportions of the upper and lower phases were selected for further trials with the addition of extract, and they were composed of 4:12:8:12 and 3:12:4:16 volumed mixtures of n-hexane, buthanol, ethanol, and water. Equal volumes of the upper and lower phases were then separately analyzed using HPLC-MS chromatography, allowing for the calculation of the partition coefficient (k), that is, dividing the peak area of a compound in the lower phase by the peak area of the same compound in the upper phase. The coefficients reflected the distribution of the leading components between the upper and lower phases, providing insights into the potential efficiency of separation under various conditions. The calculated k values for each compound guided the selection of chromatographic conditions toward the ascending operation mode.

### 2.2.2. Centrifugal Partition Chromatography-Based Fractionation of Alkaloids from the Methanolic Extracts of *Berberis vulgaris* Root and Stem

The selected solvent systems were prepared in a separatory funnel. After mixing the liquids, the biphasic solvent system was left to settle and separated into two bottles. The injected extract (100 mg) was dissolved in the smallest possible volume of a 50:50 (v/v) mixture of the upper and lower phases. The fractionation was performed using a hydrostatic CPC system equipped with a 250 mL stainless steel column, a UV detector, and a fraction collector (Spot CPC, Armen, Saint Ave, France). The separation process was conducted in the ascending separation mode. The first step involved introducing the stationary phase onto the column at a speed of 20 mL/min with 500 rpm. Next, the extract was injected along with the mobile phase and fractionated. During the analyses, the stationary phase was used interchangeably as the lower or upper one, which is precisely presented in the table with the test parameters. Rotor speed and flow rate varied in different extraction trials. The exact parameters of individual trials are presented in Table 1. All fractions were collected, monitoring UV absorption at 290/320 nm. Post-run, 2 mL of each purified fraction was filtered through a 0.1 µm pore size nylon syringe filter into autosampler vials, evaporated to dryness using an Eppendorf Concentrator Plus evaporator (Hamburg, Germany), re-dissolved in methanol, and subjected to HPLC-MS analysis using the method described above in Section 2.2.

**Table 1.** CPC chromatograph settings used for the optimization of the isolation protocol (trials 6 and 10 represent the most advantageous settings).

Trial No	Plant Material	Rotor Sppeed [rpm]	Flow Rate [mL/min]	UV Absorbance [nm]	Stationary Phase	Injected Volume [mL]	Solvent System [v/v/v/v]
1	Root <i>B. vulgaris</i>	800	8	290/320	lower	3	n-hex:BuOH:EtOH:H <sub>2</sub> O 3:12:4:16
2	Root <i>B. vulgaris</i>	1600	8	290/320	lower	3	n-hex:BuOH:EtOH: H <sub>2</sub> O 3:12:4:16
4	Root <i>B. vulgaris</i>	1600	8	290/320	upper	6	n-hex:BuOH:EtOH: H <sub>2</sub> O 3:12:4:16
3	Root <i>B. vulgaris</i>	1600	8	290/320	upper	3	n-hex:BuOH:EtOH: H <sub>2</sub> O 3:12:4:16
5	Root <i>B. vulgaris</i>	1600	8	290/320	upper	3	n-hex:BuOH:EtOH: H <sub>2</sub> O 3:12:4:16
6	<b>Root</b> <i>B. vulgaris</i>	<b>1600</b>	<b>8</b>	<b>290/320</b>	<b>upper</b>	<b>3</b>	<b>n-hex:BuOH:EtOH: H<sub>2</sub>O</b> <b>3:12:4:16</b>
7	Stem <i>B. vulgaris</i>	800	4	290/320	lower	3	n-hex:BuOH:EtOH: H <sub>2</sub> O 4:12:8:12
8	Stem <i>B. vulgaris</i>	1300	5	290/320	lower	3	n-hex:BuOH:EtOH: H <sub>2</sub> O 4:12:8:12
9	Stem <i>B. vulgaris</i>	1600	8	290/320	lower	3	n-hex:BuOH:EtOH: H <sub>2</sub> O 3:12:4:16
10	<b>Stem</b> <i>B. vulgaris</i>	<b>1600</b>	<b>8</b>	<b>290/320</b>	<b>upper</b>	<b>3</b>	<b>n-hex:BuOH:EtOH: H<sub>2</sub>O</b> <b>3:12:4:16</b>

### 2.3. Chromatographic Analysis of the Extract by HPLC-ESI-Q-TOF-MS

High-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) was used for the qualitative composition assessment of the obtained extract and fractions from the countercurrent separation, but also for the determination of partition coefficient values ( $k$ ). An HPLC chromatograph (HP 1200 series, Agilent Technologies, Santa Clara, CA, USA) equipped with a degasser, a binary pump, an autosampler, a column thermostat, and a UV-PDA detector coupled with a Q-TOF mass spectrometer with an electrospray ionization (ESI) source (6500 Series, Agilent Technologies, Santa Clara, CA, USA) was used in the study. The instrument was calibrated right before the injections, and the operating parameters were initially optimized in both negative and positive ionization modes. The following conditions were applied to all samples: gas and sheath gas flows—12 L/min, gas temperature: 350 °C, sheath gas temperature: 325 °C, fragmentor voltage: 120 V, skimmer voltage: 65 V, nebulizer voltage: 30 psig, capillary voltage: 3500 V, and collision energy: 20 and 40 V. The spectra were collected within the  $m/z$  range of 100–1000. The MS/MS spectra of alkaloids were recorded out of the two most intensive signals in a scan. Internal calibrants were used throughout the analysis to sustain the accuracy of mass measurements. In the constructed method, after the collection of one spectrum of a given  $m/z$  value, the selected peaks were excluded from fragmentation for 0.3 min to trace the fragmentation spectra of the weaker signals. The HPLC method with Zorbax Eclipse Plus stable bond RP-18 chromatographic column (150 × 2.1 mm,  $d_p = 3.5 \mu\text{m}$ , Agilent Technologies, Santa Clara, CA, USA) run under the following gradient of solvent A (0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid): 10% of B in A at 0 min, 40% of B in A at 10 min, isocratic run at 40% of B in A for the following 2 min, 95% B in A at 21–25 min. The post-run was set at 10 min, the flow rate at 0.2 mL/min, the temperature at 25 °C, and the detection wavelengths at 254, 280, 290, and 365 nm. Mass Hunter Workstation and Qualitative Navigator B.08.00 software (Agilent Technologies, Santa Clara, CA, USA) were used to record and handle the obtained data, respectively. Standards of berberine, palmatine, jatrorrhizine, and magnoflorine were injected in the same analytical conditions to provide evidence for the fragmentation of the two groups of IQAs and facilitate the identification of the isolates.

### 2.4. The Antibacterial Assay against *H. pylori*

The tested samples were dissolved in dimethylsulfoxide (DMSO) and tested for antibacterial activity against *H. pylori* ATCC 43504. Minimal inhibitory concentrations of compounds were determined using a two-fold microdilution method with the use of Mueller-Hinton broth supplemented with 7% lysed horse blood. Previously prepared bacterial inoculum of 3 McFarland standard were added to media with compound concentration ranging from 10,000 to 8 mg/L to obtain bacterial suspension of  $1 \times 10^6$  CFU/mL. After incubation at 35 °C for 72 h under microaerophilic conditions (5% O<sub>2</sub>, 15% CO<sub>2</sub>, and 80% N<sub>2</sub>), the growth of *H. pylori* was visualized with the addition 10  $\mu\text{L}$  of 0.04% resazurin. The MIC endpoint was recorded after 4 h incubation as the lowest concentration of extract that completely inhibits growth. A DMSO control and a bacterial growth control were included in the experiment. Minimal bactericidal concentrations (MBC) were obtained by culture of 5  $\mu\text{L}$  of media from each well that showed growth inhibition, from the last positive one, and from the growth control onto recommended agar plates. The plates were incubated at 35 °C for 72 h under the microaerophilic conditions. The experiments were repeated in triplicate. Representative data are presented.

### 2.5. Assay of Urease Inhibitory Activity

*H. pylori* was incubated for 72 h in the MH broth with 7% horse serum (Sigma-Merck, Darmstadt, Germany) in microaerophilic conditions. Bacterial biomass was collected by centrifugation at  $4000 \times g$  at 4 °C for 10 min; then, the cells were dissolved in an ice-cold phosphate buffer (pH 7.3) with a protease inhibitor cocktail (Sigma). Urease enzyme was prepared by disturbing *H. pylori* cells by sonication, followed by centrifugation at  $12,000 \times g$  at

4 °C for 10 min. The enzyme reaction was activated in 96 well plates by mixing the appropriate volume of 2% urea, sodium phosphate buffer solution (100 µL), different concentrations (2000–3.9 µg/mL) of propolis extract, and the reaction mixture was incubated for 15 min at 37 °C, then the concentration of ammonia was determined using the Berthelot method with phenol–hypochlorite reaction [25], which produces a colorimetric product measured at 630 nm. The pH level of the reaction was set at 7.3. The amount of ammonia is equivalent to the hydrolysis of urea using the urease enzyme. The inhibition rate (%) was calculated using the following formula:  $I\% = (1 - \text{activity with inhibitors}/\text{activity without inhibitors}) \times 100\%$ . The IC<sub>50</sub> was expressed as the concentration of inhibitor that decreased urease activity by 50% and calculated by plotting the percent of inhibition using the internet IC<sub>50</sub> Calculator (AAT Bioquest, Pleasanton, CA, USA). The experiments were performed in triplicate.

### 3. Results and Discussion

#### 3.1. HPLC-ESI-QTOF-MS/MS Fingerprinting of the Extracts

The HPLC-ESI-QTOF-MS/MS tests revealed a diverse array of isoquinoline alkaloids in the methanolic extract derived from *Berberis vulgaris*. The performed identification of alkaloids that was based on the analysis of high-resolution mass measurement, the retention time, fragmentation pattern, and literature data confirmed the presence of the representatives of isoquinoline alkaloids as the leading metabolites, with berberine, magnoflorine, jatrorrhizine, palmatine, 4-O-Methyl-N-methylcoclaurine, and demethyleneberberine in the highest concentrations. The list of identified alkaloids in both samples is presented below in Table 2, whereas the chromatograms are in the Supplementary Materials (Figure S1).

**Table 2.** The tentatively identified alkaloids in the extracts from *Berberis vulgaris* (the compounds in bold highlight the alkaloids identified based on the comparison with the standard compounds—see Supplementary Materials, Figure S3).

Ion (+/−)	Rt (min)	Molecular Formula	<i>m/z</i> Calculated	<i>m/z</i> Experimental	Error (ppm)	DBE	MS/MS Fragments	Proposed Compound	References
[M+H] <sup>+</sup>	15.3	C <sub>20</sub> H <sub>24</sub> NO <sub>4</sub>	342.1700	342.1704	−1.22	10	297.1104 265.0890	<b>Magnoflorine</b>	[26]
[M+H] <sup>+</sup>	15.4	C <sub>19</sub> H <sub>23</sub> NO <sub>3</sub>	314.1751	314.1776	−8.08	9	300.1377 269.1052 192.0853	4-O-Methyl-N-methylcoclaurine	[27]
[M+H] <sup>+</sup>	16.4	C <sub>20</sub> H <sub>21</sub> NO <sub>4</sub>	340.1543	340.1554	−3.14	11	295.1899 263.0634 235.0677	Tetrahydroberberine (canadine)	[28,29]
[M+H] <sup>+</sup>	16.9	C <sub>19</sub> H <sub>23</sub> NO <sub>3</sub>	609.2959	609.2954	−0.84	19	579.2569, 567.2530	Oxyacanthine	[27]
[M+H] <sup>+</sup>	17.4	C <sub>20</sub> H <sub>20</sub> NO <sub>5</sub>	354.1336	354.1325	3.11	12	339.1105 324.0905 280.0899	Protopine	[30]
[M+H] <sup>+</sup>	17.5	C <sub>37</sub> H <sub>40</sub> O <sub>7</sub> N <sub>2</sub>	625.2908	625.2926	−2.84	19	608.3629, 581.2192 381.2131	Northalrugosine	[31]
[M+H] <sup>+</sup>	17.9	C <sub>19</sub> H <sub>17</sub> NO <sub>4</sub>	324.1230	324.1229	−6.7	12	280.0935 175.0565	Demethyleneberberine/ isomer	[27,30]
[M+H] <sup>+</sup>	19.1	C <sub>20</sub> H <sub>19</sub> NO <sub>4</sub>	338.1387	338.1382	1.44	12	294.1114 208.0594	<b>Jatrorrhizine</b>	[26,32]
[M+H] <sup>+</sup>	19.6	C <sub>19</sub> H <sub>16</sub> NO <sub>4</sub>	322.1074	322.1085	5.56	13	250.0954 151.9525	Thalifendine	[26]
[M+H] <sup>+</sup>	19.8	C <sub>20</sub> H <sub>18</sub> NO <sub>4</sub>	336.1230	336.1231	−0.2	13	304.084, 292.0859 278.0710	Epiberberine	[26]
[M+H] <sup>+</sup>	20.2	C <sub>21</sub> H <sub>21</sub> NO <sub>4</sub>	352.1543	352.1549	−0.47	12	308.1281 248.0918	<b>Palmatine</b>	[30]
[M+H] <sup>+</sup>	20.5	C <sub>20</sub> H <sub>17</sub> NO <sub>4</sub>	336.1230	336.1231	−1.99	13	292.0983 220.1163	<b>Berberine</b>	[30]

Isoquinoline alkaloids, including MGN, possess a basic nitrogen atom within their structure. When analyzing them in the positive ionization mode, the nitrogen atoms readily accept protons, resulting in the formation of positively charged ions. That is why the fragmentation patterns and stability of isoquinoline alkaloids fit well into the positive ion mode. In the case of MGN that was of particular interest in this study, the methoxyl and hydroxyl substituents present in its structure allow for the generation of characteristic fragment ions that enable identification and analysis. The parent ion of MGN appears at the  $m/z$  of 342.17. The major fragment ions visible in the MS/MS spectra are characterized by the  $m/z$  values of 311, 297, 282, 265, and 237, corresponding to  $[M-\text{MeO}]^+$ ,  $[M-\text{C}_2\text{H}_7\text{N}]^+$ ,  $[M-\text{C}_2\text{H}_7\text{N}-\text{Me}]^+$ ,  $[M-\text{C}_2\text{H}_7\text{N}-\text{MeOH}]^+$ , and  $[M-\text{C}_2\text{H}_7\text{N}-\text{MeOH}-\text{CO}]^+$  ions, respectively [33].

Tian and co-investigators confirmed a similar fragmentation pattern. Their injections showed prominent fragment ions of MGN that emerged at  $m/z$  of 297.0, 282.1, and 265.0. The signal at  $m/z$  297.0, being the most abundant, was selected as the primary product ion, and the optimal collision energy parameters for MGN were determined to be 23 and 26 eV, respectively, to achieve maximum sensitivity in multiple reaction monitoring (MRM) transitions in the triple-quad instrument [34].

The fragmentation of berberine, similarly to MGN, occurs in the positive ionization mode. In these conditions, the molecular ion is detected as a signal with the  $m/z$  value of 336.123 from the protonated molecular ion  $[M+\text{H}]^+$ . As berberine has been widely studied, its fragmentation pattern has been well established as well. The fragmentation spectra for this alkaloid show, next to the protonated molecular ions ( $[M+\text{H}]^+$ ), also other fragments that come from a successive detachment of the small substituents, like methyl, hydroxyl, or methylene groups. Among them, we can distinguish the  $m/z$  signals of 320, 304, 292, and 278 [35].

Previously, the presence of similar isoquinolines was confirmed by other authors in the same and in different barberry species. The extract from *Berberis vulgaris*, as proved by the study by Hostalkov et al., contained, among others, berberine and palmatine [32]. Other tests carried out on *Berberis vulgaris* show a marked content of jatrorrhizine, magnoflorine, and epiberberine [26]. Also, other species of barberry are rich sources of IQAs. Singh et al. examined the qualitative composition of various barberry species from India, specifically *B. chitria*, *B. jaeschkeana*, *B. koehneana*, *B. lycium*, and *B. pseudoumbellata*. In all tested samples, the authors showed the presence of four leading compounds—berberine, palmatine, jatrorrhizine, and magnoflorine [29]—next to the presence of tetrahydroberberine, which we also identified in our extracts.

The fingerprinting of the herein-analyzed extracts that were obtained from the roots and stems of *Berberis vulgaris* showed some differences between the samples. Root extracts contained higher amounts of alkaloids than stem extracts. Table 3 highlights the quantitative differences of the main alkaloids in the tested samples that were measured as a direct comparison of the recorded peak areas of these compounds. This analysis is important for understanding and more efficient usage of the medicinal properties of the isoquinolines and also expands knowledge about the differences in the chemical composition of this plant depending on the analyzed organs.

**Table 3.** Relative quantitative peak area comparison of alkaloids identified in *Berberis vulgaris*. +++ (1 to 9)  $\times 10^7$ , ++ (1 to 9)  $\times 10^6$ , + (1 to 9)  $\times 10^5$ , tr – (1 to 9)  $\times 10^4$  ( $n = 3$ ), -: none.

Alkaloid	<i>B. vulgaris</i> Root	<i>B. vulgaris</i> Stem
Tetrahydroberberine (canadine)	tr	tr
4-O-Methyl-N-methylcochlorine	+	-
Magnoflorine	++	+
Demethyleneberberine/isomer	+	tr
Thalifendine	+	tr
Jatrorrhizine	+++	++
Epiberberine	++	+
Palmatine	++	++
Berberine	+++	+++

### 3.2. Centrifugal Partition Chromatography as a Tool for the Fractionation of Extracts

Centrifugal partition chromatography can be perceived as a preferable technique for the recovery of alkaloids. These metabolites tend to adsorb to the surface of silica gel, and their elution from classical columns is disturbed, which results in a significant decrease in the efficiency of the isolation process. Lack of solid support is a promise of a high recovery rate. CPC offers a wide range of methodological tips that can be followed when working with alkaloids. Flow rate and rotation speed adjustments are the basic ones to follow. Different authors introduce modifiers to the applied biphasic solvent systems to enhance the selectivity of the system toward alkaloids. In their study, Kotland et al. [36] utilized a CPC model based on acid-base equilibriums and interfacial mass transfer in continuously stirred tank reactors in series. This model was employed to predict full separations on a larger CPC column under optimized operating conditions and to guide the CPC user in its scale-up strategy. This methodology enables efficient design of the CPC separation process, minimizing the need for trial and error during process up-scaling [37]. Also, considering the ability of alkaloids to be present in the form of free bases soluble in organic solvents and of salts dissolved in polar liquids, the pH-zone refining mode of operation was developed to recover alkaloids from rich matrices and detach them from other metabolites with different chemical character. Nevertheless, the addition of strong acids or bases may be questionable when it comes to the extract enrichment that will be further used in foods.

An additional advantage of the CPC technique is the ability to perform analyses using reagent-grade solvents, which significantly reduces separation costs and is consistent with the principles of green chemistry. Thanks to the above features, the technique seems to be a good choice for the recovery of alkaloids from plant extracts, bearing in mind an easy upscaling procedure that is typical for liquid–liquid chromatographs.

The presented technique was used to isolate alkaloids from *Berberis vulgaris* from the roots and stems of the plant. First, the optimization of methodology was performed for the root of this species as it has been commonly used in phytotherapy as the most rich source of alkaloids.

First, the partition coefficient values were calculated to select the best solvent system for further optimization of the method’s parameters on the CPC instrument. The obtained k values for every tested method are presented below in the Table 4. Numerous reactions were carried out in search of the best proportions of selected solvents. The separation of alkaloids was tested in the following volumes of the previously mentioned separator system: 3:12:6:15; 1:14:6:15; 4:12:8:12; 3:12:4:16 (v/v/v/v).

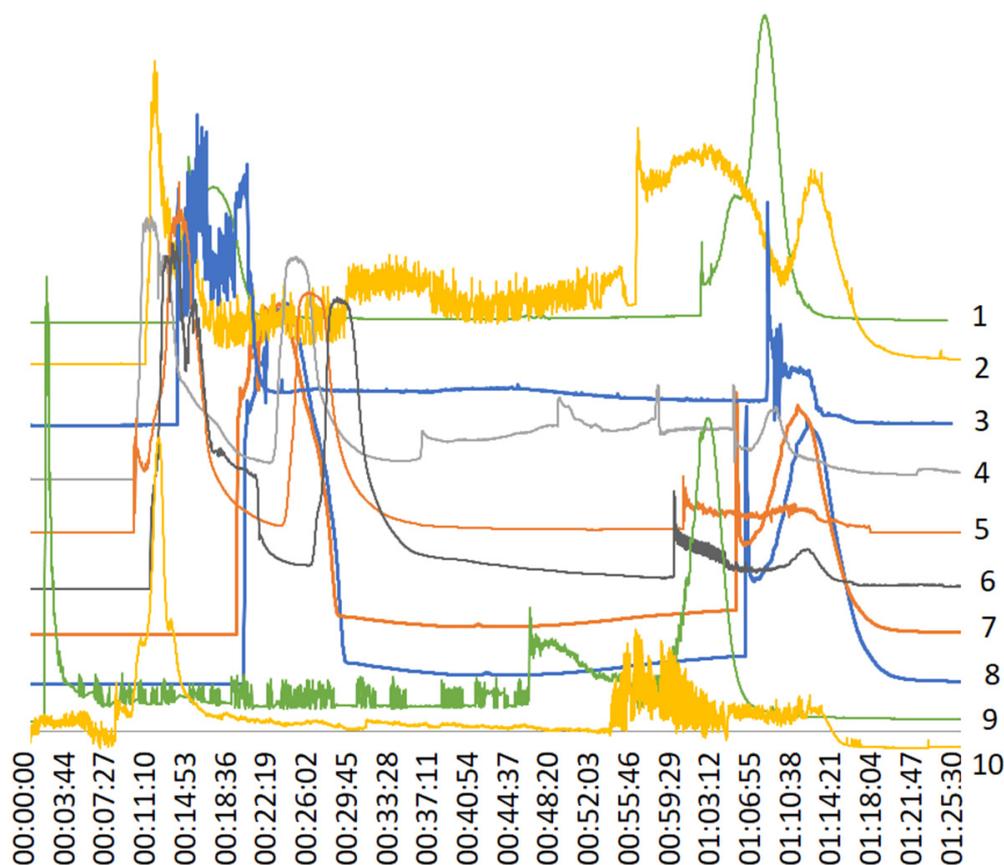
**Table 4.** The calculated k-values for the major components of the extract in the tested solvent system.

Solvent System v/v/v/v	3:12:6:15		1:14:6:15		4:12:8:12		3:12:4:16	
	Root	Stem	Root	Stem	Root	Stem	Root	Stem
<i>B. vulgaris</i>								
Berberine	1.42	0.98	1.12	0.97	0.88	4.94	0.97	1.08
Magnoflorine	0.14	0.17	0.19	1.42	1.58	0.87	0.18	0.16
Palmatine	0.22	1.78	1.45	0.98	0.92	0.44	0.58	1.32
Jatrorrhizine	0.35	0.81	0.42	0.97	1.08	0.82	0.71	0.86

To ensure an effective separation of substances during countercurrent chromatography separation, the partition coefficient values should be in the range of 0.5–2 [37]. The authors’ idea was to construct a biphasic solvent system with satisfactory k values to obtain high-purity MGN and berberine after a single injection of the total extract into the column.

Ultimately, trial 4 (3:12:4:16 v/v/v/v) was selected as the best system among others. The differences in the k values between the major alkaloids were the highest in this case, which stood for its highest efficiency. Also, the settling time of the solvents was short, and the volumes of the upper and lower systems were almost equal, which would not lead to interference with countercurrent separation at high rotational speeds.

Later, ten CPC analyses were performed using the selected solvent system with different flow rates, rotation speeds, and injection volume settings according to the parameters presented in Table 1 to find the best settings and achieve a recovery of the least studied molecule, magnoflorine. Figure 1 shows the obtained CPC chromatograms.



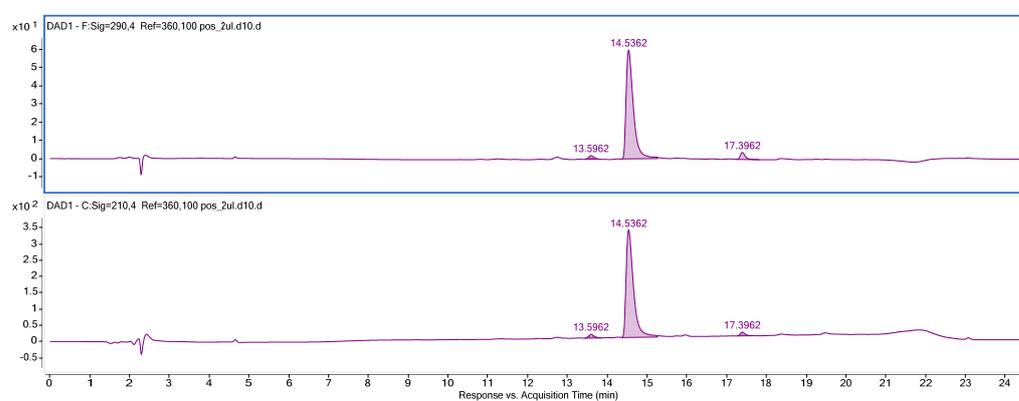
**Figure 1.** CPC chromatograms obtained for the parameters presented in Table 1 (numbers on the right refer to the numbers of runs described in the Table 1).

The introduced methodological parameters modified the elution time of both alkaloids. The fractionation of the methanolic root extract from the root of barberry was optimized first to achieve the isolation of high-purity MGN and, additionally, berberine. A favorable result was obtained by using the upper phase as the stationary phase, which significantly improved the efficiency of substance separation. When the stationary phase was the lower phase, berberine—the main alkaloid of barberry—was tailing and disturbed an efficient purification of other alkaloids, as it was present in many fractions throughout the separation. For the stem extract, two solvent systems, hexane, butanol, ethanol, and water in the proportions of 4:12:8:12 (*v/v/v/v*) and (3:12:4:16 *v/v/v/v*) were introduced. The experiment was reproduced with different rotation speeds of the rotor and flow rate settings. Undoubtedly, the highest impact on the separation efficiency was attributed to the composition of the biphasic solvent system. The rotation speed influenced the retention time of the analytes. In analysis 1, when the rotation speed was set as 800 rpm, berberine was isolated in the 16th minute and MGN in the 75th minute, while at the rotation speed of 1600 rpm, berberine was isolated in the 10th minute and MGN in the 65th minute (analysis 2). In the fractionation of the stems (analyses 7 and 9) at the rotation speeds of 800 and 1600 rpm, similar conclusions were observed. At a higher rotation speed, berberine was eluted within 18 min, whereas MGN was in the 65th minute. However, at a rotation speed of 800 rpm, berberine and MGN were eluted after 35 and 100 min. A higher flow rate and rotation speed can significantly shorten the recovery process of compounds. This

is due to a smaller volume of the stationary phase that is inside the faster rotating column. At the same time, no differences in the purity of the isolated compounds were observed.

Concerning the flow rate, similar conclusions can be observed. Increased flow rate led to a quicker elution of the alkaloids from the column. These results can be observed in analyses No 7, 8, and 9, where the flow rates were set as 4, 5, and 8 mL/min, respectively. The higher the flow value, the earlier the compounds were isolated. In the case of berberine, the elution occurred after 35, 30, and 18 min, respectively. It can be concluded that a higher flow rate results in a faster elution of compounds while maintaining high purity of isolates.

As a result of the herein-described studies on a CPC chromatograph, high-purity MGN (95.1%) and berberine (91.2%) were obtained. The most advantageous separations were recorded for injection 6 (see Figure 1) from the root extract and 10 for the fractionation of the stem. The chromatograms of the fractions containing MGN are presented in Figure 2 below, whereas the chromatograms of the extracts and berberine are presented in the Supplementary Materials (Figures S1 and S2).



**Figure 2.** The HPLC-DAD chromatograms of magnoflorine in 210 and 290 nm.

Countercurrent chromatography, as a separation technique, has been used for the isolation of alkaloids in recent years. In other studies [36,38], the researchers concluded that the rotation speed and flow rate had no effect on the purity and recovery of the compounds; however, it positively affected the productivity of the separation process by facilitating reduced run durations without compromising separation efficiency. The majority of the described trials use hydrodynamic instruments, like high-speed countercurrent chromatographs (HSCCC), that are known to be efficient for analytical scale separations. In 2020, Correa et al. carried out successful purification trials on three lycorine-type alkaloids from *Rhodolirium speciosum* using this technique in the methyl-*tert*-butyl ether/acetonitrile/water (4:1:5, *v/v/v*) system in descending mode [25]. In a study conducted by Sun et al., the isolation of alkaloids from *Lycoris cordata* was achieved using the pH-zone-refining mode of operation on a conventional HSCCC apparatus using a biphasic solvent system consisting of petroleum-ether-ethyl acetate-*n*-butanol-water (1:9.5:0.5:10 *v/v/v/v*) with the addition of 10 mM triethylamine and 10 mM hydrochloric acid. The introduction of a retainer and eluter led to a 150-fold increase in the loading capacity and resulted in a successful purification of seven alkaloids. The results of this experiment clearly demonstrated that the use of CCC provides a quick and effective method for the separation and purification of natural products [39].

Scientific literature shows various examples of a successful purification of alkaloids using hydrodynamic instruments. However, there are still gaps to be filled when it comes to the application of hydrostatic instruments, like CPC, which are more useful for industrial-scale purification protocols. Thanks to the applied technique, these two isolated compounds could be directed to the studies on the inhibition of *Helicobacter pylori* infections.

### 3.3. *Helicobacter pylori* and Urease Inhibition

Minimal inhibitory and bactericidal concentrations were determined for tested compounds (see Table 5). Berberine has revealed good anti-*H. pylori* activity, whereas MGN was of moderate action against *H. pylori* growth. Interestingly, when studying the *H. pylori* urease inhibitory effect of these compounds, it occurred to us that MGN showed a stronger potential from berberine with an IC<sub>50</sub> value of 25.5 mg/L in comparison with 729.5 mg/L value calculated for berberine. In the same test, the potential of thiourea was tested for whom the calculated IC<sub>50</sub> value was 92.7 mg/L.

**Table 5.** Antibacterial activity of magnoflorine and berberine against reference strain of *H. pylori* ATCC 43504.

Compound	MIC (mg/L)	MBC (mg/L)	MBC/MIC	Urease Inhibition IC <sub>50</sub> (mg/L)
Magnoflorine	320	640	2	25.5
Berberine	32	32	1	729.5
Thiourea				92.7

Previously, berberine was recognized as a compound with activity against *Helicobacter pylori* [40]. Evidence from a clinical trial suggests that berberine combined with standard therapy can be an option to eradicate *H. pylori* [41,42]. On the other hand, the inhibition of urease is another parameter that can predict the potential behavior of a metabolite against *H. pylori*. It is an important enzyme that enables bacteria colonization and also survival. *H. pylori* generates ammonia to counteract the acidic environment of the stomach. Inhibition of this enzyme decreases the chances of bacterial survival. Hence, urease is considered to be a critical target in the research and exploitation of antibacterial agents. Li et al. noted that berberine inhibits urease activity and urease maturation by targeting the urease active site sulfhydryl group to elicit anti-*H. pylori* effects [43]. Thiourea and its derivatives have been proven to exhibit strong inhibitory potential against urease, e.g., in the studies of Rasheed and co-investigators [44]. The fact that MGN was proven to exhibit stronger action from thiourea makes it an interesting compound for further studies. The obtained results for MGN show its potential as a urease inhibitor. Even if MGN did not exhibit strong inhibitory potential against *H. pylori* itself, the proved urease inhibitory action is important and may contribute to a disturbed colonization of stomach by this bacterium. This observation indicates a potential application of MGN in supporting the eradication of *H. pylori*.

## 4. Conclusions

*Berberis vulgaris* shrub is known as a rich source of isoquinoline alkaloids. This plant matrix was selected to perform the optimization of the fractionation protocol for the recovery of pharmacologically important isoquinolines (magnoflorine and berberine) by centrifugal partition chromatography (CPC), which is a liquid–liquid separation technique. The manuscript shows the influence of biphasic solvent system composition, flow rate, and rotation speed on the fractionation of two methanolic extracts—from the stem and the root of the shrub. A successful purification of magnoflorine and berberine enabled further studies on their impact on *Helicobacter pylori*. The obtained results show that apart from the marked activity of berberine in this field, which was previously proved, magnoflorine was found to inhibit urease—the enzyme neutralizing the acidic pH of the stomach by the bacterium—much more strongly than berberine.

**Supplementary Materials:** The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/separations11040094/s1>, Figure S1. The HPLC-DAD chromatograms of the total extract from the roots (above) and the stems (middle) of *Berberis vulgaris* and the fraction enriched in berberine (below); Figure S2. UV spectra of magnoflorine (left) and berberine (right); Figure S3. The mass spectra of the authentic standards used for identification purposes.

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