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Novel Approaches for the Analysis and Isolation of Benzylisoquinoline Alkaloids in *Chelidonium majus*#

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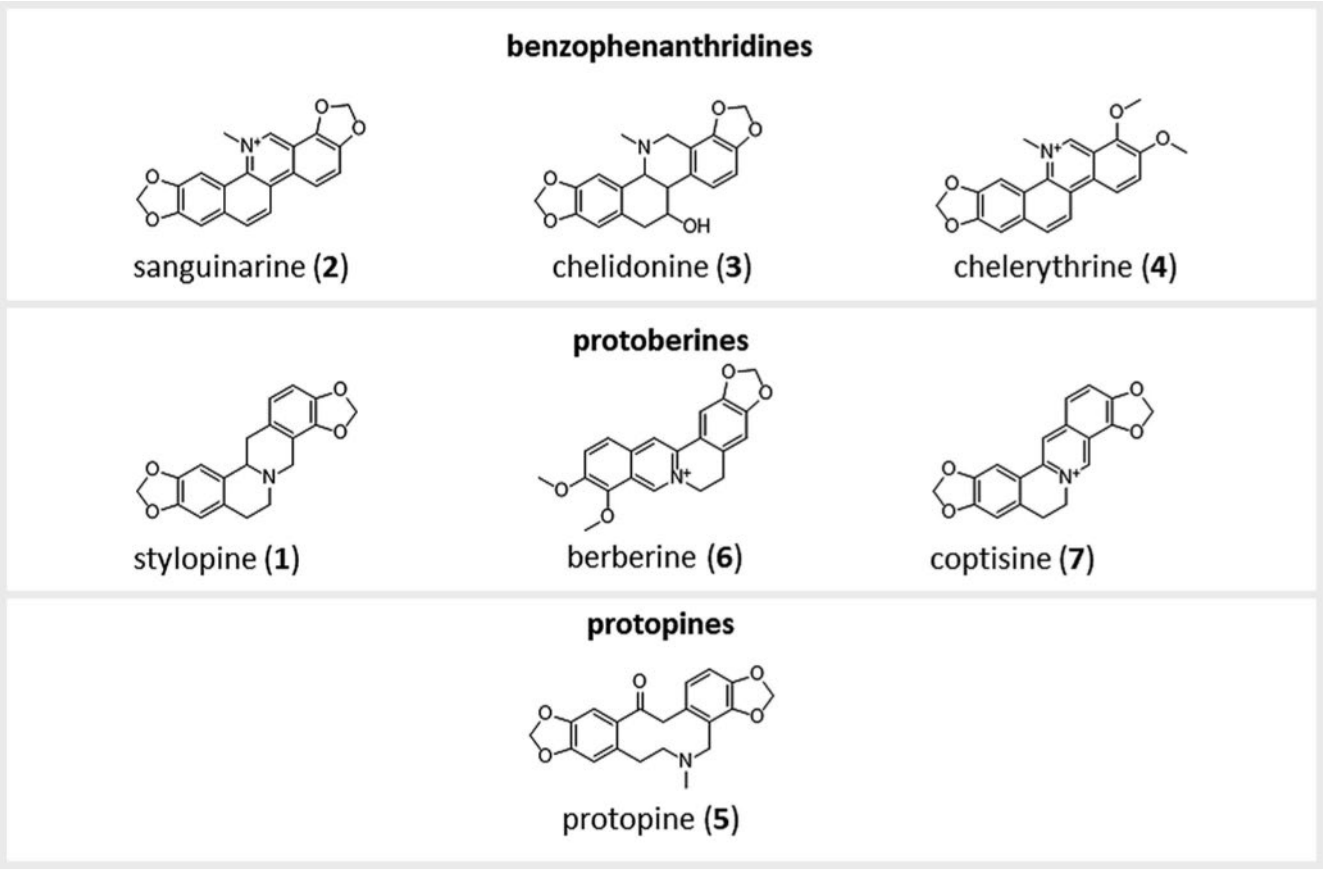
ABSTRACT

Benzylisoquinoline alkaloids are the major bioactive components in *Chelidonium majus*, a plant that has a long usage history for the treatment of gastrointestinal ailments in European and Asian phytomedicine. This study reports on the development and application of a supercritical fluid chromatography technique for the simultaneous qualitative and quantitative determination of seven benzylisoquinoline alkaloids in under six minutes using a Viridis BEH 2-EP column and a modifier comprising methanol with 30% acetonitrile and 20 mM ammonium formate. The method was fully validated according to ICH guidelines showing, e.g., excellent linearity (≥ 0.9997) and maximum deviations for intraday and interday precision of 2.99 and 2.76%, respectively. The new supercritical fluid chromatography assay was not only employed for the analysis of several *C. majus* samples but was also used for the subsequent development of a fast centrifugal partition chromatography technique, whereby five benzylisoquinoline alkaloids could be isolated within approximately 2.5 h, with only two of them, protopine and chelidonine, requiring an additional purification step. To achieve this, a solvent system composed of chloroform/methanol/0.3 M hydrochloric acid was used in descending mode. By injecting 500 mg of crude extract, stylopine (1.93 mg), sanguinarine (0.57 mg), chelidonine (1.29 mg), protopine (1.95 mg), and coptisine (7.13 mg) could be obtained. The purity of compounds was confirmed by supercritical fluid chromatography and MS.

Introduction

Chelidonium majus L., a perennial plant belonging to the Papaveraceae family, is native to Europe and Asia. Its aerial parts are used for the treatment of dyspeptic complaints, gastrointestinal spasms, and mild gall ailments, both in traditional Chinese as well as Western herbal medicine [1–3]. As a consequence, extracts of the plant are also found in commercial preparations against gastrointestinal disorders [4]. Several benzylisoquinoline alkaloids (BIAs) have been identified as the bioactive constituents in *C. majus*, belonging to three major groups: the protoberine group (stylopine, berberine, coptisine), benzophenanthridine group (sanguinarine, chelidonine, chelerythrine), and protopine group (pro-

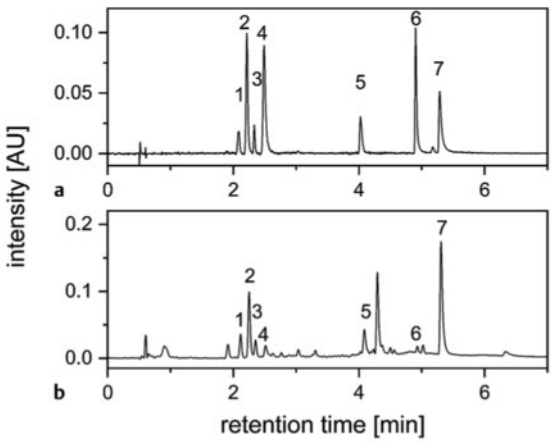
This work is dedicated to Professors Rudolf Bauer, Chlodwig Franz, Brigitte Kopp, and Hermann Stuppner for their invaluable contributions and commitment to Austrian Pharmacognosy.



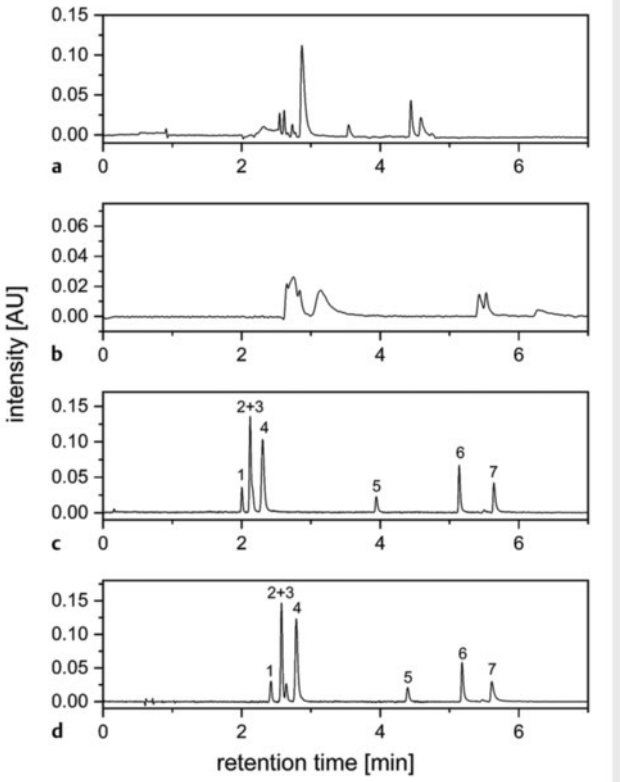
▶ **Fig. 1** Structure of investigated benzylisoquinoline alkaloids.

topine) [5, 6] (see ▶ **Fig. 1**). These compounds exhibit spasmolytic [7, 8], choleretic [5], anti-inflammatory [7, 9], antimicrobial [1, 10, 11], antiviral [12], and even *in vitro* cytotoxic and anti-migratory properties [13, 14]. Hepatotoxic effects have been reported for *Chelidonium* preparations; however, the results of individual clinical studies were divergent and the direct correlation between *C. majus* alkaloids and potential liver impairment remains unclear [1]. Currently, a daily intake of up to 2.5 mg of the total alkaloids is considered to be free of adverse effects [3]. Thus, the reliable identification and quantitation of BIAs are imperative for quality control, especially as the drug is monographed in the European Pharmacopoeia [15]. In the past, the analysis of BIAs, and especially those from *C. majus*, has been pursued from different angles, i.e., by TLC [5], CE [2], GC [16], and various HPLC- and UHPLC-based techniques, coupled either to a diode array detector MS as a detection device [5, 17–19]. However, most of the aforementioned methods require a tedious sample pretreatment, have limited sensitivity, or do not enable efficient separations in a short analysis time. A possible alternative would be supercritical fluid chromatography (SFC), an increasingly popular technique in the field of natural product analysis [20, 21]. It is considered an environmentally friendly "green technology" [20], with unique and orthogonal selectivity [22]. In SFC, the mobile phase mainly consists of supercritical carbon dioxide (CO_2), and the polarity as well as elution behavior can be adjusted with organic modifiers and various additives. In the supercritical state, beneficial properties of a gaseous and fluid mobile phase are unified, such as low viscosity and high solvation capacity [23]. SFC has already been employed for the analysis of BIAs [24, 25]; however, *C. majus* was never studied by this technique before.

The developed ultra-high performance SFC (UHPSFC) method showed to be helpful for two continuative investigations. On one hand, it was successfully used for the qualitative and quantitative analysis of several *Chelidonium* samples. On the other hand, it also supported the preparative part of the study, in which FCPC was used for the isolation of the main alkaloids in the plant. Fast centrifugal partition chromatography (FCPC) is a type of liquid-liquid chromatography that has shown to be very versatile and fast for the isolation and separation of natural products [26]. The reviews by Leitão et al. [27] and Fang et al. [28] indicate that this technique is well suitable for BIA isolation too, as, for example, evidenced by the isolation of coptisine and berberine from *Coptis chinensis* root by classical biphasic [29, 30], triphasic [31], and pH zone refining countercurrent chromatography [32, 33]. However, for *C. majus*, no respective method has been published. Furthermore, all of the aforementioned studies have utilized high-speed countercurrent chromatography (HSCCC) devices that feature a different rotor design than the here presented FCPC approach [34, 35].



▶ **Fig. 2** SFC separation of standard mix (each 62.5 µg/mL in MeOH, (**a**) and sample CM-4 (**b**) under optimized conditions. Column: Viridis BEH 2-EP column (3.0 × 100 mm; 1.7 µm); mobile phase: CO_2 (**a**) and 20 mM ammonium formate plus 30% acetonitrile in methanol (**b**); gradient elution: initial 2% B, in 1-minute steps to 3, 15, 20, 35, 40%, 2-min hold, 2-min re-equilibration; column temperature: 40 °C; ABPR: 1700 psi; flow rate: 1.2 mL/min; injection volume: 1 µL; detection at 280 nm.



▶ **Fig. 3** Influence of individual parameters on the SFC separation of standards **1**–**7** (62.5 µg/mL each) using a DEA column as the stationary phase (**a**), pure methanol as the modifier (**b**), a flow rate of 1.0 mL/min (**c**), and a different gradient profile from 2 to 40% in 5 min (**d**). All other settings were optimal (see ▶ **Fig. 2**). Detection at 280 nm.

Results and Discussion

Initially, seven stationary phases were screened for their ability to separate **1**–**7**. All had the same dimension (3 × 100 mm), UHPSFC-typical particle sizes (1.7 and 1.8 µm) and were purchased from Waters (Viridis: BEH, BEH 2-EP, HSS C18SB, CSH Fluoro-Phenyl; Torus: DIOL, DEA, 2-PIC; see **Table 1S**, Supporting Information). In order to protect the columns, a pre-filter (Waters column in-line filter) was used. Starting with a generic CO_2-methanol gradient, raising the modifier from 0 to 20% in 10 min, was already suggested due to the structure of the target compounds higher modifier concentrations as well as further additives being were required. For the optimized separation refer to ▶ **Fig. 2**.

Although in our previous UHPSFC investigations on alkaloids the DEA column was the optimum choice [36, 37], acceptable separations could not be obtained for the BIAs (▶ **Fig. 3a**). Here, the best result was achieved on a BEH 2-EP column. According to the LSER (linear solvation energy relationship) model of West et al. [38], this column also belongs to the group of polar stationary phases with polar ligands. Such columns have positive *e* and *s* terms, which indicate that they allow dipole-dipole interactions, positive but low *b* terms, which suggests potential hydrogen bonding with bases, and positive *d* terms implying interactions with ionizable species.

The next step was the optimization of the mobile phase. The polarity of supercritical CO_2 is comparable to *n*-hexane, therefore polarity adjustments with organic solvents are usually required in SFC. Methanol is the most commonly utilized modifier and also proved beneficial for this application (▶ **Fig. 3b**). All compounds eluted but they were not resolved. Thus, the addition of acetonitrile and isopropanol was evaluated. Best results were achieved when a certain percentage of acetonitrile, i.e., 30%, was added to methanol, as this modification especially improved the peak shape of coptisine. All analytes feature a basic nitrogen, thus also basic additives were evaluated. Whereas in our previous work [36, 37] the addition of NH_3 or DEA proved favorable, this was not the case for BIAs. In contrary, poor peak symmetries and coelutions were the result. Neither formic acid nor water had a positive impact either. However, by adding ammonium formate, the resolution enhanced. Different concentrations of this salt were evaluated, and 20 mM was finally select as the optimum. The exchange of this additive with ammonium acetate did not result in the same good results anymore.

When optimizing the gradient, it was observed that when setting the initial modifier concentration either too high or low (5/0%), coelution of **2** and **3** occurred. Also, omitting the stepwise increase and simply raising B from 2 to 40% in 5 min led to an overlap of **2** and **3** (▶ **Fig. 3c**). The same was observed when the gradient was too steep. Flatter gradients or ending at lower modifier concentrations (20% B) did not permit the baseline separation of all alkaloids either. Only the selected gradient could achieve this, with satisfactory peak shapes and within a short analysis time.

▶ **Table 1** Validation results for the SFC assay.

	1	2	3	4	5	6	7
Regression equation	y = 814.7 x + 1240.6	y = 3545.8 x + 7259.2	y = 673.77 x − 51.546	4543.9 x − 7505.3	y = 766.33 x − 2482.6	y = 1976.2 x − 10637	y = 1666.3 x − 10721
R^2	0.9998	0.9999	0.9998	0.9999	0.9998	0.9997	0.9997
LOD[a]	2.34	1.13	2.57	1.04	2.00	1.82	1.82
LOQ[a]	7.02	3.39	7.72	3.12	6.01	5.48	5.47
Precision							
intraday[b]	2.57	1.38	2.99	1.68	2.78	1.71	2.53
inter-day[c]	1.55	1.46	2.32	2.88	2.57	2.06	1.97
Accuracy[d]							
low spike	99.0 (1.43)	99.4 (0.87)	98.4 (1.53)	100.2 (0.79)	97.54 (0.97)	98.0 (1.25)	99.4 (0.97)
medium spike	103.2 (0.67)	98.5 (1.03)	102.6 (1.94)	101.3 (1.19)	103.0 (1.78)	101.0 (1.44)	99.3 (2.00)
high spike	100.2 (0.61)	99.74 (2.15)	97.4 (1.92)	101.2 (1.08)	97.42 (1.14)	99.3 (1.27)	98.6 (1.78)

[a] µg/mL, [b] maximum deviation within 1 day based on peak area in percent, [c] deviation over 3 days based on peak area in percent, [d] expressed as recovery rate in percent; n = 3, relative standard deviations in parentheses

Subsequently, the column temperature was optimized. Below 40 °C, the required analysis time was marginally shorter; however, coelution of **1** and **2** occurred and the peak symmetries were poor due to tailing. Above 40 °C, shorter retention times were observed and again peaks **2** and **3** could not be resolved, whilst for the other signals, the peak shape was acceptable. Therefore, the optimal temperature was 40 °C. Concerning flow rate and ABPR (Automatic Backpressure Regulator), these parameters were set to 1700 psi and 1.2 mL/min, which was a good compromise between rapid analyses and optimal resolution, as again, a coelution of **2** and **3** was also visible when the flow rate was reduced, e.g., to 1.0 mL/min (▶ **Fig. 3 d**).The injected volume was set to 1 µL and the detection wavelength to 280 nm, which agrees with the literature [5, 19].

Even if the optimal UHPSFC conditions were applied, compound **7** always showed a slight tailing effect. Nevertheless, this did not hamper the practical use of the method and was still a significant improvement in peak shape compared to chromatograms shown in the other two SFC papers with BIA context [24, 25]. For detailed figures on the evaluated parameters, see **Figs. 1S–9S**, Supporting Information

As summarized in ▶ **Table 1**, the developed assay met all ICH validation criteria. It exhibited excellent linearity with correlation coefficients always over 0.9997, covering a concentration range from 7.8 up to 10 000 µg/mL. In case of compounds **2** and **4**, the linear range even expanded to 3.9 µg/mL. Limit of detection (LOD) and limit of quantitation (LOQ) were determined by calculation and were always below 2.57 and 7.72 µg/mL, which are typical values for UHPSFC-DAD assays. In the case of the two aforementioned analytes, lower values could be achieved (e.g., LOD ≤ 1.13 µg/mL). Concerning precision, the maximum intraday and inter-day deviations were 2.99% (**3** on day 1) and 2.88% (**4**), respectively. In terms of recovery rates, good results were obtained as well, as accuracy of the assay was in the range of − 2.59% (**5**, high spike) to + 3.16% (**1**, medium spike). All examined peaks showed to be pure, with no signs of coelution.

To confirm the practical suitability of the UHPSFC method, ten *C. majus* samples (four commercially available and six collected ones) were analyzed. BIAs were assigned according to their retention time and UV spectra being congruent with those of standard compounds. It was observed that the qualitative profile of alkaloids in the samples was homogeneous, whereas their quantity was variable; respective results are summarized in ▶ **Table 2** (for a graphical overview, see also **Fig. 10S**, Supporting Information). All target analytes were present in all of the samples, and the main compound, in agreement with the literature [5], was always **7** (coptisine). The second most abundant benzylisoquinoline alkaloid was either **5** (protopine, samples CM-1 and CM-2) ranging from 0.68 to 2.51 mg/g, **3** (chelidonine, CM-3, CM-4, CM-C2, CM-C4) between 0.87 and 3.20 mg/g, or **1** (stylopine, CM-C1, CM-C5 and CM-C6). The alkaloid found in the lowest concentrations was always **4** (chelerythrine, concentrations 0.10–0.85 mg/g), except for sample CM-C4 (**6**, berberine, 0.34 mg/g). Interestingly, the latter revealed a significantly higher content of **1** (6.14 mg/g), which was twice as high as all the other specimens. As the samples were collected in the same year and season, the underlying reason for this deviance (soil, exposure to sunlight, etc.) can only be speculated. The total BIA content ranged from 9.02 to 21.04 mg/g of dry plant material, which seems considerably higher than the values defined in the European Pharmacopoeia (minimum requirement 6 mg/g of total BIAs) [15]. The compendial determination is based on a colorimetric assay, which tends to underestimate the actual content of alkaloids in the plant [5]. However, our results are in good agreement with those of previously published LC-DAD studies, which, e.g., reported coptisine contents ranging from 5.71 to 11.67 [5] and 2.97 to 8.13 mg/g [18] (current study: from 4.08 to 9.32 mg/g for both compounds).

▶ **Table 2** Quantitative evaluation of *C. majus* samples by SFC. All values expressed in mg/g of dry material, relative standard deviation (%) in parentheses.

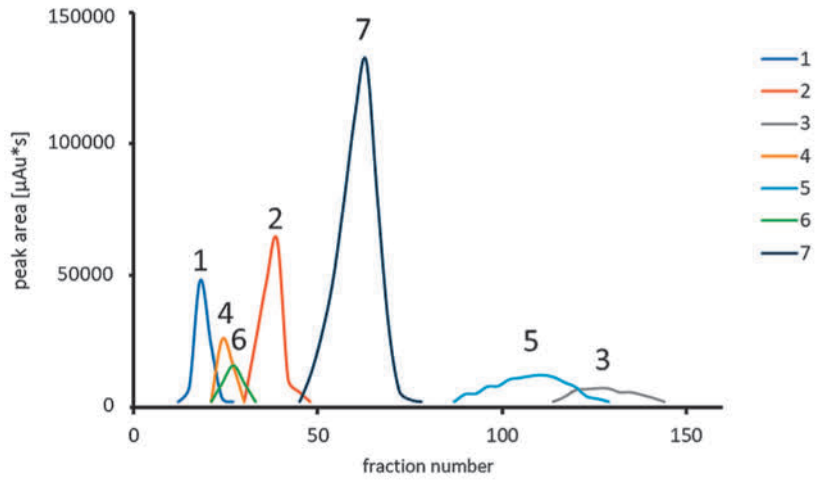
	Substance							
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Σ of BIAs**
CM-1	1.84 (0.64)	0.51 (0.45)	1.25 (1.09)	0.22 (0.54)	1.91 (0.47)	0.24 (0.84)	6.00 (0.91)	11.98
CM-2	2.43 (0.82)	0.25 (0.61)	0.86 (1.26)	0.16 (0.99)	2.51 (0.44)	0.38 (0.84)	4.08 (1.35)	10.68
CM-3	1.28 (0.59)	0.18 (1.51)	1.31 (1.76)	0.14 (1.48)	0.72 (1.48)	0.21 (1.09)	5.17 (0.98)	9.02
CM-4	0.39 (1.15)	0.34 (0.52)	3.20 (0.69)	0.21 (0.89)	2.42 (0.74)	0.25 (0.89)	4.88 (1.21)	11.68
CM-C1	1.01 (1.39)	0.73 (0.66)	0.87 (1.25)	0.17 (0.44)	0.79 (1.15)	0.70 (0.37)	8.83 (0.65)	13.10
CM-C2	1.35 (0.81)	0.82 (0.74)	1.84 (0.56)	0.13 (1.04)	1.04 (0.43)	0.62 (0.27)	5.03 (1.10)	14.63
CM-C3	0.44 (1.60)	1.26 (1.71)	1.22 (0.72)	0.09 (1.44)	0.69 (1.65)	0.53 (0.92)	7.80 (0.70)	12.02
CM-C4	6.14 (1.55)	0.61 (0.74)	2.87 (0.35)	0.85 (0.93)	0.91 (1.24)	0.34 (0.86)	9.32 (1.13)	21.04
CM-C5	1.41 (0.71)	0.50 (0.80)	1.18 (0.83)	0.10 (1.62)	1.30 (0.55)	0.41 (0.80)	5.22 (1.84)	10.12
CM-C6	1.77 (0.56)	1.60 (1.31)	1.46 (0.56)	0.12 (0.74)	0.68 (0.94)	0.54 (0.61)	8.05 (1.22)	14.22

▶ **Table 3** Selection of FCPC solvent systems. Partition coefficients (*K*) were calculated using the modified shake flask workflow as described in section "Determination of partition coefficients (*K*)".

System	**Ratio v/v**	**Target compounds and their *K* values**						
		1	**2**	**3**	**4**	**5**	**6**	**7**
Hexan : EtOAc : MeOH : H_2O	7 : 3 : 6 : 4	0.12	1.93	0.42	0	0.39	0.03	0.04
Hexan : EtOAc : MeOH : H_2O	3 : 7 : 4 : 6	0.29	64.87	28.86	0.24	7.25	0.15	0.87
EtOAc : Butanol : H_2O	4 : 6 : 10	0.5	∞	27.38	10.13	7.28	0.84	1.42
$CHCl_3$: MeOH : H_2O	10 : 3 : 7	5.18	○	0.01	○	0.02	4.06	○
MTBE : ACN : H_2O	4 : 1 : 5	0.22	∞	23.86	○	21.31	0.09	∞
Hexan : EtOAc : MeOH : H_2O + 1 % AA	1 : 1 : 1 : 1	2.92	○	0.15	○	○	○	14.11
$CHCl_3$: MeOH : 0.1 N HCl	7 : 3 : 4	0.50	1.30	3.14	1.03	3.46	1.10	3.23
$CHCl_3$: MeOH : 0.2 N HCl	7 : 3 : 4	0.31	0.92	2.18	0.76	2.42	0.76	1.47
$CHCl_3$: MeOH : 0.3 N HCl	*7 : 3 : 4*	*0.21*	*0.50*	*2.98*	*0.31*	*2.40*	*0.33*	*1.56*
DCM : MeOH : 0.3 N HCl	7 : 3 : 4	0.27	0.86	3.09	0.31	3.32	0.34	1.45

○ All analytes in the lower phase, ∞ all analytes in the upper phase; the selected system is in italics

In regard to the preparative part of this study, a precondition for a successful FCPC separation is the selection of a suitable biphasic solvent system (see ▶ **Table 3**). The selection is based on partition coefficients (*K*), which should be between 0.1 and 8 for one-step elution [39] and between 0.25 and 4 ("sweet spot") for optimal operation, i.e., separation in short run times [40]. To fully resolve two compounds, the separation factor α needs to be over 1.5 ($\alpha = K_2/K_1$). Naturally, also possible matrix effects have to be taken into account. To investigate the partition behavior of the target alkaloids, several classical systems defined in the G. U. E. S.S workflow [40], e.g., HEMWat (*n*-hexane/ethyl acetate/methanol/water), ChMWat (chloroform/methanol/water), EBuWat (ethyl acetate/1-butanol/water), or terAcWat (MTBE/acetonitrile/water), were evaluated. Besides, the review by Fang et al. [28] provides a good overview of the variety of solvent systems already applied for alkaloid purification by FCPC. Reported HEMWat systems with an acidic additive (*n*-hexane/ethyl acetate/methanol/1 % acetic acid solution in equal amounts) [30] did not enable the desired separation. Neither did a pH-zone refinement approach as described in [33]. More promising results were obtained with ChMWat systems, substituting water with diluted hydrochloric acid [29, 41]. Accordingly, both the amounts of the different solvents as well as the acid concentration were varied, with the outcome that a mixture of chloroform/methanol/0.3 M hydrochloric acid (7 : 3 : 4) was best suited (see ▶ **Table 3**). This system exhibited fast phase separation (11 s settling time) and *K* values in the acceptable or even sweet spot range.



▶ **Fig. 4** Reconstructed fractogram of the FCPC separation using a biphasic solvent system comprising chloroform : methanol:0.3 M hydrochloric acid in a ratio of 7 : 3 : 4 (descending mode). Other parameters are sample size: 500 mg crude extract; rotor size: 250 mL; rotation speed: 1300 rpm; flow rate: 6 mL/min; S_f: 70.0 %.

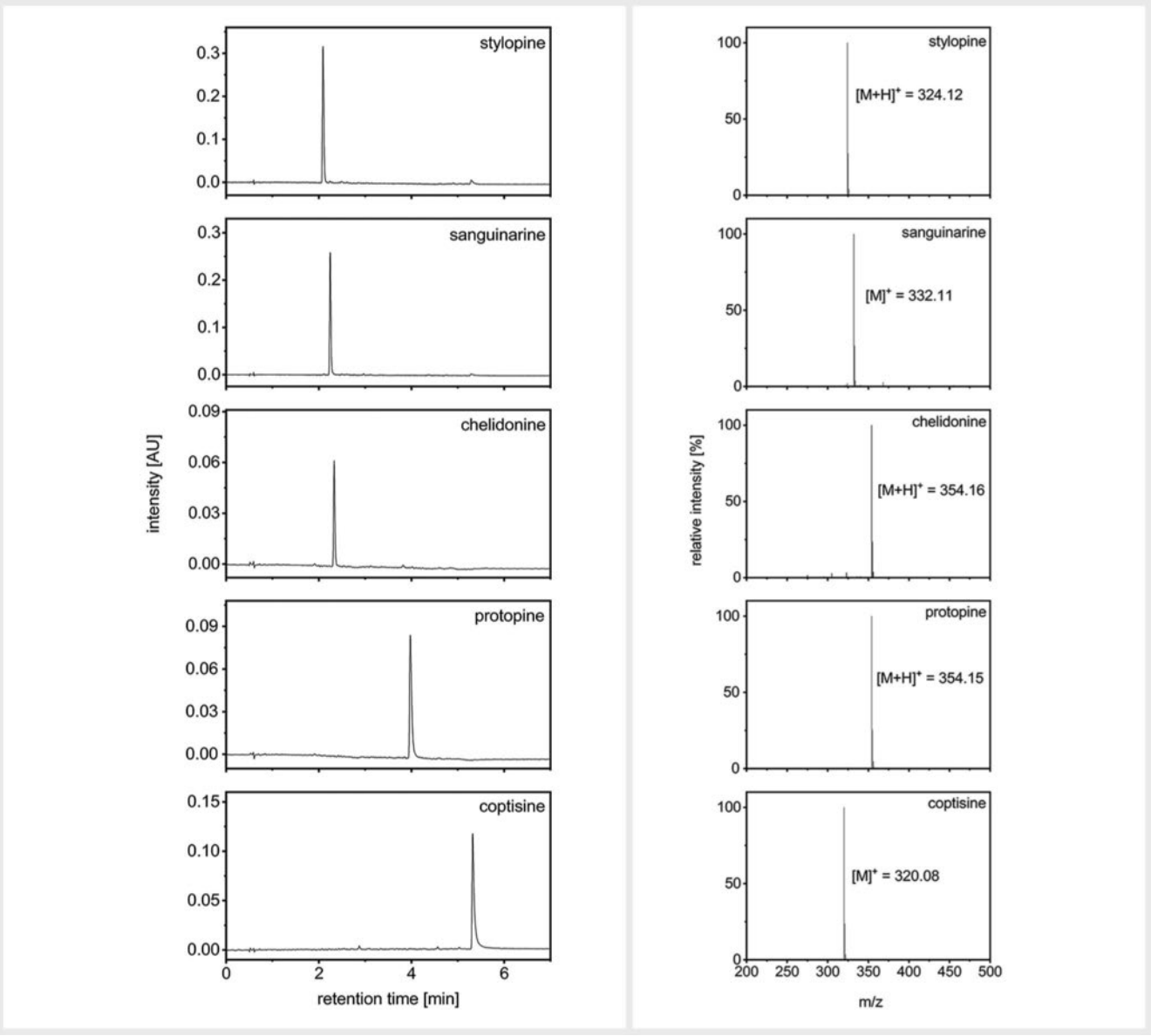
By increasing the hydrochloric acid concentration to 0.3 M (compared to 0.2 M in the literature [29]), the critical α-value for chelidonine/protopine could be increased from 1.10 to 1.24, yet it still remained below 1.5, required for baseline resolution. The use of basic additives was considered as well; both ammonia and diethylamine either prolonged the settling time or resulted in decreased solubility of the compounds. For toxicological and environmental reasons, a change from chloroform to dichloromethane was attempted, but chloroform was clearly advantageous in terms of separation efficiency. For an overview of all evaluated systems, please refer to **Table 3S**, Supporting Information.

In order to save solvents, the method was developed on an analytical scale device with 50 mL rotor. Here, a rotation speed of 900 rpm and a flow rate of 1.2 mL/min permitted a good stationary phase retention (S_f 61.5 %) with very low bleeding. Once optimized, the method was transferred to the preparative-size instrument with 250 mL rotor volume. Here, the flow rate was simply upscaled to 6 mL/min, whilst the rotation speed set could be further increased (1300 rpm). These changes resulted in a stable backpressure of 68 bar, an even better stationary phase retention (S_f of 70.0 %), and overall enhanced performance. With this instrument, 500 mg of crude *Chelidonium* extract could be separated in one run of just 150 min. The sample (CM-1) was prepared as stated in section “plant material and sample preparation”. After the separation was completed, extrusion started. Several dark colored fractions eluted, containing only matrix compounds but no alkaloids (confirmed by UHPSFC).

Selected fractions were evaporated and analyzed by UHPSFC. Based on the peak area of the individual alkaloids, a fractogram could be constructed in order to monitor the efficiency of the achieved separation (▶ **Fig. 4**). It indicated that the isolation of selected *Chelidonium* alkaloids, i.e., stylopine (**1**), sanguinarine (**2**), and coptisine (**7**), was possible, with good purity. However, as expected from preliminary testing, chelidonine (**3**) and protopine (**5**) partially coeluted and had to be subjected to further purification. One possible approach to do so might be 2D FCPC using the HEMWat-system (7 : 3 : 5 : 5) with an α of 1.53 as a second dimension. However, due to the relatively small amount of the respective fraction, it was decided to employ column chromatography for the final purification. Both alkaloids could readily be separated on a short silica column (10 × 150 mm) using acetone as the mobile phase (see **Fig. 11S**, Supporting Information). Besides the above-mentioned alkaloids, berberine (**6**) and chelerythrine (**4**) were also constituents of the extract; they were present in a low amount only, so their separation by FCPC was not attempted. The purity of all isolated compounds was assured by UHPSFC and HPLC-MS. UHPSFC-MS would have been an option too, but this technique was not available. Overall, from 500 mg crude extract, 1.93 mg stylopine (**1**) (purity 88.7 %, yield 69 %), 0.57 mg sanguinarine (**2**) (purity 92.6 %, yield 74 %), 1.29 mg chelidonine (**3**) (purity 94.4 %, yield 68 %), 1.95 mg protopine (**5**) (purity 95.27 %, yield 67 %), and 7.13 mg coptisine (**7**) (purity 92.2 %, yield 78 %) could be obtained. Purities determined by UHPSFC-DAD were always above 95 %; the stated values refer to LC-MS data (see also ▶ **Fig. 5**).

As the content of alkaloids in *C. majus* was low, enhancing the isolation yields by an enrichment step prior to FCPC separation seemed to be an interesting alternative. For BIAs, Yin et al. [42] have presented a respective protocol. By following it, the overall amount of alkaloids could be increased and the purity slightly enhanced; yet the yield of coptisine was approx. 30 % lower than expected. As coptisine is the main alkaloid in *Chelidonium*, this enrichment step was not considered further, even if it showed to be beneficiary for some of the other constituents.

To sum up the outcome of this study, an analytical UHPSFC assay as well as a preparative FCPC technique for the comprehensive investigation of BIAs in crude extracts of *C. majus* were developed in this study. The UHPSFC method not only permitted the reliable



▶ **Fig. 5** Confirmation of identity and purity of the isolated compounds based on SFC chromatograms and LC-MS spectra. SFC conditions were the same as in ▶ **Fig. 2**. LC conditions: Phenomenex Synergi Max-RP (3.0 × 150 mm; 4.0 µm) column; gradient: in 8 min from 0 to 16 % B, in 15 min to 20 % B, then changing to 95 % B and holding for 5 min. Column temperature: 50 °C; flow rate: 1.0 mL/min; injection volume: 5 µL. MS conditions: ESI positive; drying gas flow: 9.0 L/min; vaporizer pressure: 40 psi; drying gas temperature: 350 °C; capillary voltage: 4.5 kV.

and fast quantitation of seven bioactive compounds in a complex sample matrix, i.e., the crude plant extract, but also showed to be a helpful analytical tool when setting up the FCPC procedure and confirming the purity of the isolated alkaloids.

The separation of alkaloids in *C. majus* by UHPSFC has not been described before; yet the here presented approach has several advantages compared to previously reported UHPSFC assays for comparable analytes or HPLC/UHPLC methods for *Chelidonium*. Not only was the separation time considerably shorter (7 compared to at least 15 min [25]), it also showed enhanced separation efficiency, combined with a sample saving injection volume of 1 µL, the environmentally friendly "green" character of UHPSFC, and did not require a complex mobile phase setup (e.g., deep eutectic solvents as in [25]). Overall, all validation parameters were within the required limits and the quantitative results for real samples were in good agreement with the literature (9.02–21.04 mg/g compared to 8.90–17.01 mg/g in [18]). Concerning the already published UHPSFC techniques for BIAs, the LOD and LOQ were similar [24] or even lower [25] (coptisine, **7**). The respective values were also comparable to established HPLC assays but higher than those reported for UHPLC using a DAD. The possible hyphenation of UHPSFC to MS may drastically decrease LOD and LOQ values and also enable the annotation of further alkaloids. Regardless, the chosen detection mode, UHPSFC, is definitely an interesting alternative for this analytical problem, surpassing established protocols in some aspects and showing good practical applicability.

Although the overall BIA content in *C. majus* seems relatively low, by FCPC, it was possible to isolate five alkaloids (stylopine **1**, sanguinarine **2**, chelidonine **3**, protopine **5**, and coptisine **7**) with a satisfactory yield (≥ 67 %) and purity in unmatched speed. Only two compounds, chelidonine and protopine, required a second, but easy to perform, purification step. The separation was accomplished in just under 2.5 h without needing any specific sample pretreatment. A water-rich acidic phase that best exploits the basic character of the investigated BIAs was the optimum choice and such systems are quite common in countercurrent separations. However, they cannot be applied for standard isolation techniques like silica gel column chromatography as they would corrupt the solid stationary phase [35]. These aspects, together with the convenient scale-up options of FCPC, ease of operation, neglectable sample loss, and high loading capacity, serve as convincing indicators for the efficiency and practical benefits of FCPC, a technique that had never before been considered for the isolation of alkaloids from *C. majus*.

Materials and Methods

Chemicals and reagents

All organic solvents (methanol, acetonitrile), except chloroform, which was purchased from ChemLab, and the additives (formic acid, acetic acid, hydrochloric acid, ammonia, diethylamine) had HPLC quality and were acquired from VWR International. Ammonium formate was from Serva. HPLC grade water was prepared in house at the Department of Pharmacognosy by a Sartorius Arium purification system. The 4.5 grade carbon dioxide (purity ≥ 99.995 %) required for UHPSFC analyses was obtained from Messer.

Standards of the seven alkaloids (see ▶ **Fig. 1**) were acquired from different suppliers: stylopine (**1**), sanguinarine (**2**), chelerythrine (**4**), and coptisine (**7**) were from MedChemExpress (distributed via THP chemicals) and chelidonine (**3**), protopine (**5**), and berberine (**6**) from Sigma-Aldrich. They all had a purity of more than 98 %. The compounds were well soluble in methanol and the respective solutions stable for at least 4 weeks if stored at 4 °C (as confirmed by repeated UHPSFC analyses).

Plant material and sample preparation

The analyzed plant samples were either purchased (Alfred Galke; Kottas Pharma GmbH) or collected by the authors in Tyrol, Austria, 2023 (see **Table 2S**, Supporting Information for further details). The samples were morphologically authenticated by Stefan Schwaiger, air-dried, finely milled, and stored protected from light and moisture. Voucher specimens are available upon request.

For UHPSFC analysis, 200 mg of each sample were extracted four consecutive times with 2.5 mL of methanol/0.05 M HCl (8 : 2), each in an ultrasonic bath (Bandelin Sonorex) at ambient temperature for 15 min. This protocol was adapted from Zielinska et al. [43], who already evaluated the optimal extraction solvents for *Chelidonium* alkaloids; also, Gu et al. [18] used the same solvents. The extract was centrifuged (7 min, 1500 *g*), and the clear supernatants were combined and filled to volume with the extraction solvent in a 10-mL volumetric flask. An aliquot of 1 mL was taken, evaporated under an air stream, and redissolved in 500 µL of methanol. Before analysis, all sample solutions were filtered through a 0.45-µm syringe filter (PFTE; Macherey-Nagel). Efficiency of the protocol was confirmed by extracting one sample (CM-C3) a fifth time and analyzing this solution for possible remnants of BIAs. Respective levels were below 1 % of the initial concentration, therefore, the extraction was considered to be exhaustive.

For FCPC experiments, the same extraction protocol was used but up-scaled, i.e., using 3.0 g of pulverized drug and extracting it 4 times with 37.5 mL of methanol/0.05 M HCl (8 : 2). The final volumetric adjustment was omitted and instead the obtained liquid extract was evaporated and freeze-dried. Additionally, an alkaloid enrichment was performed as described by Yin et al. [42]. An aliquot of the dried crude extract (1.0 g) was redissolved in 30 mL water and the pH adjusted to 2.0 with conc. hydrochloric acid. After staying overnight, the solution was filtered, and the pH value changed to 10.0 with 1 M sodium hydroxide solution. This solution was partitioned with chloroform (3 × 50 mL), and the lower phase was evaporated under reduced pressure and lyophilized again.

Ultra-high performance supercritical fluid chromatography instrumentation

For all UHPSFC experiments, an Acquity UPC^2 system from Waters was used, comprising a binary solvent manager, a column oven, convergence and sample manager as well as a PDA detector. The operating software was Empower 3, release 2 (Waters).

An optimal separation of the seven BIAs from *C. majus* was achieved on a Viridis BEH-2EP column (3.0 × 100 mm; 1.7 µm particle size) from Waters. The mobile phase was CO_2 (A) and 30 % acetonitrile plus 20 mM ammonium formate in methanol (B). Elution was conducted at a flow rate of 1.20 mL/min in gradient mode, starting with 2 % B, and increasing in one-minute steps each to 3, 15, 20, 35, and finally 40 % B. This composition was kept for 2 min (total run time 7 min). Each run was followed by a 2-min re-equilibration step with the initial solvent composition. Column temperature, ABPR, and injection volume were adjusted to 40 °C, 1700 psi, and 1 µL, respectively. The detection wavelength was set to 280 nm, which is in agreement with the literature [5, 19]. The optimal UHPSFC separation of standards and a typical sample solution is shown in ▶ **Fig. 2**.

Method validation

As the suitability of an analytical assay has to be confirmed by validation, the respective ICH guideline [44] was followed. In order to determine linearity and establish calibration curves, a stock solution of all standard compounds was generated by dissolving 2.0 mg of **1**–**7** in 2.0 mL methanol using a volumetric flask. This solution (1.0 mg/mL) was then serially diluted 1 : 1 with methanol. The assessment of the regression parameters was done in Microsoft Excel 2019. LOD and LOQ were calculated, whereby the former was expressed as 3.3 times the standard deviation of the y-intercept divided by slope; the LOQ corresponded to three times this value. Peak purity (selectivity) was indicated by a symmetric peak shape and the peak purity option in the software; none indicated any co-elutions. Precision was evaluated both intra- and in-

ter-day. On each of 3 consecutive days, five individual solutions of sample CM-C3 were prepared as described in section "Plant material and sample preparation" and subsequently analyzed. Intraday precision was determined by comparing the results of the same day. For inter-day, those of all 3 days were evaluated. A series of spiking experiments was performed to establish the methods accuracy. Three concentration levels of **1–7** were selected and spiked individually to the dry sample CM-C3 prior to extraction: low spike (+ 20 µg/mL), medium spike (+ 40 µg/mL), and high spike (+ 80 µg/mL). By comparing the theoretically calculated and practically determined analyte concentrations, the percentual recovery rates could be estimated. All measurements were done in triplicate.

Determination of partition coefficients (*K*)

K values are important parameters in FCPC method development. They were determined using the shake-flask method, whereby 600 µL each of the upper and lower phase of a pre-equilibrated two-phase system were added to approximately 20 mg of crude extract (sample CM-1) in an HPLC vial. After vigorously vortexing for 2 min and phase separation, an aliquot of 300 µL of each phase was removed and dried under a stream of air. The residues were dissolved in 300 µL methanol and analyzed by UHPSFC. *K* values reflect the ratio of respective alkaloid peak areas in the upper and lower phase, as determined at 280 nm. For a one-step elution, the range should be from 0.1 to 8, and best results are usually obtained in the "sweet spot" range from 0.25 to 4 [40].

Fast centrifugal partition chromatography experiments

For the initial screening, an analytical FCPC instrument from Kromaton with 50 mL rotor was used. The sample loop size was 5 mL, rotation speed adjustable up to 3000 rpm (900 rpm was selected), and the solvent delivered by a Merck-Hitachi L-7100 pump (flow rate 1.25 mL/min). All final preparative experiments were performed on a Gilson CPC 250 device with a rotor capacity of 250 mL. Rotation speed was set to 1300 rpm. The rotor module was coupled to a Gilson-PLC unit consisting of a quaternary pump (flow rate 6 mL/min), 10-mL sample loop, and a UV detector (280 nm); fractions were collected in 1-min intervals. Every third tube was additionally analyzed by UHPSFC, i.e., the solvent was evaporated and the residue redissolved in methanol prior to analysis.

The best results were obtained with a biphasic system comprising chloroform, methanol, and 0.3 M hydrochloric acid in a ratio of 7 : 3 : 4. All solvents were mixed in a separatory funnel by vigorously shaking for several minutes. After complete partition, the upper and lower phases were separated and degassed by sonication right before FCPC use. The upper aqueous phase was used as stationary, whereas the lower organic phase served as the mobile phase (descending mode). Prior to injection, the system was filled with the stationary phase and equilibrated with the mobile phase at the selected rotation speed and flow rate. Stationary phase retention volumes (S_f) were monitored by measuring the displaced stationary phase volume during equilibration.

The sample (500 mg of crude CM-1 extract for the 250 mL rotor size instrument) was dissolved in a 1 : 1 mixture of the upper and lower phase (2 mL each) by sonication for 5 min and then centrifuged (5 min, 1500 *g*). As a residue formed in the phase interface, the upper and lower phases were removed, and the solid was extracted once more with each 1 mL of both phases. This avoided possible losses of target compounds during extraction (confirmed by UHPSFC analysis). The sample solution (final volume 6 mL) was then injected via a sample loop into the equilibrated FCPC system.

HPLC-MS analysis

HPLC-MS analyses were performed on an Agilent 1260 Infinity II instrument, hyphenated to a Bruker amaZon iontrap-MS. Separation conditions were as follows: the mobile phase was composed of water with 0.2 % acetic acid (A) and acetonitrile (B). A Phenomenex Synergi Max-RP (3.0 × 150 mm; 4.0 µm particle size) column was used as the stationary phase, employing the following gradient: in 8 min from 0 to 16 % B, in 15 min to 20 % B, then changing to 95 % B, and holding this composition for 5 min. Column temperature, flow rate, and injection volume were 50 °C, 1.0 mL/min, and 5 µL, respectively. ESI was used in the positive ionization mode, with the drying gas flow being 9.0 L/min, vaporizer pressure 40 psi, drying gas temperature 350 °C, and a capillary voltage of 4.5 kV. This method was developed in-house but not validated, as it served for monitoring purposes only.

Supporting information

A detailed representation of the impact of individual parameters in the UHPSFC method development, a graphic overview of the quantitation results, and optimal TLC conditions for the separation of chelidonine and protopine as well as tables summarizing the evaluated stationary phases, the investigated plant samples, and tested FCPC systems are available as Supporting Information.

Contributors' Statement

M. G. and M. Z. designed the approach, M. Z. guided L. B. and J. M. and prepared the first draft of the manuscript. L. B. conducted the UHPSFC experiments, J. M. the FCPC experiments. S. S. advised the FCPC development and identified the collected plant material. M. G. supervised M. Z., supported his work and finalized the paper. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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