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Marinobufagenin extraction from *Rhinella marina* toad glands: Alternative approaches for a systematized strategy

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Marinobufagenin is a bufadienolide compound detected mainly in skin and parotoid gland secretions of *Rhinella marina* (L.) toad. Bufadienolides regulate the Na^+/K^+-ATPase pump by inhibiting the cardiotonic steroid dependent-site and act as cardiac inotropes with vasoconstrictive properties. Marinobufagenin and other bufadienolides, such as telocinobufagin and bufalin, are thought to be found endogenously in mammals in salt-sensitive hypertensive states such as essential hypertension, congestive heart-failure, and preeclampsia. The role of marinobufagenin as antimicrobial agent and its cytotoxic potential have also been recognized. The particular interest around marinobufagenin prompts us to consider the *Rhinella marina* toad venom as a possible source for molecules with pharmacological and/or diagnostic potential. In this article, two different approaches of extraction and purification of marinobufagenin from *Rhinella marina* (L.) venom are studied: (i) Preparative thin-layer chromatography combined to mass spectrometry and/or ultraviolet detection and (ii) solid-phase extraction coupled with fractionation on high-performance liquid chromatography. Different chromatographic conditions are tested for each approach. The solid-phase extraction combined with high-performance liquid chromatography fractionation approach was preferred as it offered a greater yield, was less time-consuming and allowed us to selectively isolate marinobufagenin. Both protocols aim to provide efficient and convenient methods for toad venom extraction, based on an easily automatable and systematized strategy.

KEYWORDS
high-performance liquid chromatography, marinobufagenin, thin-layer chromatography, toad venom

1 | INTRODUCTION

For 4000 years, venomous secretions of amphibians have been part of the traditional medicine in Egyptian, South-American, and Asian civilizations [1,2]. One of the most well-known example from the traditional Chinese medicine is *Ch'an Su* (Venenum bufonis), a remedy composed of dried toad skins in the form of a cake from the toad species *Bufo bufo gargarizans* or *Bufo melanostictus*. It is still extensively used today for its anti-inflammatory, cardiotonic, diuretic, local anesthetic, and hemostatic properties [3–5]. Skin and gland secretions from toads constitute a rich source of various compounds such as biogenic amines, alkaloids, some peptides, bufadienolides, and bufotoxins [6,7].

Bufadienolides from animal origin are steroid compounds containing a δ-lactone ring at carbon C_{17} of ring D mainly synthetized through the mevalonate-independent pathway, with cholesterol as precursor (see Figure 1 [8]. Bufotoxins are amino acid-dicarboxylic acid esters at the C_3 position of the bufagenins. The presence of amino acid residues provides a hydrophilic basic character to the bufotoxins. This work will

Article Related Abbreviations: EtOAc, ethyl acetate; MBG, marinobufagenin; MeOH, methanol

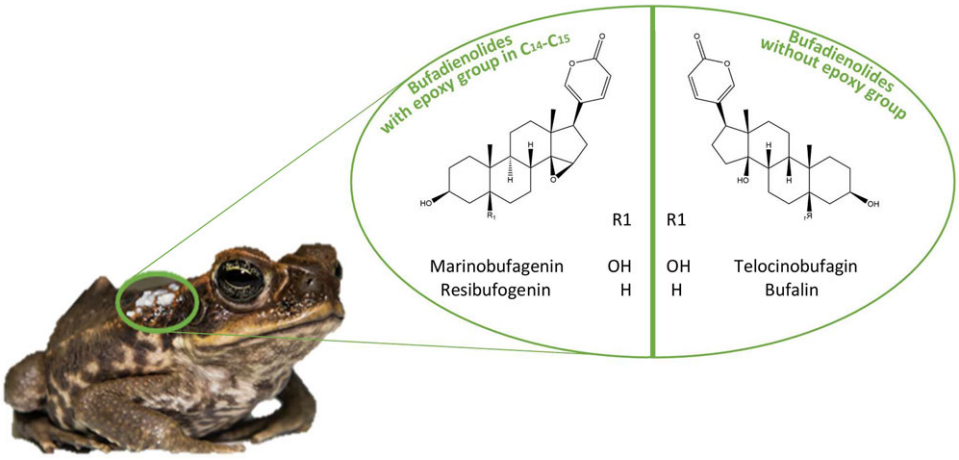

FIGURE 1 *Rhinella marina* toad with venom dripping from the rand of its parotoid glands. Chemical structure of the four main bufadienolides from *Rhinella marina (L.)* toad venom. Bufadienolides are steroids containing δ-lactone ring at carbon C_{17} of ring D. Two different types of bufadienolides are shown: A) Bufadienolides with epoxy group in C_{14}-C_{15}. B) Bufadienolides without an epoxy group in C_{14}-C_{15}

focus on one bufadienolide in particular, marinobufagenin (MBG).

Marinobufagenin is a bufadienolide that regulates the Na^+/K^+-ATPase pump by inhibiting the cardiotonic steroid dependent-site [1,9–11] and has gained a growing interest as a potential endogenous biomarker for cardiovascular pathologies such as salt-sensitive hypertensive states [12–15], congestive heart-failure [16,17], and preeclampsia [18–21]. MBG as well as other bufadienolides in biological fluids is becoming a real concern. Several methods, mostly designed for therapeutic drug monitoring of Traditional Chinese Medicine in plasma and/or serum, have already been published but are all facing the same issue: the tricky access to pure standard material for the setup and validation of the analytical methods for MBG measurement [4,14,22–24]. MBG is also found in the skin and parotoid gland secretions of *Bufonidae* toad species and especially in the secretions of the *Rhinella marina (L.)* toad. Venom issued from the parotoid glands of this toad is thus considered as the main source for MBG standard extraction and purification [25,26]. Besides MBG, the secretions of *Rhinella marina (L.)* toads contain three other bufadienolides: telocinobufagin, bufalin, and resibufogenin (see Figure 1 [4,27].

Bufadienolides and bufotoxins are usually extracted from dried or fresh toad secretions/skins following a solvent extraction treatment. Solvents mainly used are alcohols (e.g., methanol, ethanol), chlorinated organic solvents (*e.g.*, chloroform), and non-chlorinated organic solvents (*e.g.*, ethyl acetate). Different techniques have already been described to separate and isolate MBG from the other compounds: preparative thin-layer chromatography, classical column chromatography (on silica gel, Sephadex-LH 20 and also Amberlite resin), preparative HPLC with various column chemistries (*e.g.*, un-grafted silica gel, octadecyl grated, positively charged stationary phases), flash column chromatography [4, 28–33].

This publication deals with the development of two different approaches for MBG extraction and purification from *Rhinella marina* (L.) parotoid gland venom: the first one is based on preparative TLC coupled to MS and/or UV detection and scratching of the corresponding spot for subsequent purification. As this technique suffers from a marked diffusion phenomenon of the compounds of interest on silica, several conditions tested on non-preparative silica plates are presented to enhance resolution. The second approach uses direct fractionation on HPLC with automatic fraction collector preceded by a solid phase extractive pre-purification step on weak cation exchange cartridges. Both protocols aim to provide a small-scale non-preparative method that generates a sufficient quantity of MBG for analytical use.

2 | MATERIAL AND METHODS

2.1 | Preparative TLC

Two milliliters of *Rhinella marina* (L.) toad venom crude extract (the extraction method is exposed in Supporting Information I) was deposited on a preparative TLC plate over 13 cm. After migration in ethyl acetate (EtOAC) and drying at room temperature, the most intense spot visible in UV at 254 nm, corresponding to MBG, was scratched. The scraped silica was extracted twice with methanol (MeOH) by vortexing and ultrasonication for 30 min. After centrifugation, the supernatant was filtered on a Millex® filter and evaporated. The residue was further extracted with dichloromethane to avoid silica contamination. After ultrasonication, supernatant was removed, filtrated, and evaporated. The viscous yellow

residue was dissolved in MeOH to constitute the stock solution of MBG (All chemicals and materials used are described in Supporting Information I).

2.2 | TLC Instrumentation

An automatic TLC sampler was used to deposit the samples (Camag® Automatic Sampler 4, Switzerland) on TLC plates. Several conditions of migration were tested as listed in Table 1. The spots were visualized under UV light at a wavelength of 254 nm. A TLC-MS interface (Camag® TLC-MS Interface 2, Switzerland) was used to analyze the spots visible on the TLC plate after migration. The TLC-MS interface is connected to a HPLC pump (Agilent® Technologies Inc, California, USA) so that an extraction solvent (MeOH:Milli-Q Water 0,1% formic acid) can be sent to the mass spectrometer system with a flow rate of 0.2 mL/min.

Mass spectra were recorded using an Advion Expression CMS simple quadrupole (Advion®, Ithaca, USA). Ionization was performed at 3500 V. Only positive ions were detected. The following operating conditions were used: capillary temperature of 250°C, 180 V capillary voltage, 20 V cone voltage, and desolvation gas temperature of 200°C. Data was acquired over a m/z range between 150 and 900 (Spot analysis on C_{18}-grafted TLC plates is given in detail in Supporting Information I).

2.3 | Sample preparation for HPLC purification

The SPE pre-treatment was carried out using Waters® SPE MCX 150 mg cartridges (6 mL). The sample is constituted by 5 mL of a mix of ultrapure water with the methanolic extract of toad venom (90:10) spiked with 80 µL of the corresponding working solution of prednisolone at 1 mg/mL as an internal standard (IS). After vortex and centrifugation, the sample was loaded onto the SPE MCX cartridge previously conditioned with 6 mL of acetonitrile and equilibrated with 6 mL of milli-Q water. A first wash (Wash1) was performed with three times 2 mL of a mix of ACN/H_2O 10:90 with 2% formic acid. A second wash (Wash2) was performed with three times 2 mL of 100% pure acetonitrile. The final elution was performed with 6 mL of NH_4OH 4% in MeOH. Each wash and eluate was collected in an eppendorf tube, evaporated under a gentle stream of nitrogen and reconstituted in 1 ml of MeOH/H_2O 50:50. A crude methanolic venom extract was diluted with H_2O (1:1) prior to UHPLC-UV-QDa analysis so as to obtain a complete venom profiling and thus evaluate the efficiency of the SPE step without quantitative comparison considering that the analyzed extract is diluted at 50% and that the sample extracted by SPE is diluted at 10%.

2.4 | HPLC-fraction collector instrumentation

The liquid chromatography system consisted of a Waters® instrument composed of a Waters® 626 binary pump, a Waters® 6008 Controller, a Waters® 717 plus 48-vial autosampler and equipped with a Waters® Fraction collector III (Waters®, Milford, USA). The separation was performed on a Luna C18 column (250 × 4.6 mm, i.d.; particle size: 5 µm) from Phenomenex®. The column and the autosampler were maintained at room temperature. UV detection was carried out using a Waters® 2996 PDA detector (Waters®, Milford, USA). Control of the whole system and data acquisition were managed via Empower® software (Waters, version 1). The delayed collection time corresponding to the fraction collector tubing dead volume is defined, thanks to the injection of 0.1 M solution of $K_2Cr_2O_7$ in 0.005 M H_2SO_4.

The mobile phases were composed of A = milli-Q water with 0.1% formic acid and B = acetonitrile. Gradient elution was used according to the following method: 20% of mobile phase B from 0 to 1 min, gradually increased to 42% B at 22 min, going from 42 to 60% B in 21 min and set at 95% B at 43.1 min and maintained isocratic for 5 min (for column washing), 20% B at 48.1 min and isocratic 20% B until 52 min (for column re-equilibration). The injection volume was 20 µL and the mobile phase flow rate was set at 1 mL/min. UV detection wavelength was set at 296 nm, the wavelength of choice for bufadienolide compounds [29]. The fraction of interest is evaporated to dryness under reduced pressure, at 60°C using a rotary evaporator (BUCHI, Rotavapor R-210, New Castle, USA). After vortex and sonicating, each single fraction was analyzed by QTOF mass spectral analysis (Conditions detailed in Supporting Information I).

2.5 | LC analysis

The LC system consisted of a Waters® Acquity UHPLC H-Class instrument (Waters, Milford, USA) and was equipped with a quartenary pump and a 2 × 48-vial autosampler. The separation was performed on a CSH C18 column (100 × 2.1 mm, i.d.; particle size: 1.7 µm) from Waters®. Column temperature was set to 40°C and the thermostated autosampler was maintained at 10°C. The instrument was equipped with a PDA and QDa detector allowing MS detection (Waters®, Milford, USA), equipped with an electrospray ionization source operating in positive ionization mode (ESI +) and a simple quadrupole. The cone voltage was set at 15 V. Control of the whole system and data acquisition were managed via Empower® software (Waters, version 3.1).

The mobile phases were composed of A = milli-Q water with 0.1% formic acid and B = acetonitrile with 0.1% formic acid. Gradient elution was used according to the following method: 20% of mobile phase B from 0 to 0.5 min, gradually increased to 30% B at 2 min, going from 30 to 35% B

TABLE 1 Selected TLC conditions for toad venom compounds with MS data obtained for MBG identification and the corresponding Rf

Selected TLC conditions	MBG Identification	Rf_{MBG}
Stationary phase: Silica plate Mobile phase: Ethyl acetate 100% Deposit solvent: Methanol	401.3 m/z $[MBG + H]^+$ 423.3 m/z $[MBG + Na]^+$ 455.3 m/z $[MBG + Na + MeOH]^+$	0,30
Stationary phase: Silica plate Mobile phase: Ethyl acetate 100% Deposit solvent: Ethyl acetate	401.2 m/z $[MBG + H]^+$ 423.2 m/z $[MBG + Na]^+$ 455.3 m/z $[MBG + Na + MeOH]^+$	0,46
Stationary phase: C_{18}-grafeted silica plate Mobile phase: ACN/H_2O (80:20) Deposit solvent: Methanol	401.2 m/z $[MBG + H]^+$ 455.3 m/z $[MBG + Na + MeOH]^+$	0,53

in 4 min, and from 35 to 60% B in 3 min and set at 95% B at 10 min, 20% B at 12 min and maintained isocratic until 14 min (for column re-equilibration). The injection volume was 10 µL and the mobile phase flow rate was set at 0.450 mL/min. UV detection wavelength was set at 296 nm. QDa full mass scan was allowed over a wide range of masses going from 100 to 800 Da.

3 | RESULTS AND DISCUSSION

3.1 | TLC and compound identification

Preparative TLC in 100% EtOAc performed on a crude *Rhinella marina* (L.) toad venom methanolic extract showed a significant band broadening. The spot corresponding to MBG (boxed in black) is dispersed. Band broadening decreases resolution and separation of the venom components. Purification is hindered by the quantity of scratched silica as by the presence of other venom components. The different TLC conditions tested for crystallized *Rhinella marina* (L.) toad venom extracts are listed in Supporting Information II Table 1 which also gathers the observations that ensued: migration based on the Rf, separation resolution based on the distance between spots and band broadening (width). Non-preparative TLC plates were used to avoid the use of expensive material, to save time and to limit our extract consumption. The spots present on the TLC plates after migration were identified using mass spectrometry. This was done only for the plates that gave the best results (Table 1) and the obtained plates are shown in Figure 2 Following the MS analysis, many sodium and MeOH adducts are observed. This could be explained through the fact that sodium can often be found in tubing and glass ware. As for the MeOH adducts, this could originate from non-optimal source conditions [34]. Also, as on the MS spectrum of Figure 2, many parasite peaks are present, a blank solution (small quantity of scratched C_{18}-grafted silica extracted with methanol) has been injected (Mass spectrum: Supporting Information III Figure 1). Many peaks visible on Figure 2 are also present in the blank's mass spectrum but no MBG peak, proving the contamination of the extracted sample with reversed phase silica.

To conclude on this part, the final conditions adopted are: deposition in MeOH on an un-grafted silica plate with mobile phase consisting of 100% EtOAc, as shown in Figure 2. This method is the one that gave the least diffusion and the best separation of venom components; and was applied to preparative TLC to isolate MBG from the rest of the complex venom matrix. However, due to diffusion phenomenon, the scratched extract requires an additional purification step on HPLC with manual peak collection to obtain pure MBG. Moreover, the risk of material loss increases with the number of steps needed to obtain a pure compound. Preparative TLC is thus a tedious and time-consuming purification technique. In addition, the extraction yields remain low as only 2.4 mg of MBG was collected from 200 mg of dry *Rhinella marina* (L.) toad venom.

3.2 | HPLC fractionation

This second approach was also considered as an alternative to the preparative TLC. At each step of the SPE protocol, washes and eluates are collected to be analyzed by UHPLC-UV-QDa to identify which compounds are retained and which are not. Figure 3 shows the UHPLC-UV-QDa chromatograms obtained after each step of SPE protocol: chromatogram A corresponds to the crude venom extract analysis obtained prior SPE pre-treatment and will give us an indication about the solid phase extraction efficiency and its potential for pre-purification of the venom. The first two peaks eluted, in green, are the most intense ones and correspond to the main compounds of the venom, the biogenic amines. The next compounds, shown in red, are the more polar bufotoxins. And finally free hydrophobic bufagenins, shown in black, are eluted. A last bufagenin coelutes at Rt = 3.5 min and corresponds to arenobufagin, but became visible only when bufotoxins were removed by SPE. Preliminary peaks identification was based on parent ion $[M+H^+]$ mass to charge ratio compared to published detailed LC-QTOF and HPLC-ESI-MS/MS analyses of several *Rhinella marina* (L.) toad

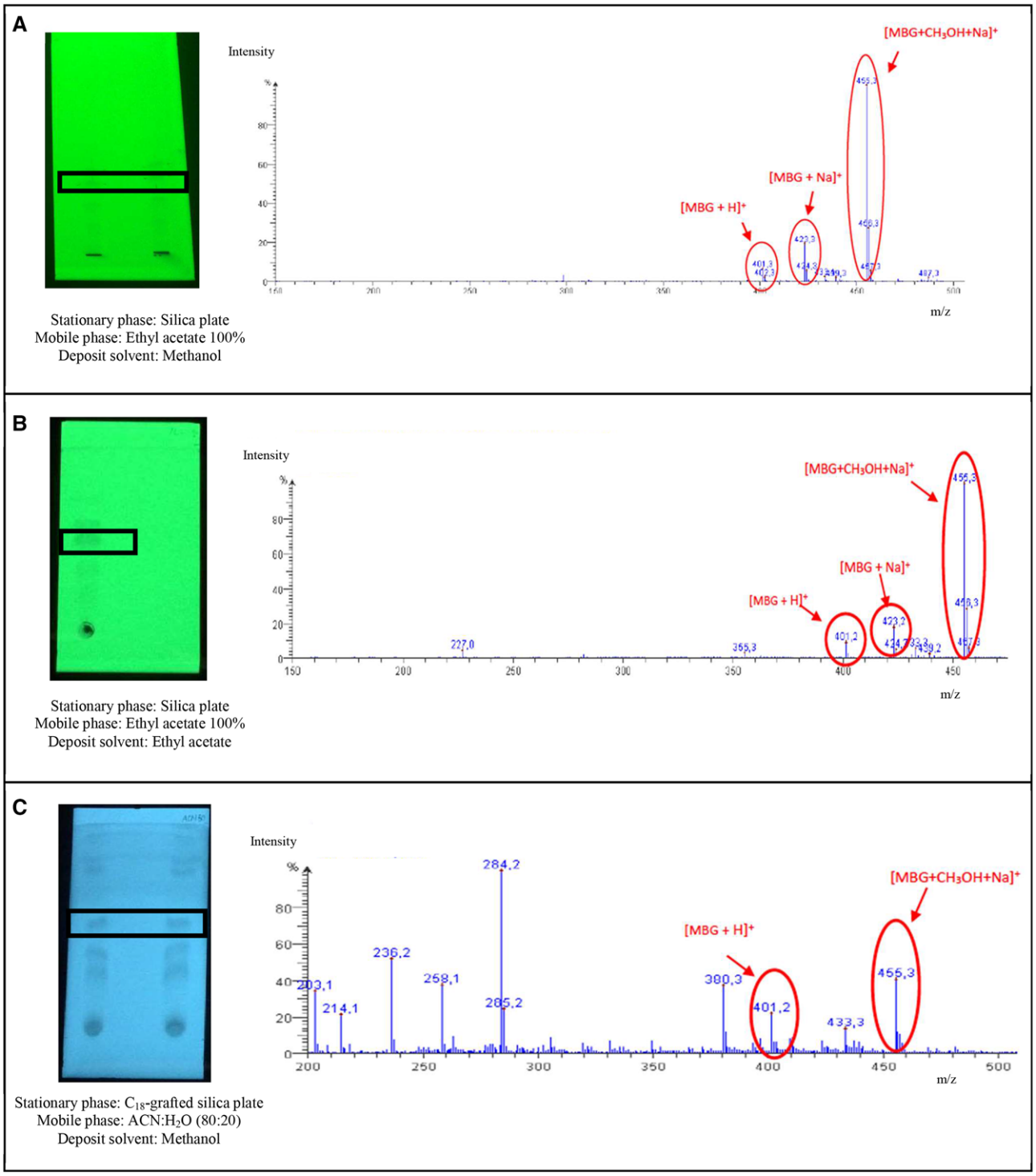

FIGURE 2 TLC plates obtained after migration of *Bufo marinus* toad extracts in several conditions as presented in Section 3.1 and Table 1: Spots corresponding to MBG are boxed on each TLC plate. A) 401.3 m/z $[MBG + H]^+$; 423.3 m/z $[MBG + Na]^+$; 455.3 m/z $[MBG + Na + MeOH]^+$. B) 401.2 m/z $[MBG + H]^+$; 423.2 m/z $[MBG + Na]^+$; 455.3 m/z $[MBG + Na + MeOH]^+$. C) 401.2 m/z $[MBG + H]^+$; 455.3 m/z $[MBG + Na + MeOH]^+$

venom specimens [25–27,35–38]. The m/z ratio of parent ion, the relative abundance and the eluting order matched the already published research work mentioned above. Previous chromatographic analyses also showed improper chromatograms with overlapping peaks between bufotoxins and bufogenins, even with slow organic gradients [25,27,36,38]. Co-elution of bufogenins and bufotoxins is also observed on the chromatogram obtained after UHPLC-UV analysis of our crude toad venom extract. This phenomenon emphasizes the need of a hydrophilic pre-chromatographic purification step to

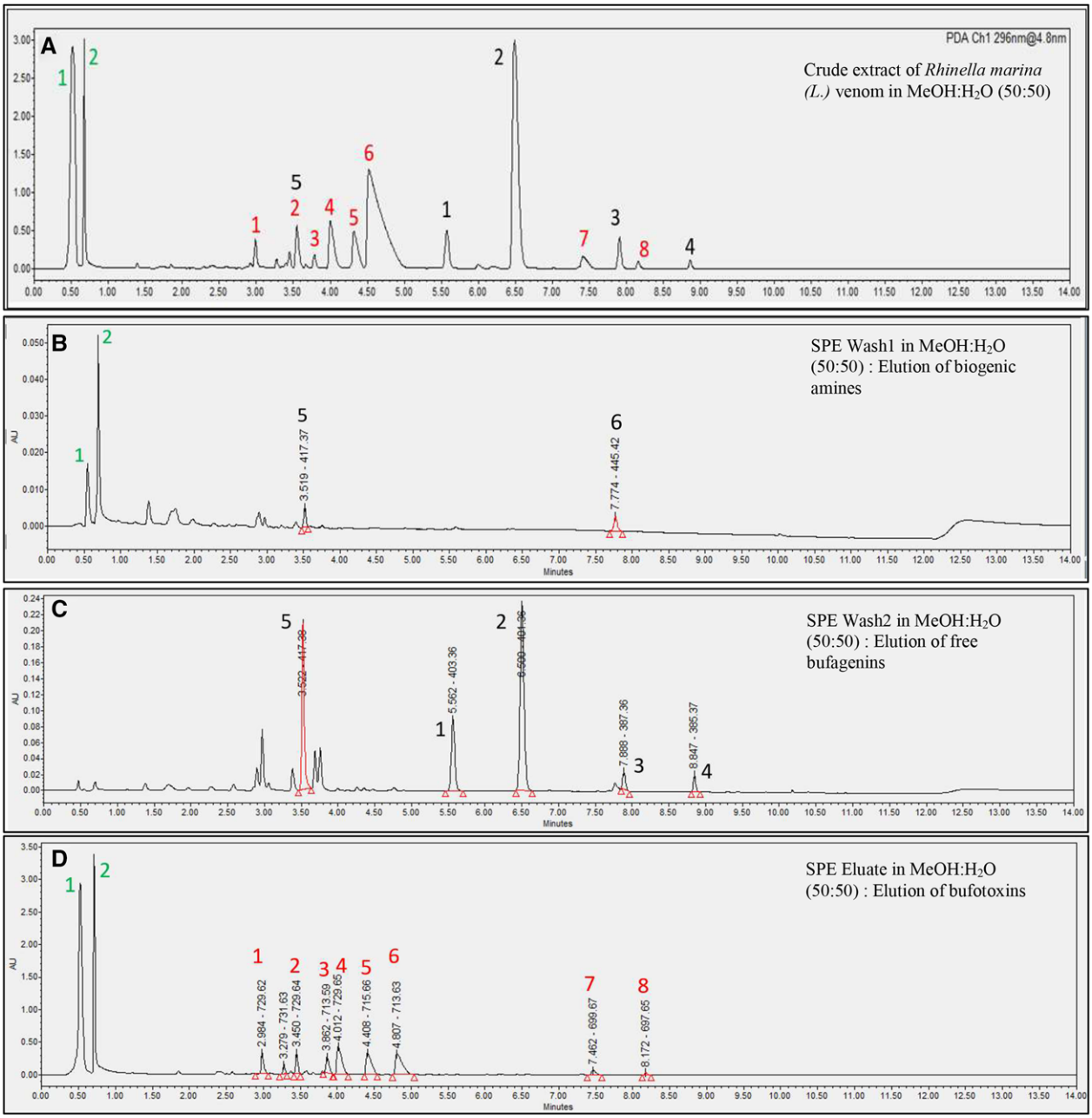

FIGURE 3 UHPLC-UV analyses of: A) Crude methanolic extract of Bufo marinus toad venom: **Green** – 1: dehydrobufotenin ($[M+H]^+ = 203.2$ m/z); 2: suberoyl arginine ($[M+H]^+ = 331.2$ m/z). **Red** – 1: arenobufotoxin ($[M+H]^+ = 729.4$ m/z); 2: marinobufagin-3-adipate-arginine ($[M+H]^+ = 685.2$ m/z); 3: telocinobufagin-3-pimeloyl-arginine ($[M+H]^+ = 701.4$ m/z); 4: marinobufagin-3-pimeloyl-arginine ($[M+H]^+ = 699.2$ m/z); 5: telocinobufotoxin ($[M+H^+] = 715$ m/z); 6: marinobufotoxin ($[M+H^+] = 713$ m/z); 7: bufalitoxin ($[M+H]^+ = 699.2$ m/z); 8: resibufotoxin ($[M+H]^+ = 697.2$ m/z). **Black** – 1: TBC ($[M+H]^+ = 403.2$ m/z); 2: MBG ($[M+H]^+ = 401$ m/z); 3: bufalin ($[M+H]^+ = 387.2$ m/z); 4: resibufogenin ($[M+H]^+ = 385.2$ m/z); 5: arenobufagenin ($[M+H]^+ = 417$ m/z, coeluting @ 3.5 min). B) Wash1: **Green** – 1 & 2: see Figure 3 . **Black** – 5: see Figure 3 ; 6: bufotalin ($[M+H]^+ = 445.2$ m/z). C) Wash2: **Black** – 1 to 5: see Figure 3. D) Eluate: **Green** – 1 & 2: see Figure 3 . **Red** – 1 to 8: see Figure 3

eliminate bufotoxins and other more hydrophilic compounds and avoid coelution. This strategy is based on the separation of natural compounds combining two different polarities, hydrophilic and reversed phase, and allows for excellent orthogonality and extraction of targeted compounds from natural sources [39]. Chromatogram B is obtained by analyzing the first wash of the cartridge with the ACN/H_2O 1:9 solution enriched with 2% formic acid. We could identify more polar compounds such as the two main biogenic amines and the more polar arenobufagin and bufotalin that are already eluting during this step, but no other bufagenins could be identified. The low organic content of the Wash1 allows to

perform a first efficient wash of the venom by eluting very polar compounds without eluting hydrophobic compounds. This assures us that more hydrophobic bufagenins such as MBG are still retained on the cartridge. Chromatogram C corresponds to the Wash 2 step that allows the elution of the more hydrophobic compounds i.e. the bufogenins. The five main bufadienolides, of which MBG, from *Rhinella marina* (L.) toad venom could be identified in the Wash 2 eluate. The absence of peaks corresponding to bufotoxins suggests that these compounds are still retained on the cartridge, thanks to their positive charge. Finally, elution step with MeOH enriched with NH_4OH 4% enabled to elute bufotoxins and all biogenic amines not eluted as seen on chromatogram D. The use of a basic-specific mixed-mode cation exchanger SPE sorbent, Waters Oasis MCX®, allowed for the selective retention of bufotoxins based on their basic character and allowed us to separate hydrophobic compounds of the venom from more hydrophilic ones.

The Wash 2 eluate from SPE pre-purification is then collected, evaporated, and concentrated to be injected on the HPLC-UV-fraction collector device for purification. The optimized HPLC gradient is slow, which, combined to a long column, allows an optimal separation of the hydrophobic venom constituents. Resolution of peaks is sufficient to collect them separately, thanks to the automatic fraction collector with a window time error of 10 s (see Supporting Information IV Figure 1). The fraction of interest, fraction 1, (Supporting Information IV Figure 1: peak boxed in green (Rt = 32.785 min)) is collected in a 10 mL collection tubes. After evaporation, 1.36 mg of dried residue were obtained from 76.3 mg of *Rhinella marina* (L.) toad venom. The residue was dissolved in MS-grade MeOH so as to obtain a solution at 1 µg/mL. QTOF mass spectral analyzes of the collected fraction allowed us to identify pure MBG with a peak at 401.21 m/z ($[MBG+H]^+$) and the peaks corresponding to its adducts (see Supporting Information IV Figure 1). This method has numerous advantages compared to preparative TLC: minimizing losses, avoiding contamination of silica from TLC-plates, increased extraction efficiency as less crude venom was used, increased purity of isolated compounds, and less time-consuming. Moreover, automation with on-line SPE combined to HPLC-UV-fraction collector is conceivable allowing for higher throughput, minimal error, and extra time-saving.

4 | CONCLUDING REMARKS

Two approaches were used to isolate and purify marinobufagenin, from *Rhinella marina* (L.) toad venom. The objective was to develop a small-scale non-preparative isolation technique to attain a sufficient quantity of pure MBG for analytical use.

Preparative TLC was first tested with the use of several TLC conditions to enhance the purification potential by reducing the band broadening phenomenon of this method but no noteworthy improvement was seen. By combining SPE on cation-exchange cartridges, to eliminate bufotoxins and other basic components, with automated HPLC-UV guided fraction collection, we could successfully extract MBG with a high degree of purity. This bufadienolide is a promising active compound for diagnostic approaches in the hypertensive states linked to volume expansion and salt-sensitivity. In this context, access to a MBG standard of high purity will be a huge issue in the coming years considering the need to possess specific, sensitive, and robust analytical MBG dosage method allowing quantifications as low as possible. The antimicrobial and newly discovered cytotoxic properties of MBG rendering its purification in large quantities is even more important. This could be attained by scaling up the developed techniques.

As a perspective, the implementation of such sorbent to on-line SPE coupled to HPLC-UV or 2D-HILIC/RPLC followed by automated fraction collection to *Rhinella marina* (L.) venom extracts should enable to isolate promising pharmacologically active compounds with higher throughput and purity rates and on a bigger scale. Other techniques to isolate compounds from natural sources could also be considered, such as counter-current chromatography, extraction on macroporous resins combined to preparative HPLC [40,41].

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CONFLICT OF INTEREST

The authors have declared no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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