Published online: 2020-03-18

Core Imprinting: An Alternative and Economic Approach for Depleting Pyrrolizidine Alkaloids in Herbal Extracts

OPEN ACCESS

Authors
Thomas Kopp[1, 2], Mona Abdel-Tawab[1], Boris Mizaikoff[2]

Affiliations
1 Central Laboratory of German Pharmacists, Eschborn, Germany
2 Institute of Analytical and Bioanalytical Chemistry, Ulm University, Ulm, Germany

Key words
Pyrrolizidine alkaloids, core imprinting, *Mentha piperita*, *Matricaria chamomilla*, *Chelidonium majus*, *Glycyrrhiza glabra*

received 13.10.2019
revised 17.02.2020
accepted 18.02.2020

Bibliography
DOI https://doi.org/10.1055/a-1121-4868
Planta Med Int Open 2020; 7: e26–e33
© Georg Thieme Verlag KG Stuttgart · New York
ISSN 2509-9264

Correspondence
Thomas Kopp MSc
Central Laboratory of German Pharmacists
Carl-Mannich-Straße 20
65760 Eschborn
Germany
Tel.: + 49 6196 937 958, Fax: + 49 6196 937 810
t.kopp@zentrallabor.com

Supporting Information for this article is available online at: http://www.thieme-connect.de/products.

ABSTRACT

Due to the high toxicity of pyrrolizidine alkaloids, in 2011, the German Federal Institute of Risk Assessment recommended that their daily intake limit should be no more than 0.007 µg/kg body weight. The risk of ingesting these substances in herbal preparations, either from their inherent presence in plants or through contamination with pyrrolizidine alkaloid-containing weeds, should not be underestimated. A promising molecular imprinted polymer was developed previously to minimise exposure to these compounds. Due to the high costs of the template and the risk of template bleeding, an alternative and more economic pyrrolizidine alkaloid depleting strategy is still required. Core imprinting, which focuses on the most important structural element in the target molecule, was investigated using triethylamine and tetraethylammonium as easily available and cheap alternative templates. The suitability of core imprinting was demonstrated using a pyrrolizidine alkaloid standard solution if an excess of an alternative template compared to monocrotaline was used for imprinting. Matrix trials in pyrrolizidine alkaloid-spiked *Mentha piperita*, *Chelidonium majus*, *Glycyrrhiza glabra*, and *Matricaria chamomilla* extracts containing *Echium vulgare* revealed better pyrrolizidine alkaloid binding than demonstrated for the original molecular imprinted polymer. Echimidine and echimidine-N-oxide were depleted in the range of 31.8–70.0 and 26.1–45.1 %, respectively. However, solvent-dependent differences in pyrrolizidine alkaloid binding and inherent plant analytical marker compounds were observed. Hence, binding of analytical marker compounds was better minimised in methanolic than in ethanolic extracts. The present study reveals core imprinting to be an economic alternative approach for depleting pyrrolizidine alkaloids in plant extracts.

ABBREVIATIONS

BfR	Federal Institute of Risk Assessment
CI	core imprinting
MIP	molecular imprinted polymer
NIP	non-imprinted polymer
PA	pyrrolizidine alkaloid
PAN	pyrrolizidine alkaloid-n-oxide
TEA	triethylamine

Introduction

PAs are secondary plant constituents with a sporadic taxonomic occurrence in plants of the families Asteraceae, Boraginaceae, and Fabaceae. They are believed to function as protection against herbivores and other predators [1, 2]. More than 500 different PAs have been identified and are distributed in a wide variety of more than 6000 plant species [2]. Recently, PAs and their corresponding N-oxides have become a controversial issue due to their hepatotoxic, pneumotoxic, genotoxic, and cytotoxic properties [1, 3–6].

Because of the high toxicity of these substances, in 2011, the BfR in Germany introduced a daily recommended intake of no more than 0.007 µg/kg body weight [2]. Plant extracts used for pharmaceutical purposes may be affected by PAs in two ways. On the one hand, extracts may contain inherently produced PAs, or they become contaminated with PA-containing weeds due to inattentive harvesting. MIPs may provide a solution to this problem, as they can be used to deplete PAs without affecting the analytical marker compounds in herbal extracts. MIPs are already widely implemented in science for specific purposes, such as in the processing of human or veterinary samples, where they are used to detect different pharmaceutical ingredients in serum, plasma, and urine [7, 8], or in an environmental sample, where they are used to determine traces of triazines [9–11]. MIPs are used in food analysis, where they have become increasingly important, for example, in the extracting and analysing of triazines and ochratoxine in fruits, corn, juice, wine, and wheat [10–13]. With all of this in mind, in a previous study, a monocrotaline-based MIP was developed to minimise the PA content in plant extracts. We have shown that PAs can be depleted in a matrix contaminated with echimidine and echimidine-N-oxide from natural sources without affecting the analytical marker compound [14]. The applied template was useful for creating selective cavities, which are responsible for binding of the target molecule.

However, monocrotaline is very expensive, and due to possible template bleeding, an alternative approach for molecular imprinting was developed. Based on the observed extended binding of further alkaloids in *Chelidonium majus* L. (Papaveraceae), it was assumed that binding mainly occurs at the nitrogen centre. Therefore, CI was investigated as an alternative approach for producing MIPs in an economic way. Suitability was tested, in the first step, in a methanolic standard solution containing different PAs and their corresponding N-oxides. In the second step, matrix trials were carried out on *Mentha piperita* L. (Lamiaceae), C*helidonium majus* L. (Papaveraceae), *Glycyrrhiza glabra* L. (Fabaceae), and *Matricaria chamomilla* L. (Asteraceae). For each plant matrix, both methanolic and ethanolic extracts were prepared to test for possible solvent-dependent binding. Furthermore, the analytical marker compounds of the three individual extracts were determined to verify the selectivity of the CI.

Results

First, the suitability of the imprinted polymers was tested in a methanolic PA standard mix containing 26 pyrrolizidine alkaloids in the form of a mixture of free bases and their N-oxides (PANs), with alkaloid present in a concentration of 500 ng/mL, corresponding to a total amount of 13 µg/mL of PAs. In these experiments, the monocrotaline imprinted polymer developed earlier revealed an absolute PA binding capacity of 8829.6 ng/20 mg with a selective binding capacity of 145.5 ng/20 mg, where the selective binding capacity is calculated by subtracting the absolute binding capacity of the NIP from that of the imprinted one. In the initial approach in creating selective cavities via alternative imprinting, triethylamine was used in a two- (TEA2) and five- (TEA5) molar excess compared to the monocrotaline template. However, comparing TEA2 with the NIP resulted in a negative balance (− 207.5 ng/20 mg), indicating that some selective cavities were missing, whereas an increased selective binding was observed for TEA5 (213.1 ng/20 mg). In order to increase the selectivity, further polymers were developed by adding a two-fold excess of tetraethylammonium to TEA2 and a four-fold excess to TEA5, resulting in the formation of TTEA2/2 and TTEA5/4, respectively. Again, the lowest excess of template (TTEA2/2) resulted in only a moderate increase in the absolute binding capacity compared to that of NIP and still showed a negative balance for selective binding (− 147.1 ng/20 mg). However, the selectivity was substantially improved to 314.9 ng/20 mg using larger amounts of triethylamine and tetraethylammonium (TTEA5/4). This indicates that applying greater amounts of alternative templates may lead to better selectivity being achieved in the binding of PAs, even if the absolute binding capacities were not found to differ significantly between the MIP and alternative templates (▶ **Table 1**). The binding and selective binding values of each PA in the standard mix can be found in (▶ **Table 1S** and **2S**), Supporting Information.

To verify the suitability of the developed polymers for the depletion of PAs in the plant matrix, eight different plant extracts were prepared from *M. piperita*, *G. glabra*, *C. majus*, and *M. chamomilla* using both methanol and ethanol as extraction solvents. To simulate PA contamination, each plant matrix was spiked with the same volume of a PA-containing extract taken from *E. vulgare*. The absolute PA binding capacities for the different polymers are summarised in ▶ **Table 1**.

Based on the previously increased selectivity observed for the polymers imprinted with higher amounts of the alternative templates trimethylamine and tetraethylammonium, matrix trials were carried out using TEA5 (following TEA) and TTEA 4/5 (following TTEA). In the spiked methanolic extract of *M. piperita,* the amount of echimidine was determined to be 22.4 ng/mL and that of echimi-

▶ **Table 1** Overview of the absolute PA binding capacities observed for the monocrotaline (MIP), trimethylamine (TEA), mixed mode trimethylamine tetraethylammonium (TTEA), and non-imprinted polymers (NIP). The number after the abbreviation reflects the excess amount of template used, i.e., TEA2 shows twice the molar excess of triethylamine compared to that of monocrotaline, and TTEA2/2 shows twice the molar excess of triethylamine and the same molar excess of tetraethylammonium compared to the monocrotaline template. The selective binding capacity was calculated by subtracting the value of the absolute binding of NIP from the absolute values of the respective imprinted polymers.

	NIP [ng/20 mg]	MIP [ng/20 mg]	TEA2 [ng/20 mg]	TEA5 [ng/20 mg]	TTEA2/2 [ng/20 mg]	TTEA5/4 [ng/20 mg]
Sum	8684.1 ± 93.9	8829.6 ± 87.6	8476.6 ± 96.8	8897.2 ± 80.6	8537.0 ± 64.3	8999.0 ± 110.1
Free bases	4640.4 ± 64.0	4703.7 ± 60.5	4483.9 ± 61.0	4697.0 ± 60.6	4512.9 ± 50.1	4743.1 ± 71.6
N-oxides	4043.7 ± 69.0	4125.9 ± 62.7	3992.7 ± 77.2	4200.1 ± 55.1	4024.1 ± 39.6	4256.0 ± 83.8
Selectivity	–	145.5 ± 128.4	–	213.1 ± 123.7	–	314.9 ± 144.7

dine-N-oxide was 246.0 ng/mL. Applying an imprinted polymer, the absolute binding capacity for echimidine and echimidine-N-oxide ranged from 6.8 ng/20 mg (MIP) to 10.8 ng/20 mg (TTEA) and from 64.2 ng/20 mg (MIP) to 81.5 ng/20 mg (TEA), respectively, corresponding to increased binding values of 58.8 and 26.9 % for echimidine and echimidine-N-oxide, respectively, compared to the absolute binding of the monocrotaline imprinted polymer (▶ **Table 2**). In line with this, the selective binding capacity of echimidine was significantly increased from 0.5 ng (MIP) to 4.5 ng/20 mg using TTEA and for echimidine-N-oxide, from 9.6 ng/20 mg (MIP) to 26.9 ng/20 mg using TEA, corresponding to 41.6 and 33.0 % of the total bound amounts of free base and N-oxide, respectively (▶ **Tables 3** and ▶ **4**). Regarding the initial echimidine and echimidine-N-oxide content spiked into the extract of *M. piperita*, the PAs were found to be successfully depleted by up to 48.2 and 33.1 %, respectively, using CI (▶ **Table 4**).

The determination of PAs spiked into the ethanolic extract of *M. piperita* resulted in a content of 25.5 ng/mL for echimidine and 286.3 ng/mL for echimidine-N-oxide, with absolute binding capacities ranging from 6.1 ng/20 mg (MIP) to 8.1 ng/20 mg (TTEA) for echimidine and between 68.4 ng/20 mg (TEA) and 74.7 ng/20 mg (MIP) for its N-oxide (▶ **Table 2**). Comparing the absolute binding of echimidine with that of the conventional MIP, an increased binding of 32.7 % was achieved. In the case of echimidine-N-oxide, the depletion of the PA using the alternative imprinted polymer was comparable to that with the monocrotaline imprinted ones. However, for the selective binding of echimidine, an increase from 4.4 ng (MIP) to 4.8 ng/20 mg (TTEA) was observed, making up 59.3 % of the total binding capacity of TTEA (▶ **Tables 3** and ▶ **4**). Comparing the absolute binding with the initial spiked concentration, it was found that the amount of echimidine was depleted by up to 31.8 % and that of N-oxide by up to 26.1 % (▶ **Table 4**).

Because of the suppressing effects, which result from the accompanying substances in the spiked methanolic extract of *C. majus*, a content of only 2.0 ng/mL was determined for the echimidine free base and 97.9 ng/mL for its corresponding N-oxide. Nevertheless, the absolute binding capacity for echimidine increased nearly three-fold from 0.5 ng/20 mg (MIP) to 1.4 ng/20 mg

▶ **Table 2** Overview of the absolute binding capacities of echimidine and echimidine-N-oxide determined in methanolic and ethanolic *M. chamomilla*, *G. glabra*, *C. majus*, and *M. piperita* extracts using different polymers (NIP, non-imprinted polymer; MIP, monocrotaline imprinted polymer; TEA, triethylamine imprinted polymer with a five-fold excess of template; TTEA, combined imprinted polymer with a five-fold excess of triethylamine and a four-fold excess of tetraethylammonium).

	M. piperita methanolic		***C. majus methanolic***		***G. glabra methanolic***		***M. chamomilla methanolic***	
	Free base [ng/20 mg]	**N-oxide [ng/20 mg]**	**Free base [ng/20 mg]**	**N-oxide [ng/20 mg]**	**Free base [ng/20 mg]**	**N-oxide [ng/20 mg]**	**Free base [ng/20 mg]**	**N-oxide [ng/20 mg]**
NIP	6.3 ± 1.7	54.6 ± 19.0	0.6 ± 0.2	35.3 ± 3.3	4.0 ± 2.6	51.1 ± 22.9	5.0 ± 1.9	55.9 ± 10.1
MIP	6.8 ± 1.6	64.2 ± 20.4	0.5 ± 0.2	36.2 ± 3.2	5.8 ± 1.9	58.3 ± 20.3	6.7 ± 2.4	65.9 ± 17.0
TEA	9.4 ± 0.8	81.5 ± 10.7	1.1 ± 0.2	39.4 ± 2.6	7.5 ± 2.0	77.4 ± 19.7	7.6 ± 1.9	69.7 ± 11.4
TTEA	10.8 ± 0.9	73.8 ± 15.0	1.4 ± 0.2	41.0 ± 4.2	9.7 ± 1.9	90.6 ± 19.1	8.1 ± 1.9	80.0 ± 11.4
	M. piperita ethanolic		***C. majus ethanolic***		***G. glabra ethanolic***		***M. chamomilla ethanolic***	
	Free base [ng/20 mg]	**N-oxide [ng/20 mg]**	**Free base [ng/20 mg]**	**N-oxide [ng/20 mg]**	**Free base [ng/20 mg]**	**N-oxide [ng/20 mg]**	**Free base [ng/20 mg]**	**N-oxide [ng/20 mg]**
NIP	3.2 ± 0.6	44.9 ± 4.9	0.4 ± 0.2	34.2 ± 2.7	7.0 ± 0.9	73.6 ± 8.3	6.9 ± 1.1	56.3 ± 11.3
MIP	6.1 ± 0.6	74.7 ± 11.3	0.5 ± 0.2	38.7 ± 2.4	9.7 ± 1.4	94.6 ± 9.9	10.1 ± 0.8	77.5 ± 8.9
TEA	7.1 ± 0.6	68.4 ± 13.4	1.0 ± 0.2	45.1 ± 3.3	9.9 ± 2.6	93.8 ± 16.6	12.1 ± 1.4	101.1 ± 12.1
TTEA	8.1 ± 0.8	73.6 ± 10.0	0.9 ± 0.2	44.4 ± 3.3	10.9 ± 1.3	97.6 ± 16.0	10.6 ± 0.5	92.2 ± 7.1

▶ **Table 3** Overview of the selective binding capacities of echimidine and echimidine-N-oxide in methanolic and ethanolic solutions expressed as ng per 20.0 mg of polymer material. MIP refers to the monocrotaline imprinted polymer that was used as a reference. The best imprinted polymer represents the polymer that yielded the best results, which was either TEA (five-fold excess of triethylamine for imprinting) or TTEA (five-fold excess of triethylamine and four-fold excess of tetraethylammonium for imprinting).

Free base *methanolic*	**MIP [ng]**	**Best [ng]**		**N-oxide *methanolic***	**MIP [ng]**	**Best [ng]**	
M. piperita	0.5 ± 2.3	4.5 ± 1.9	TTEA	*M. piperita*	9.6 ± 27.9	26.9 ± 21.8	TEA
C. majus	0.0 ± 0.3	0.9 ± 0.2	TTEA	*C. majus*	0.9 ± 4.6	5.7 ± 5.3	TTEA
G. glabra	1.7 ± 3.3	5.7 ± 3.2	TTEA	*G. glabra*	7.2 ± 30.6	39.5 ± 29.9	TTEA
M. chamomilla	1.7 ± 3.0	3.0 ± 2.7	TTEA	*M. chamomilla*	10.0 ± 19.8	24.1 ± 15.3	TTEA
Free base *ethanolic*	**MIP [ng]**	**Best [ng]**		**N-oxide *ethanolic***	**MIP [ng]**	**Best [ng]**	
M. piperita	4.4 ± 0.8	4.8 ± 1.0	TTEA	*M. piperita*	29.9 ± 12.4	28.7 ± 11.2	TTEA
C. majus	0.1 ± 0.3	0.5 ± 0.3	TTEA	*C. majus*	4.5 ± 3.7	10.9 ± 4.3	TEA
G. glabra	2.7 ± 1.7	3.9 ± 1.6	TTEA	*G. glabra*	21.1 ± 12.9	24.0 ± 18.0	TTEA
M. chamomilla	3.2 ± 1.3	5.2 ± 1.7	TEA	*M. chamomilla*	21.2 ± 14.4	44.8 ± 16.6	TEA

(TTEA) and that of echimidine-N-oxide increased by 13.3 % from 36.2 ng/20 mg (MIP) to 41.0 ng/20 mg (TTEA) (▶ **Table 2**). Regarding the selective binding value of the free base, this was in the range of 0.0 ng/20 mg (MIP) to 0.9 ng/20 mg (TTEA), indicating that 64.3 % of the echimidine free base is bound due to selective interactions when using TTEA. The selective binding of the N-oxide increased from 0.9 ng/20 mg (MIP) to 5.7 ng/20 mg (TTEA), making up 13.9 % of the total binding capacity, which may be attributed to selective cavities (▶ **Tables 3** and ▶ **4**). In terms of the initial spiked PA concentration, echimidine was depleted in a methanolic solution of *C. majus* by 70.0 % and its N-oxide by 41.9 % when the mixed mode polymer TTEA was applied (▶ **Table 4**).

As in the case of the spiked methanolic extract, low amounts of echimidine (2.2 ng/mL) and echimidine-N-oxide (109.6 ng/mL) were also determined in the spiked ethanolic extract of *C. majus* due to the suppressing effects of the matrix. Nevertheless, the absolute binding capacity of echimidine was enhanced two-fold from 0.5 ng/20 mg (MIP) to 1.0 ng/20 mg (TEA) and that of the N-oxide by 31.9 % from 34.2 ng/20 mg (MIP) to 45.1 ng/20 mg (TTEA) (▶ **Table 2**). At the same time, the selective binding of echimidine was increased from 0.1 (MIP) to 0.5 ng/20 mg (TTEA) and from 4.5 ng (MIP) to 10.9 ng/20 mg (TEA) for echimidine-N-oxide, corresponding to a selective share of 60.0 % for the free base and 24.2 % of the N-oxide, which can be attributed to selective binding cavities (▶ **Tables 3** and ▶ **4**). In summary, echimidine was depleted by up to 45.5 % and echimidine-N-oxide up to 45.1 % with respect to the initial spiked concentration when alternative approaches were used (▶ **Table 4**).

Spiking the methanolic extract of *G. glabra* with the PA-containing solution resulted in 29.6 ng/mL of echimidine and 275.9 ng/mL of echimidine-N-oxide content. The absolute binding capacity was increased from 5.8 ng/20 mg (MIP) to 9.7 ng/20 mg (TTEA), corresponding to a 67.2 % higher binding in the case of alternative imprinting. Regarding the N-oxides, binding was improved from 58.3 ng/20 mg (MIP) to 90.6 ng/20 mg (TTEA), indicating a 32.8 % higher capacity of the mixed mode imprinted polymer (▶ **Table 2**). The selective binding capacity ranged between 1.8 (MIP) and 5.7 ng/20 mg (TTEA), indicating that in the case of TTEA, 58.8 % of echimidine can be selectively bound. In the case of echimidine-N-oxide, maximum selective binding was achieved with TTEA, resulting in 43.5 % (39.5 ng/20 mg) that is bound to selective cavities (39.5 ng/20 mg) (▶ **Tables 3** and ▶ **4**). In terms of the initial concentration of PAs spiked into a methanolic extract of *G. glabra*, both the free base and the N-oxide were depleted by up to 32.8 % using TTEA (▶ **Table 4**).

In the spiked ethanolic solution, echimidine was determined to have a concentration of 34.0 ng/mL and its N-oxide a concentration of 296.4 ng/mL. Absolute binding of echimidine was increased from 9.7 ng/20 mg (MIP) to 10.9 ng/20 mg (TTEA), corresponding to 12.4 % higher binding compared to the monocrotaline imprinted polymer. An N-oxide maximum binding of 97.6 ng/20 mg was again achieved using the polymer imprinted with both templates, equating to an increased binding of just 3.2 % (▶ **Table 2**).

Similarly, the selective binding capacity of echimidine increased from 2.7 ng/20 mg (MIP) to 3.9 ng/20 mg and that of its N-oxide from 21.2 ng/20 mg to 24.0 ng/20 mg (TTEA) when TTEA was applied, corresponding to 32.9 and 24.6 % selective binding, respectively (▶ **Tables 3** and ▶ **4**). In line with this, echimidine and echimidine-N-oxide were depleted by up to 32.1 and 32.9 %, respectively, from the initial spiked *E. vulgare* extract (▶ **Table 4**).

The determination of PAs in the spiked methanolic *M. chamomilla* extract resulted in 23.2 ng/mL of echimidine and 222.3 ng/mL of echimidine-N-oxide content. Using an MIP, 6.7 ng/20 mg was bound, but 8.1 ng/20 mg was bound by TTEA, which equates to a 20.9 % higher binding capacity resulting from the use of the alternative approach. In the case of the N-oxide, a 21.4 % increase in the binding capacity from 65.9 ng/20 mg (MIP) to 80 ng/20 mg was achieved using TTEA (▶ **Table 2**). The selective binding capacity was shown to be in the range of 1.7-3.0 ng/20 mg (37.3 % of the total binding) for echimidine and 10.0 ng/20 mg-24.1 ng/20 mg for echimidine-N-oxide, corresponding to 30.1 % of the total binding (▶ **Tables 3** and ▶ **4**). All in all, 34.9 and 36.0 % of the initially spiked echimidine and echimidine-N-oxide, respectively, were successfully depleted from the spiked methanolic *M. chamomilla* extract with TTEA (▶ **Table 4**).

Spiking *E. vulgare* in an ethanolic solution of *M. chamomilla* resulted in 30.8 ng/mL echimidine and 276.2 ng/mL echimidine-N-oxide content. In this case, 19.8 % more echimidine and 30.5 % more echimidine-N-oxide were bound by TEA compared to the binding with the monocrotaline imprinted polymer (▶ **Table 2**), where the selective binding capacities ranged from 3.2 to 5.2 ng/20 mg for echimidine (48.8 % of the total binding) and 21.2 to 44.8 ng/20 mg for echimidine-N-oxide (44.3 % of the total binding) (▶ **Tables 3** and ▶ **4**). Regarding the PA-spiked ethanolic solution, a depletion of 39.3 % of echimidine and 36.6 % of echimidine-N-oxide was achieved with TEA (▶ **Table 4**).

In summary, it was shown that the free bases were depleted to within the range of 31.8-70.0 % and their N-oxides from 26.1–45.1 % when alternative CI approaches were applied. The selective binding values were found to be in the range of 32.9–64.3 % for the free bases and 13.9–44.3 % for their N-oxides (▶ **Table 4**). Furthermore, it was shown that the highest absolute binding capacities were achieved for echimidine and echimidine-N-oxide when the combined imprinted polymer (TTEA) was used in both spiked methanolic and ethanolic extracts. Regarding the selective binding, it was shown for the monocrotaline imprinted polymer that echimidine and its N-oxide were better bound in ethanolic solution. Whereas the selective binding of the free PA bases revealed no significant solvent dependency, the N-oxide binding increased in ethanolic solutions using the alternative approaches (▶ **Table 3**).

To figure out if the selectivity of the applied alternative imprinting approaches is still sufficient in the presence of complex plant matrices, the representative analytical marker compounds *C. majus*, *G. glabra*, and *M. chamomilla* were additionally quantified (▶ **Table 5**).

In spiked methanolic and ethanolic *C. majus* extracts, 0.05 and 0.13 mg/mL chelidonic acid contents were determined, respectively. The alternative imprinted polymers exerted no effect in both types of solutions on the chelidonic acid content, thus proving the selectivity of these alternative imprinted polymers towards PAs in the presence of chelidonic acid. Similarly, the glycyrrhizic acid content was determined in spiked solutions of *G. glabra* (methanolic

▶ **Table 4** Determined echimidine and echimidine-N-oxide contents in the different extract matrices expressed as ng/mL. Additionally, the best depletion results for the free PA bases and the N-oxides obtained with TEA/TTEA are presented for each plant matrix, showing the absolute depleted PA amount (expressed as a percentage relative to the initial PA concentration in the respective plant matrix) and the respective share of selective binding calculated by comparison with the non-imprinted polymer.

Free base methanolic	**Start [ng/mL]**	**Depleted (absolute) [%]**	**Selective share [%]**		**N-oxide methanolic**	**Start [ng/mL]**	**Depleted (absolute) [%]**	**Selective share [%]**	
M. piperita	22.4 ± 0.8	48.2	41.6	TTEA	*M. piperita*	246.0 ± 9.6	33.1	33.0	TEA
C. majus	2.0 ± 0.2	70.0	64.3	TTEA	*C. majus*	97.9 ± 1.0	41.9	13.9	TTEA
G. glabra	29.6 ± 1.8	32.8	58.8	TTEA	*G. glabra*	275.9 ± 18.9	32.8	43.5	TTEA
M. chamomilla	23.2 ± 1.9	34.9	37.3	TTEA	*M. chamomilla*	222.3 ± 10.1	36.0	30.1	TTEA
Free base ethanolic					**N-oxide ethanolic**				
M. piperita	25.5 ± 0.5	31.8	59.3	TTEA	*M. piperita*	286.3 ± 4.6	26.1	31.8	TTEA
C. majus	2.2 ± 0.2	45.5	60.0	TTEA	*C. majus*	109.6 ± 1.6	45.1	24.2	TEA
G. glabra	34.0 ± 0.4	32.1	32.9	TTEA	*G. glabra*	296.4 ± 2.0	32.9	24.6	TTEA
M. chamomilla	30.8 ± 0.4	39.3	48.8	TEA	*M. chamomilla*	276.2 ± 7.0	36.6	44.3	TEA

▶ **Table 5** Overview of the determined contents of representative analytical marker compounds in methanolic and ethanolic *G. glabra*, *C. majus*, and *M. chamomilla* extracts before and after PA depletion using different imprinted polymers.

	Glycyrrhizic acid		**Chelidonic acid**		**Apigenin 7-glucoside**	
	Methanolic extract [mg/mL]	**Ethanolic extract [mg/mL]**	**Methanolic extract [mg/mL]**	**Ethanolic extract [mg/mL]**	**Methanolic extract [mg/mL]**	**Ethanolic extract [mg/mL]**
Before depletion						
Pure extract without polymers	0.09 ± 0.00	0.15 ± 0.00	0.05 ± 0.00	0.13 ± 0.00	33.0 ± 0.86	29.0 ± 0.16
After depletion						
NIP	0.09 ± 0.00	0.15 ± 0.00	0.05 ± 0.00	0.12 ± 0.01	35.4 ± 5.17	27.1 ± 0.44
MIP	0.09 ± 0.00	0.14 ± 0.00	0.05 ± 0.00	0.13 ± 0.01	37.7 ± 0.50	28.1 ± 0.40
TEA	0.09 ± 0.00	0.14 ± 0.00	0.05 ± 0.00	0.14 ± 0.00	35.6 ± 1.09	26.9 ± 0.28
TTEA	0.09 ± 0.00	0.14 ± 0.00	0.05 ± 0.00	0.13 ± 0.00	33.2 ± 2.45	25.6 ± 1.26

0.09 mg/mL and ethanolic 0.15 mg/mL). No significant effects were observed on the concentration of glycyrrhizic acid upon treating the extracts with TEA or TTEA. When quantifying apigenin 7-glucoside, the content in a spiked methanolic extract of *M. chamomilla* was found to be 33.0 mg/mL and 29.0 mg/mL in a spiked ethanolic extract, showing that although the apigenin 7-glucoside content in the methanolic extract was not affected by the process of PA depletion, the content was slightly reduced by a maximum of 3.4 mg/mL in the ethanolic extract.

Discussion

Based on the assumption resulting from the work carried out in our previous study [14], in which binding of PAs to imprinted polymers was found to mainly occur at the tertiary nitrogen functionality of the necine base, this study focussed on test CI. In this new and alternative approach, imprinting templates were used, addressing only one structural element of the target molecule. In this study, several core imprinted polymers were prepared using triethylamine or a mixture of triethylamine and tetraethylammonium as templates. These two templates were chosen to address both the tertiary nitrogen function of the free bases and the quaternary nitrogen functionality of the N-oxides. Trials in methanolic PA standard solution revealed that insufficient selective cavities were created upon the applying of a two-fold or four-fold excess of the alternative templates, compared to a monocrotaline imprinted reference polymer. Further investigation led to the discovery that an additional increased molar excess (five-fold or nine-fold) of core templates resulted in increased PA binding. It is assumed that PAs are better depleted with these polymers because of the increased number of selective cavities that are present as a result of the increased amount of template used, as it is known that increasing the amount of template can have the positive effect of inducing the formation of recognition centres in the imprinted polymer [15]. Tetraethylammonium was chosen as an additional template to investigate if the N-oxides can be better bound if an additional quaternary nitrogen function is present in the template. However, no significant binding of echimidine-N-oxide was observed in the trials. Overall, the created polymers showed positive characteristics in terms of the absolute binding of echimidine (up to 70 %) and echimidine-N-oxide (up to 45 %). To evaluate the quality of binding in more detail, the selective share (binding MIP − binding NIP) needs to be de-

termined. Despite considering only the best results in the eight combinations of solvent and characteristics of the plant matrices (accompanying substances, concentration of leading compounds), the selective share for echimidine was found to vary from 32.9 to 64.3 % and for echimidine-N-oxide from 13.9 to 44.3 %. The high difference of the results, determined in this study, can be explained by the fact that imprinted polymers bind substances by means of electrostatic recognition in a so-called key/lock mechanism. The more specific these cavities are, the lower the possibility for interactions with other substances. PA-free bases (tertiary amine), as an example, fit better in the cavities created by triethylamine than the N-oxide (quaternary amine) and therefore have a higher selective share.

The opposite is also true in that if the cavities are created less specific, as done in our studies or via CI, the binding of target molecules is not preferred, as shown for MIPs, which were imprinted using more specific templates. This means that other competitive binding can take place based on electrostatic interactions. Because of this decreased selectivity, the share of non-selective PA binding is, in most cases, above 50 % for the core imprinted polymers. In our previous study, the selective share was found to be below 10 %, where it was shown that the low selective share was caused by alkaloids found in the solution, which fill the selective cavities [14]. In fact, there are two possibilities that influence the share of selective and non-selective binding. On the one hand, cavities are not that specific and target molecules bind to other molecule positions or resins in the polymer. On the other hand, there are substances in solutions that are also preferentially bound, thus blocking the cavities for the target molecule. Another aspect to be considered is that during the process of binding PAs in herbal extracts, analytical marker compounds are not affected during this process. No depletion of chelidonic acid and glycyrrhizic acid was observed in the ethanolic and methanolic extracts using the different polymers. However, apigenin 7-glucoside, present in a 300-fold higher concentration in the extract, was slightly depleted by the polymers. Nevertheless, it was clearly shown in the present study that the approach of CI may lead to satisfying results with regard to PA depletion. CI has many advantages compared to molecular imprinting with monocrotaline. Hence, the alternative templates applied here are available in higher quantities and are cheaper than PAs. Moreover, the risk of contamination of herbal extracts with PAs caused by column bleeding is additionally reduced using core templates. All of these factors facilitate economic scale-up. At the same time, it is apparent that PA binding by imprinted polymers in plant extracts may be affected by a large number of factors, which is why it has to be optimised for the respective plant extract.

Materials and Methods

Chemicals and solvents

All solvents, chemicals, and reagents used for determination of PAs, chelidonic acid, glycyrrhizic acid, and apigenin 7- glucoside were purchased from Carl Roth, Merck, or Sigma-Aldrich in MS grade. Chemicals and reagents applied for imprinting were purchased by Sigma and Merck in the highest purity available. Intermedine (purity > 99 % by HPLC), lycopsamine (purity > 99 % by HPLC), retrorsine (purity > 99 % by HPLC), senecionine (purity > 99 % by HPLC), senecyphilline (purity > 99 % by HPLC), senkirkine (purity > 98 % by HPLC), erucifoline (purity > 98 % by HPLC), senecivernine (purity > 95 % by HPLC), erucifoline-N-oxide (purity > 95 % by HPLC), retrosine-N-oxide (purity > 99 % by HPLC), senecyphilline-N-oxide (purity > 99 % by HPLC), senecivernine-N-oxide (purity > 99 % by HPLC), and chelidonic acid (purity > 98 % by HPLC) used as reference standards for quantification were purchased in the highest purity available from Phytolab. Apigenin 7-glucoside (purity > 94 % by HPLC) and glycyrrhizic acid (purity > 72 % by HPLC) were supplied by EDQM.

Pyrrolizidine alkaloid standard mix

Different PAs were weighed in separate flasks, dissolved in methanol, and made up to a volume with a concentration of 1 mg/mL. These different solutions were then combined and diluted to obtain a PA standard mix containing each PA in a concentration of 500 ng/mL.

Plant material

Samples of *E. vulgare* (TKO-02-2018), *C. majus* (TKO-01-2018), *M. piperita* (TKO-05-2018), *G. glabra* (TKO-03-2018), and *M. chamomilla* (TKO-04-2018) were donated by Dr. Klaus Denzel, Holzheim. Voucher specimens are stored at ZL (Zentrallaboratorium Deutscher Apotheker, Eschborn, Germany). Samples were ground using a conventional blender, sieved (< 710 µm), and stored in brown glass bottles at 20 °C.

Echium stock solution

For the preparation of *Echium* extract, 2.0 g of sample material was weighed into a test tube. Then, 20.0 mL of extraction solvent, a mixture of water and methanol (1:1) containing 1 % formic acid, were added. The test tube was shaken for 15 min and centrifuged at 4000 rpm (1761 g). The supernatant was collected, and the sample was extracted a second time with a further 20.0 mL in the same way as above. Finally, the organic phases were combined.

Matrix solutions

To compare the binding behaviour of the imprinted material in the different matrix solutions, all of the plants were extracted in the same way using ethanol and methanol. Each plant was extracted for 30 min in an ultrasonic bath in 50.0 mL of solvent using 0.5 g of plant material. The solutions were filtered using a conventional folded paper filter. Finally, 45 mL of each solution was spiked with 0.5 mL of *E. vulgare* extract stock solution.

Imprinting

Bulk imprinting, as a basic method for the production of an adsorptive material, was used in this study. To create the polymers, according to a previous study [14], acrylic acid (functional monomer), chloroform (porogen), ethylene dimethacrylate (cross-linker), and azodiisobutyronitrile (initiator) were used. The template was dissolved in chloroform (9.6 mL) followed by the addition of functional monomer (640 mg), cross-linker (4.5 mL), and initiator (80 mg). Reactive oxygen was removed by treating the mixture in an ultrasonic bath for 15 min. After this, chain growth was initiated at 70 °C, and this temperature was maintained for 24 h. Following this meth-

od, different polymers were produced. To investigate the effect of CI, a monocrotaline-based (400 mg) MIP was created. Further polymers were synthesised for comparison, such as TEA2 (390 mg of triethylamine), TTEA2/1 (390 mg of triethylamine/205 mg of tetraethyl ammonia), TEA5 (625 mg of triethylamine), and TTEA5/4 (625 mg of triethylamine/410 mg of tetraethyl ammonia) (n = 3), resulting in 18 polymers (including an NIP).

Processing polymers

The newly formed monoliths were removed from the test tubes, ground roughly using a blender, finely ground into powders using a pestle, and sieved (20 µm). To remove the template, the polymers were washed three times with 25 mL of water and three times using the same volume of methanol. Each washing step included shaking the tubes for 60 min, spinning the mixture at 4000 rpm (1761 g) for 10 min, decanting the supernatant, and repeating this procedure. After this, the washed material was dried in an oven at 70 °C for 12 h and stored as produced in a test tube.

Binding trials

Binding was tested using a PA standard mix (500 ng/mL). For this purpose, 1.0 mL of the standard solution was pipetted in a 10-mL Sarstedt tube containing 20 mg of the processed polymer. Afterwards, the mixture was shaken for 1 h, spun at 4000 rpm (1761 g), and decanted. For each polymer (n = 3), binding trials were carried out (n = 3), resulting in a total of 54 measurements. Means and standard deviations were calculated on the basis of the nine measurements carried out for each polymer. In the next step, all associated polymers (n = 3) were combined to achieve a representative mixed sample containing each type of polymer for use in the matrix trials. For *M. piperita*, *C. majus*, *G. glabra*, and *M. chamomilla*, binding trials were carried out in triplicate in methanolic and ethanolic solutions (eight matrix solutions). Hence, testing of NIP, MIP, TEA5, and TTEA resulted in a further 96 binding trials. After processing, the supernatant was quantified using liquid chromatography tandem mass spectrometry (LC-MS/MS).

Determination of the pyrrolizidine alkaloids

All of the solutions were analysed using HPLC using an Agilent 1200 Series HPLC apparatus equipped with an Agilent triple quadrupole 6410 Series MS detector and quantified against external standards. The validated method [16] was suitable for use in separating 28 PAs, including their corresponding N-oxides. A binary gradient with 5 mM aqueous (mobile phase A) and methanolic (mobile phase B) ammonia formate buffers was applied using a C_{18} column (Hypersil Gold, 150 × 2.1 mm; 1.9µm) at a flow rate of 0.4 mL/min for separation. Elution of the PAs was performed by increasing the percentage of mobile phase B from 5 to 100 % within 35 min. For elution of the accompanying substances, the column was washed for 10 min with 100 % B. The column temperature was set to 40 °C, and the injection volume used was 10 µL. Ionisation of the analytes was induced by electrospray ionisation in the positive ion mode. Mass filtering was performed in multiple reaction monitoring. The dwell time for detection of the specific fragments (quantifier/qualifier) was set to 20 ms. Retention times, precursor ions, and product ions are listed in (▶ **Tables 3S**), Supporting Information.

Determination of chelidonic acid

The determination of chelidonic acid was carried out using an Agilent 1200 HPLC apparatus equipped with an Agilent 1200 series DAD and quantified at 210 nm against external standards. Samples were separated on a C_{18} column (Luna, 250 mm × 4.6 mm; 5 µm) at room temperature. A binary gradient composed of tetrabutylammonium hydrogen sulfate (0.1 %) in water (mobile phase A) and acetonitrile (mobile phase B) was used. The starting gradient used 1.0 mL per minute as a flow rate, with 5 % mobile phase B. This composition was used 1 min, after which it was increased to 100 % mobile phase B within 40 min. The injection volume was set to 10 µL, and the method was validated according to ICH guidelines.

Determination of glycyrrhizic acid

Glycyrrhizic acid was quantified using an Agilent 1200 HPLC apparatus equipped with an Agilent 1200 series DAD. Separation was performed on a C_{18} column (ReproSil, 125 mm × 4 mm; 5 µm) using a mixture of glacial acetic acid, acetonitrile, and water (6:30:64 v/v/v) as a mobile phase. Chromatography was performed under isocratic conditions at a flow rate of 1.5 mL/min and a column temperature of 20 °C. The injection volume was 10 µL. Samples were quantified against monoammonium glycyrrhizate as an external standard within a range of 0.065–0.195 mg/mL.

Determination of apigenin 7-glucoside

The apigenin 7-glucoside content was analysed using an Agilent 1200 HPLC apparatus equipped with an Agilent 1200 series DAD and quantified at 340 nm against an external single point standard at a concentration of 0.0125 mg/mL. Separation was performed using a C_{18} column (Luna, 250 mm × 4.6 mm; 5 µm) at room temperature as a stationary phase using a binary gradient with a flow rate of 1 mL/min. The gradient was started with 25 % mobile phase B (0.5 % phosphoric acid in acetonitrile) and 75 % mobile phase A (0.5 % phosphoric acid in water). These conditions were used for 9 min, then the percentage of mobile phase B was increased to 75 % within 10 min. To elute the accompanying substances, the system was rinsed for 5 min. The injection volume was 20 µL.

Supporting information

The absolute binding values for each PA, each produced polymer, and the calculated selective binding are given in two different tables in the Supporting Information. Furthermore, the retention times, precursors, and product ions are stated.

Acknowledgements

T. K. expresses his honest gratitude to Ms. Rebecca Gröner for supporting this research with HPLC measurements of the leading compounds. Further, he wants to thank Dr. Sebastian Jäger for his support and the discussions during writing this manuscript. This project was funded by AiF (Arbeitsgemeinschaft industrieller Forschung).

Conflict of Interest

The authors declare that they have no conflict of interest.

References

[1] Roeder E. Medicinal plants in Europe containing pyrrolizidine alkaloids. Pharmazie 1995; 50: 83–98

[2] Analytik und Toxizität von Pyrrolizidinalkaloiden sowie eine Einschätzung des gesundheitlichen Risikos durch deren Vorkommen in Honig. Available at: https://www.bfr.bund.de/cm/343/analytik-und-toxizitaet-von-pyrrolizidinalkaloiden.pdf, Accessed January 7, 2019

[3] Colegate SM, Gardner DR, Betz JM, Fischer OW, Liede-Schumann S, Boppré M. Pro-toxic 1,2-Dehydropyrrolizidine Alkaloid Esters, Including Unprecedented 10-Membered Macrocyclic Diesters, in the Medicinally-used *Alafia cf. caudata* and *Amphineurion marginatum* (Apocynaceae: Apocynoideae: Nerieae and Apocyneae). Phytochem Anal 2016; 27: 257–276

[4] Hartmann T. Chemical ecology of pyrrolizidine alkaloids. Planta 1999; 207: 483–495

[5] Mattocks AR. Toxicity of pyrrolizidine alkaloids. Nature 1968; 217: 723–728

[6] Bush LP, Fannin FF, Siegel MR, Dahlman DL, Burton HR. Chemistry, occurrence and biological effects of saturated pyrrolizidine alkaloids associated with endophyte-grass interactions. Agric Ecosyst Environ 1993; 44: 81–102

[7] Andersson LI, Paprica A, Arvidsson T. A highly selective solid phase extraction sorbent for pre- concentration of sameridine made by molecular imprinting. Chromatographia 1997; 42: 57–62

[8] Theodoridis G, Manesiotis P. Selective solid-phase extraction sorbent for caffeine made by molecular imprinting. J Chromatogr A 2002; 948: 163–169

[9] Ferrer I, Lanza F, Tolokan A, Horvath V, Sellergren B, Horvai G, Barceló D. Selective trace enrichment of chlorotriazine pesticides from natural waters and sediment samples using terbuthylazine molecularly imprinted polymers. Anal Chem 2000; 72: 3934–3941

[10] Chapuis F, Pichon V, Lanza F, Sellergren S, Hennion MC. Optimization of the class-selective extraction of triazines from aqueous samples using a molecularly imprinted polymer by a comprehensive approach of the retention mechanism. J Chromatogr A 2003; 999: 23–33

[11] Cacho C, Turiel E, Martin-Esteban A, Pérez-Conde C, Cámara C. Characterisation and quality assessment of binding sites on a propazine-imprinted polymer prepared by precipitation polymerisation. J Chromatogr B Anal Technol Biomed Life Sci 2004; 802: 347–353

[12] Maier NM, Buttinger G, Welhartizki S, Gavioli E, Lindner W. Molecularly imprinted polymer-assisted sample clean-up of ochratoxin A from red wine: merits and limitations. J Chromatogr B Anal Technol Biomed Life Sci 2004; 804: 103–111

[13] Turiel E, Tadeo JL, Cormack PAG, Martin-Esteban A. HPLC imprinted-stationary phase prepared by precipitation polymerisation for the determination of thiabendazole in fruit. Analyst 2005; 130: 1601–1607

[14] Kopp T, Abdel-Tawab M, Khoeiklang M, Mizaikoff B. Development of a Selective Adsorbing Material for Binding of Pyrrolizidine Alkaloids in Herbal Extracts, Based on Molecular Group Imprinting. Planta Med 2019; 85: 1107–1113

[15] Yan H, Row KH. Characteristic and synthetic approach of molecularly imprinted polymer. Int J Mol Sci 2006; 7: 155–178

[16] Schulz M, Meins J, Diemert S, Zagermann-Muncke P, Goebel R, Schrenk D, Schubert-Zsilavecz M, Abdel-Tawab M. Detection of pyrrolizidine alkaloids in German licensed herbal medicinal teas. Phytomedicine 2015; 22: 646–656