Journal of Chromatography A 1763 (2025) 466460

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Expanding the use of green solvents for the isolation of melittin from honeybee venom

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ARTICLE INFO

Keywords:
Green metrics
Green solvents
Honeybee venom
Melittin purification
Preparative liquid chromatography

ABSTRACT

The intrinsic complexity of natural products, which are made of different chemical constituents, poses significant challenges in their direct therapeutic use and their application as a source of new drug candidates, primarily due to the difficulty of isolating high-value bioactive compounds. One notable example of such complex matrix is bee venom, which contains a large variety of bioactive molecules, including melittin, alongside many allergenic compounds. The latter must be depleted to enable its safe clinical application. To this aim, purification methods need to be developed based on advanced preparative chromatographic approaches. However, one major drawback is the employment of toxic solvents, such as acetonitrile (ACN) and the generation of large amount of waste.

In this context, this work aims to advance the green transition of melittin isolation through the development of more sustainable chromatographic methods able to recover as much product as possible, while simultaneously reducing the solvents consumption and enhancing the safety for operators. In this study, ACN is replaced with greener alternatives, including two alcohols, such as ethanol (EtOH) and isopropanol (IPA), as well as a mixture of dimethyl carbonate (DMC) and IPA in order to enhance process sustainability. The four methods were compared and evaluated based on four performance parameters: purity, recovery, productivity, and solvent consumption. To assess the greenness of each method, different green metrics were also employed. Most of them demonstrate that the calculated scores are much more favorable for DMC and alcohols, confirming their suitability as green alternative to ACN.

1. Introduction

Bee venom is a solution excreted by honeybees (*Apis Mellifera)* to defend themselves. As the majority of (bioactive) natural products, it is a complex mixture of different constituents, which contribute to enhancing its therapeutic potential thanks to synergistic interactions [1]. In detail, it is made of more than 18 compounds, including enzymes, peptides, lipids, and small molecules such as amino acids [2–5]. It is widely used in traditional medicines as in apitherapy and acupuncture treatments. Its various therapeutic properties (anti-inflammatory, anti-cancer, antimicrobial, neuroprotective) are mainly given by melittin, a peptide made of 26 amino acids, which constitutes over 50 % of the honeybee venom [6–9]. According to these activities, bee venom can be considered as a potential active pharmaceutical ingredient (API) in the pharmaceutical and cosmetic industry for treating different skin diseases such as atopic dermatitis, acne vulgaris, androgenetic alopecia, wound healing, facial wrinkles and vitiligo [10].

Among all the other components present in bee venom, phospholipase A2 (PLA-2), hyaluronidase and apamin can induce allergic reactions (immunoglobulin E response) in sensitive individuals [11]. Indeed, PLA-2, an enzyme, is the primary allergen, and the most harmful component of bee venom; hyaluronidase, another enzyme, facilitates venom penetration by increasing tissue permeability and widening blood vessels, thereby promoting blood flow. The latter one, apamin, is a

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https://doi.org/10.1016/j.chroma.2025.466460
Received 29 July 2025; Received in revised form 9 October 2025; Accepted 10 October 2025
Available online 11 October 2025

biologically active peptide, functioning as a neurotoxin. The presence of these compounds has significantly restricted the therapeutic application of crude bee venom [4,12–15], making the production of purified bee venom an essential step. In this context, high amounts of pure melittin may be helpful, not only to comprehensively access melittin bioactive properties, but also for the production and the design of new melittin-based peptides (or melittin analogues) able to improve melittin characteristics and enhance its positive effects [11,16]. However, the purification of bee venom remains a challenging task, characterized by a series of complicated separation steps and demanding analytical procedures. These processes involve the utilization of various separation techniques such as gel filtration, affinity chromatography, and ion-exchange chromatography methods. However, these methods are not able to completely separate melittin from allergens, hence more than one chromatographic step is required. Researchers have also tried to increase both the yield and purity of melittin by introducing intermediate steps during the purification process. The utilization of intermediate step, such sulfitolysis and cyanogen bromide cleavage for removing remaining PLA-2 after gel filtration, can be time consuming and in addition, the overall yield (lower than 25 %) turned out to be still not satisfactory [13]. Also flash chromatography was used, for melittin purification achieving high purity (>99 %). However, the overall yield reached only 63 %. Additionally, the significant use of solvent and time was a notable aspect of this method [17]. Recently, different strong cation exchange chromatographic (CEX) procedures under different pH conditions and using sodium phosphate buffer were employed for the purification of melittin in one step achieving high purity and yield. However, this study highlights that using CEX for melittin purification can lead to several challenges due to pH sensitivity, ionic strength and buffer composition, which play a critical role in separation efficiency. These aspects can significantly influence the final yield and purity, hence requiring an extensive optimization for the purification process [18]. For all these reasons the most used technique is preparative reversed-phase liquid chromatography (RPLC), thanks to its selectivity, versatility, and resolution [19–22]. However, preparative RPLC requires large column dimensions and high flow rates, meaning that significant amounts of solvents and samples are typically utilized in this process [23,24]. This technique employs a hydrophobic stationary phase, and a mobile phase made of an aqueous solution and an organic modifier. The retention of compounds with high molecular weight, such as proteins and peptides, is significantly impacted by variations in the organic modifier percentage [25,26]. Hence, employing gradient elution is crucial for effectively separating products from impurities. Nevertheless, procedures involving the purification of biologically active compounds, particularly in preparative chromatography, are often associated with considerable solvent consumption and energy demand during downstream processes.

Moreover, such techniques have long been directed at the utilization of non-green solvents, particularly in the last stage of purification, which can generate quite a lot of solvent waste and account for above 50 % of the overall costs of production [27]. Hence, the environmental repercussions of solvents used in chromatographic analyses should not be overlooked. Acetonitrile (ACN) is the predominant organic modifier for purifying biomolecules in RPLC conditions due to its strong elution capabilities, low cut-off, low viscosity and its high miscibility with water [28]. However, ACN has some disadvantages that restrict its use, such as its toxicity and challenges associated with waste disposal. For instance, considering human health and environmental factors, ACN is considered unsuitable for extensive use, based on the International Conference on Harmonization (ICH) Q3C (guideline for residual solvents in pharmaceutical) that classified this solvent as toxic and not recommended [29]. However, selecting the appropriate solvent and alternative organic modifier as a mobile phase is crucial in initiatives promoting green chemistry as it can greatly affect the environmental impact and sustainability of a chemical process [30,31]. Greener alternatives to ACN that can be employed in RP are alcohols, such as ethanol (EtOH) and isopropanol (IPA), dimethyl carbonate (DMC), ethyl lactate, cyrene, propylene carbonate. DMC has recently been shown to be suitable for the purification of different peptides under RP conditions with similar performance to ACN [32,33]. Moreover, the elution strength of DMC was demonstrated to be roughly 2.5 times higher than ACN, with a noticeable reduction in the total amount of organic modifier used [34,35]. Despite the undoubted advantages in terms of sustainability and overall greenness, these solvents present some limitations. Regarding the two alcohols, they both present higher viscosities compared to ACN, which lead to a higher back pressure, while DMC has a high cut-off (220 nm) and a limited solubility in water (10 % v/v) [36–38].

The present study is intended to propose an alternative procedure for melittin isolation from crude bee venom, with enhanced environmental compatibility with increased greenness compared to currently available methods. In detail, the feasibility of different organic modifiers has been evaluated in terms of final yield, total purity and organic solvent consumption. The overall greenness was then assessed through the application of different green metrics. The initial study was conducted using EtOH and IPA as alternatives to ACN. The best-performing solvent was then mixed with DMC to improve the elution strength of the mobile phase, permitting the reduction of the organic solvent volume used for the preparative methods, thus increasing the sustainability of the entire procedure.

2. Green metric systems

The principles of green analytical chemistry (GAC), summarized in 12 items known as “SIGNIFICANCE” [39], state that the utilization of toxic reagents and solvents should be annihilated or superseded. Despite the availability of numerous green metrics that offer both qualitative and quantitative evaluation of the greenness of analytical processes, such as Analytical Greenness (AGREE), Analytical Method Greenness Score (AMGS), Chloroform-oriented Toxicity Estimation Scale (ChlorTox Scale), Chemical Hazard Evaluation for Management Strategies (CHEMS-1), Green Analytical Procedure Index (GAPI), RGB Additive Color Model, Blue applicability grade index (BAGI), National Environmental Methods Index (NEMI), just to name a few, none of these metrics are specifically designed for preparative chromatography. Hence, only the metrics considering the type, quantity and hazard of solvents used were employed in this work, since all other experimental parameters were kept constant (for further details see Section 3.5).

2.1. AGREE

By considering the whole 12 GAC principles, AGREE is one of the recent multifactorial green metric methodologies. These guidelines are used to determine a “greenness mark” (from 0 to 1), which is then displayed as a circle pictogram. Furthermore, parameters can be given weights to help the user distinguish between them. Every score is placed around the circular pictogram and its effect is shown by the breadth of its respective section, while the greenness of the procedure in each principle is visually represented using a red-yellow-green color scale. The overall score is displayed in the middle, with dark green hues and values near 1 suggesting that the evaluated process is more environmentally friendly [40].

A modified version of AGREE has been recently presented for the application to preparative chromatography [41]. This metric takes into account performance parameters, such as purity, recovery and productivity, allowing for the direct comparison between different purification methods.

2.2. AMGS

AMGS, unlike most green metrics, which are designed for the evaluation of chemical synthesis products, is specifically introduced for chromatographic separation-based techniques by the American Chemi-

cal Society's Green Chemistry Institute Pharmaceutical Roundtable (ACS-GCI-PR). This system takes into account the instrument energy usage as well as the solvent and waste safety, health, and environmental (SHE) assessment [42]. The calculation is based on the following Eq. (1), where R is the number of replicate analyses that the method takes, t_a is the analysis time, t_c is the cycle time (the interval between the end of one analysis and the beginning of the next), F is the flow rate, S is the SHE index of the mobile phase solvent(s), C is the mobile phase solvent(s) cumulative energy demand, E is the energy consumption of the apparatus, and N is the number of the sample analytes. An online version of the AMGS calculator is provided by the ACS, which facilitates calculations [43]. Lower values indicate a greener method.

$$AMGS = R\frac{(t_a + t_c)[F(S + C) + E]}{N} \tag{1}$$

2.3. ChlorTox

This green metric utilizes an open access database, ChlorTox Base, consisting of hundreds of chemicals and their data from the hazards identification section of their material safety data sheet (MSDS) [44]. Eq. (2.1) is the base of the assessment, representing the hazard of the target compound (CH_{sub}, where the subscript *sub* means "substance of interest") divided by that of chloroform (CH_{CHCl3}) multiplied by the mass of the target compound (m_{sub}). To calculate CH_{sub}, Eq. (2.2) is used, where N_{cat} is the number of hazards of a given category multiplied by the weight reflecting the degree of potential danger, including 1 for category 1, 0.75 for category 2, 0.5 for category 3, and 0.25 for category 4 [45]. The degree of hazard for chloroform is calculated as 5.75, according to the data provided in the MSDS supplied by Sigma-Aldrich [46].

$$ChlorTox = \frac{CH_{sub}}{CH_{CHCl_3}} m_{sub} \tag{2.1}$$

$$CH_{sub} = 1{\cdot}N_{cat1} + 0.75{\cdot}N_{cat2} + 0.5{\cdot}N_{cat3} + 0.25{\cdot}N_{cat4} \tag{2.2}$$

2.4. CHEMS-1 model

CHEMS-1 model was first presented in 1997 to include hazards associated with volatility of chemicals [47]. Nevertheless, Tobiszewski and Namieśnik developed the model to score the solvents frequently used in analytical labs using information extracted from MSDS, considering the solvents' potential for chemical exposure as well as their toxicity to people and the environment [48]. As it can be deduced from Eq. (3.1), the total analytical hazard value (taHV) is the sum of several factors related to the nature of the chemicals employed, like oral toxicity (HV_{ORAL}), inhalation toxicity (HV_{INH}), carcinogenicity (HV_{CAR}), other hazardous effects (HV_{HE}), aquatic acute toxicity (HV_{FA}), and aquatic chronic toxicity (HV_{FC}) multiplied by exposure-related parameters, such as biodegradability (HV_{BOD}), hydrolysis (HV_{HYD}), bioconcentration (HV_{BCF}), and volatility (HV_{VOL}). The total analytical hazard value is calculated for each solvent independently and can be multiplied for the volume of the solvent to assess the procedure hazard value, pHV (Eq. (3.2)), defined as:

$$taHV = (HV_{ORAL} + HV_{INH} + HV_{CAR} + HV_{HE} + HV_{ORAL} + HV_{FA} + HV_{FC}){\cdot}(HV_{BOD} + HV_{HYD} + HV_{BCF} + HV_{VOL} \tag{3.1}$$

$$pHV = (taHV_1){\cdot}V_1 + \ldots + (taHV_n){\cdot}V_n \tag{3.2}$$

where the indexes 1, …, n indicate the different solvents.

3. Material and methods

3.1. Sample preparation

Lyophilized honeybee venom was purchased from a beekeeper in Iran. The venom was collected from bees through electrical stimulation using low-voltage currents (20–30 V) applied under the hive in order to stimulate bees to release venom onto a glass plate beneath a fine wire mesh. Venom is then collected periodically and then lyophilized. It is worth noting that this method is not harmful to the bees.

The honeybee venom powder was dissolved in 5 % organic modifier to obtain a concentration of 1 g/L based on powder weight and stirred for 30 min at room temperature. The solution was then filtered using a 0.22 μm Nylon filter prior to injection.

3.2. Materials

ACN, EtOH, and IPA were purchased from Carlo Erba Reagents (Rodano, Milano, Italy), trifluoroacetic acid (TFA) was purchased from Merck-Sigma Aldrich (St. Louis, MI, USA), while DMC (purity > 99 %) was purchased from ThermoFisher Scientific (Waltham, Massachusetts, USA). A high-purity (>97 %) standard of melittin was obtained from MedChemExpress (Monmouth Junction, NJ, USA).

3.3. Key parameters

The purpose of preparative chromatography is to process large sample amounts to isolate or purify the target product. The eluate is collected in fractions and analyzed offline to determine four performance parameters, to assess purification efficiency and effectiveness. These parameters include purity, recovery, productivity, and solvent consumption and are calculated for each fraction.

Purity refers to the proportion of the target product within the total output expressed as a percentage area and it is defined in Eq (4). It is calculated by dividing the area of the target product (A_{target}) peak by the sum of all peak areas (A_{total}) in a chromatogram obtained through analytical chromatography of every fraction:

$$Purity\ (\%) = \frac{A_{target}}{A_{total}}{\cdot}100 \tag{4}$$

Yield, or recovery, expressed as a percentage (Eq. (5)), is calculated by comparing the mass of the recovered product ($m_{collected}$) in a given pool or fraction, to the mass of the product injected ($m_{injected}$)

$$Recovery\ (\%) = \frac{m_{collected}}{m_{injected}}{\cdot}100 \tag{5}$$

Eq. (6) represents productivity that evaluates the quantity of the target product obtained ($m_{collected}$) over time per unit volume of the column, CV, measured as the geometrical volume, and where time is the duration of the preparative run.

$$Productivity\ (g/L/h) = \frac{m_{collected}}{CV \cdot time} \tag{6}$$

The last parameter to be considered is the solvent consumption, which represents the total volume of solvent used to purify the mass collected, $m_{collected}$. The lower the solvent consumption, the more economically advantageous and environmentally favorable the process. It is defined in Eq. (7):

$$Solvent\ Consumption\ (L/g) = \frac{Total\ volume}{m_{collected}} \tag{7}$$

3.4. Investigation of retention behavior under linear conditions

The retention behavior of melittin (1 g/L of pure standard) was evaluated at different mobile phase compositions by varying the type of organic modifier. ACN, EtOH, IPA, and a mixture of DMC and the best performing alcohol were tested. All measurements were performed with an Agilent 1100 Series HPLC system (Agilent, Santa Clara, CA, USA) equipped with a binary solvent pump, a column thermostat, an auto-sampler, and a photodiode array detector. The detection wavelength was 214 nm (melittin maximum absorption) and the flow rate was 1.5 mL/min. The temperature was set at 25 °C. A YMC-Triart Prep C18-S column (250×4.6 mm) with a particle size of 10 μm was used as the stationary phase. The injection volume was 8 μl, while the dead volume of the column was determined by injecting uracil.

The retention factor, k, is defined as:

$$k = \frac{V_r - V_0}{V_0} \tag{8}$$

where V_r the retention volume and V_0 the dead volume of the column.

3.5. Purification conditions

Melittin isolation was performed using a Contichrom CUBE 30 system (YMC ChromaCon, Zurich, Switzerland) equipped with a detector set to 280 nm at the column outlet and a Foxy R1 fraction collector. The column used for purification scopes is the same as the linear studies, namely a YMC-Triart Prep C18-S (250×4.6 mm) column with a particle size of 10 μm and a column volume of CV = 4.15 mL. A solution prepared as described in Section 3.1, with a concentration of 1 g/L based on powder was injected using a dedicated pump at a flow rate of 1 mL/min. The loading was set at 1 % of the CV, corresponding to 41.5 mg of dried bee venom. The mobile phases employed for melittin purification are as follows: mobile phase A (MPA) consists in water + 0.1 % TFA, while mobile phases B (MPB) consist of pure ACN or EtOH or IPA or a mixture of DMC/IPA/water (25/25/50 % v/v/v) with the addition of 0.1 % TFA. For the latter one, the addition of IPA as a cosolvent was used to increase the solubility of DMC in water, since only 10 % of DMC (its solubility limit in water) was not sufficient to elute melittin. Regarding the MPB using DMC/IPA/water, the mixture was sonicated for one hour until the DMC was fully dissolved and the bubbles were removed. For all the methods, the duration was the same and the steps were as follows: 2 CV of equilibration set at 5 % MPB (flowrate = 1.5 mL/min), 10 CV of loading (flowrate = 1.0 mL/min), 6 CV of gradient elution (flowrate = 1.0 mL/min), 2 CV of stripping at 100 % MPB (flowrate = 1 mL/min), and 2 CV of re-equilibration. During the gradient elution, fractions were collected periodically every minute. To assess the reproducibility of the methods, three replicates were performed.

The performance of the green organic modifiers was assessed through the comparison with ACN.

3.6. Off-line analysis

Bee venom feed and all fractions were analyzed using the same system described in Section 3.4. A YMC-Triart Bio C18-S column (150×4.6 mm, 5 μm) was used as stationary phase. MPA was water + 0.1 % TFA and MPB consisted in ACN + 0.1 % TFA, the flow rate employed for the analysis was set at 1 mL/min. The gradient program was developed based on literature data [49–52], to obtain the separation of apamin, PLA2 and melittin: from 20 % to 80 % MPB in 10 min, 100 % MPB for 4 min and then the column is equilibrated for 5 min at 20 % MPB. The injection volume was 3 μl. The standard solution was prepared by dissolving the melittin standard in water. A calibration curve was obtained in the range of 0.1 to 2.5 g/L. The bee venom feed chromatogram is reported in Fig. 1. The initial chromatographic purity of melittin was 60 %, calculated with Eq. (4).

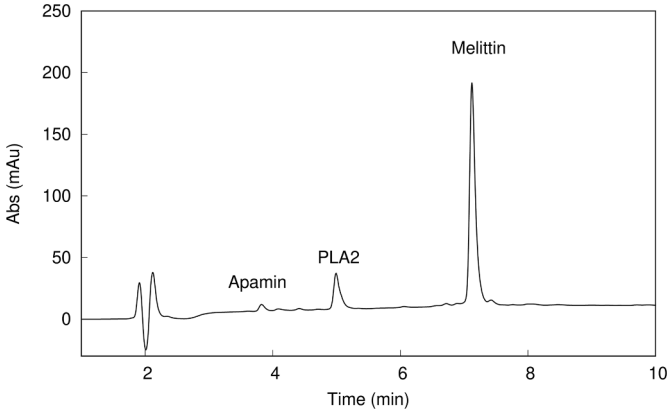

Fig. 1. Analytical chromatogram of 1 g/L bee venom feed at 214 nm.

4. Result and discussion

4.1. Investigation of the retention behavior

In the first part of the study, the dependence of the retention factor of melittin (Sect. 3.4), expressed as ln$k$, on the fraction and type of organic modifier in the mobile phase, ϕ, was initially evaluated for the three most common organic solvents used in RP, i.e. ACN, EtOH, and IPA. From the results, reported in Fig. 2, it can be observed that the elution strength of the solvents is EtOH<ACN<IPA. IPA was then chosen as a co-solvent for DMC to further increase its elution strength, since the solubility limit of DMC in water is around 10 % which was not sufficient to promote melittin elution. The applicability of the mixture DMC/EtOH was recently demonstrated in [53], while the mixture of DMC/IPA was used for the first time for the purification of large polypeptides (up to 32 amino acids) [33]. In the present work, a mixture of 25/25/50 % v/v/v IPA/DMC/water was considered as MPB. It is worth noting that 25 % IPA was useful also to improve the miscibility of DMC with water, from 10 % up to 25 %. As expected, the curve of DMC/IPA, reported in Fig. 2, shows the highest elution strength if compared to the other solvents. It is worth mentioning that on the x-axis, the total percentage of organic phase (meaning DMC+IPA in the same amount) is reported.

The higher elution strength observed by adding DMC to IPA allows to achieve the same retention as for other pure organic modifiers but with a substantial reduction in the volume of organic required. Indeed, a retention factor of 1 for melittin (ln$k$ = 0) is obtained using 60 % EtOH, 45 % ACN, 40 % IPA and only 27 % DMC/IPA, as reported in Fig. 2.

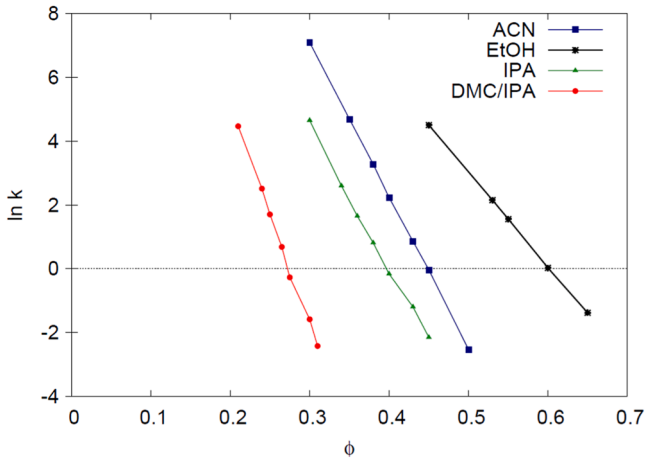

Fig. 2. Dependence of the retention factor (ln k) of melittin on the fraction of organic modifier (ϕ) in the mobile phase, using DMC/IPA (red points), IPA (green triangles), ACN (blue squares) and EtOH (black cross).

Therefore, a combination of DMC and IPA is beneficial in terms of organic modifiers consumption, especially in the large-scale process, to improve the sustainability and greenness of the process.

4.2. Evaluation of the purification process

Based on retention data in Fig. 2, initial and final gradient conditions were chosen to keep melittin retention constant under semi-preparative conditions. The variation of the organic modifier ($\Delta\phi$) during the gradient was set as follow: for ACN from 0.35 to 0.5; EtOH from 0.45 to 0.70; IPA from 0.30 to 0.45 and DMC/IPA from 0.21 to 0.31. It is worth mentioning that all other experimental parameters, i.e. loading, duration, flow rate, etc., were kept constant for the four purifications.

Results of the four purifications are reported in Fig. 3 (A-D), where it is possible to notice that when using ACN the peak shape is sharper, while for the other solvents, broader peaks and lower intensities are observed. As a matter of fact, lower efficiencies are obtained with alcohols as organic modifiers due to their large viscosity which limits the surface mobility of the analyte (smaller sample diffusion) [35].

The fractions comprising the main peak were collected and subsequently analyzed offline (see Section 3.6). From the fraction analysis, the so-called Pareto Curves can be constructed and are shown in Fig. 4A. These graphs show the purity-yield trade-off, where a decrease in purity (calculated with Eq. (4)) is observed by widening the collection window, i.e. increasing the yield (Eq. (5)) [54,55]. The relevant data are summarized in Table 1. Concerning alcohol-based mobile phases, from Fig. 4A, it can be stated that the same performance in terms of final purity and yield is obtained for EtOH and IPA, as already demonstrated in [32]. ACN leads to better results with respect to pure alcohols (the curve is more shifted towards right), while the curve of DMC/IPA crosses all the other three curves. More in detail, the mixture DMC/IPA leads to the highest purity for recovery $\leq$ 70 %, conversely, at the maximum recovery the overall purity is the lowest. This can probably be attributed to a change in the elution order of impurities by changing the organic modifier. Nevertheless, the four Pareto curves differ by only 1 % and 3 % at the two extremities, i.e. at the smallest and largest yield, respectively. Indeed, in the first point (on the left), i.e. at the smallest yield, the maximum purity is achieved with the combination of DMC/IPA (98.5 %), while ACN, IPA and EtOH can reach slightly smaller purities in the range 97.5–98 %. In the last point (on the right), i.e. at the highest yield ($\approx$ 100 %), almost 96 % purity is obtained with ACN, while alcohol-based mobile phases lead to 95.1, 94 % and 93.2 % purity for IPA, EtOH and DMC/IPA, respectively. This indicates that ACN leads to better performance when the total recovery of melittin is required, even if it differs by only 3 % in purity compares to DMC/IPA mixture. Nevertheless, since the scope of the work was to remove allergens (weak impurities like apamin and PLA2) from the bee venom, new Pareto curves were constructed omitting fractions containing these impurities (Fig. 4B). Since there is no regulation about the maximum amount of these allergens in melittin, the fractions containing a total concentration of weak impurities $\geq$ 0.1 g/L (calculated based on the melittin calibration curve) were discarded for the new Pareto curves. Based on these new Pareto curves, the maximum achievable purity, yield, productivity and solvent consumption for the four solvents have been calculated and are listed in Table 1. It is then possible to notice that with the purifications carried out with ACN and EtOH there is loss in terms of melittin mass of about 10 mg, achieving a comparable final yield of about 50 %, while IPA and DMC/IPA the melittin loss is around of 3 mg, allowing to reach a yield of 87.2 % and 82.6 % respectively. Therefore, between ACN and DMC/IPA at a fixed purity of 96.8 % a difference in yield of about 39.6 % is obtained, the same result happened with EtOH. This indicates that, with ACN and EtOH, the front of melittin peak is more largely overlapped with the impurity peaks, meaning that in these cases allergens are mostly contained in the more concentrated melittin fractions. In Fig. 5 the analytical chromatogram of the purest fraction obtained with DMC/IPA overlapped with the feed is shown. As can be seen,

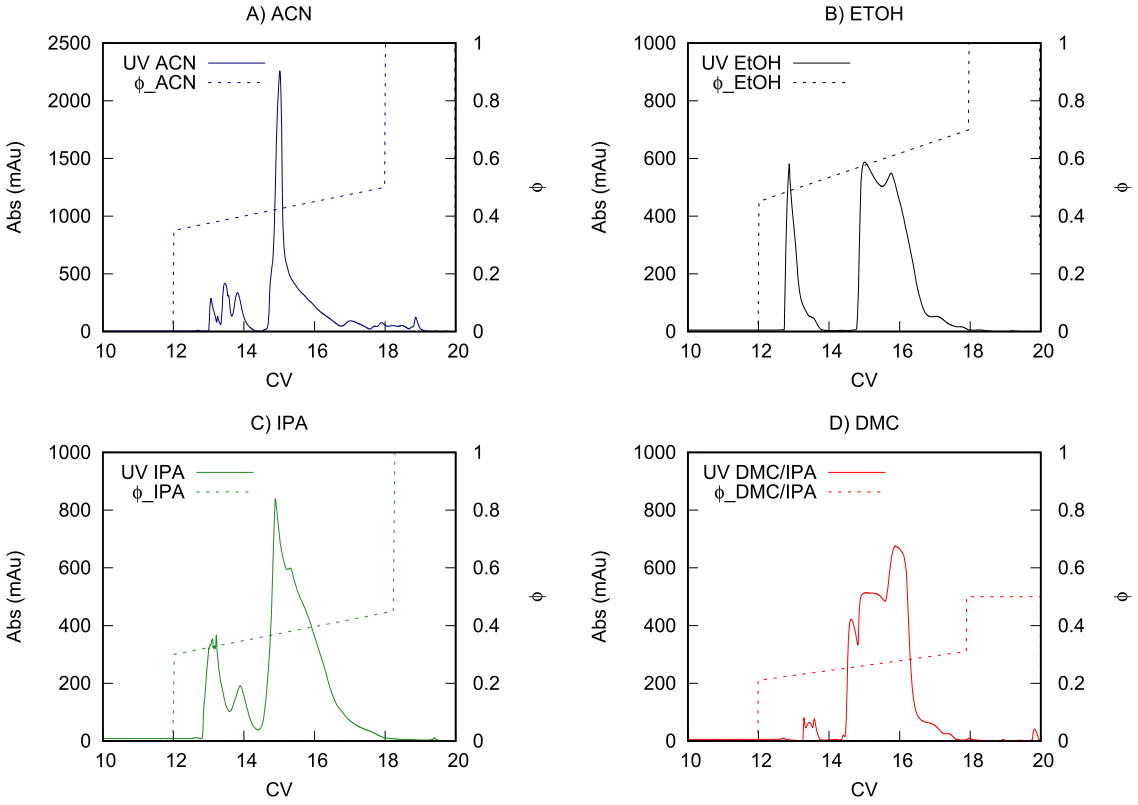

Fig. 3. Preparative chromatograms related to bee venom purification using ACN (A), EtOH (B), IPA (C), and DMC/IPA (D) as organic modifiers, melittin is the main peak eluting at 15–16 CV. The dotted line represents the gradient, while the solid line represents the UV profile at 280 nm.

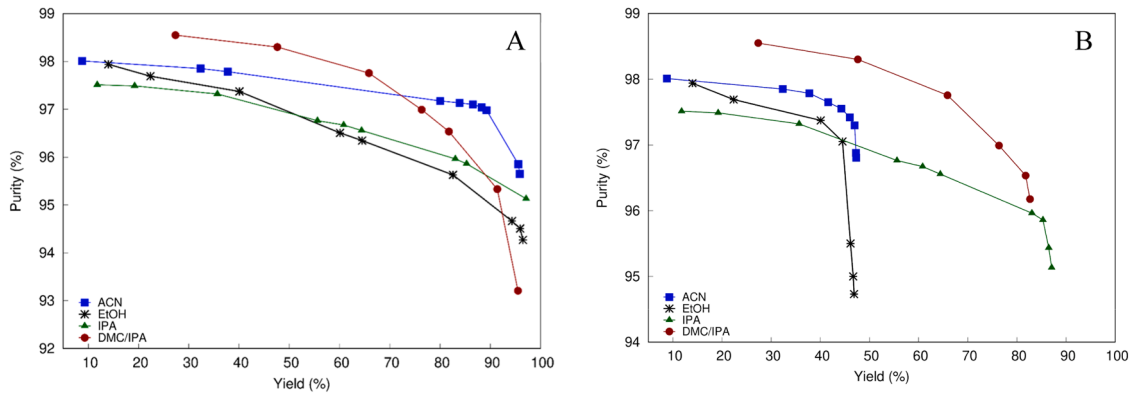

Fig. 4. A) Pareto curves for the four organic modifiers used in the purification process: DMC/IPA (red), ACN (blue), IPA (green), and EtOH (black); B) new pareto curves for the four organic modifiers built on the fractions that does not contain weak impurities (PLA2, apamin).

Table 1
Comparison of purification performance of the four organic modifiers based on yield, purity, productivity, and solvent consumption, using data derived from the overall Pareto Curve and the Pareto Curve where the fraction containing the allergens are not taken into account. Regarding the first one, purity and productivity are calculated both at maximum yield reached and at the first point of the pareto curve (i.e. the purest one).

	Pareto Curve of the Overall purification (Fig. 4A)				Pareto Curve not counting PLA-2 and Apamin (Fig. 4B)			
Organic modifier	Yield (%)	Purity (%)	Productivity (g/L/h)	Organic solvent Consumption (L/g)	Yield (%)	Purity (%)	Productivity (g/L/h)	Organic solvent Consumption (L/g)
EtOH	13.9	97.9	0.46	1.3	46.8	94.7	1.5	2.6
	99.0	94.0	3.17					
IPA	11.7	97.5	0.39	0.9	87.1	95.1	2.9	1.1
	97.1	95.1	3.45					
ACN	8.7	98.0	0.312	1.0	47.3	96.8	1.7	2.1
	95.8	95.6	3.41					
DMC/IPA	27.3	98.5	0.94	0.6	82.6	96.1	2.8	0.7
	95.5	93.2	3.34					

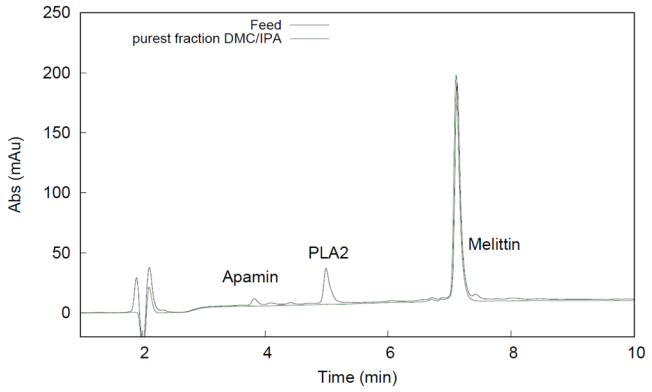

Fig. 5. Overlay of analytical chromatograms of the feed (black line) and the purest fraction obtained with DMC/IPA (green line) as mobile phase modifier.

the two allergens were effectively depleted from the mixture.

For the sake of comparison, in Fig. S1 the purest fraction obtained with DMC/IPA is overlapped with the purest one in ACN, the feed and the blank.

Solvent consumption was calculated based on the volume of organic modifiers used (Eq. (7)). From data in Table 1, it can be observed that the highest solvent consumption, as expected, was obtained when using EtOH, since this solvent has the lowest elution strength. In particular, solvent consumption increased by 27 % if compared to ACN. On the other hand, for IPA and the mixture DMC/IPA, which have a higher elution strength, the consumption was reduced by −13 % and −40 %, respectively. This aspect becomes even more evident when examining the results obtained with the new Pareto curves. For DMC/IPA and IPA, the values were approximately the same due to the final yield being around 83 % and 87 %, whereas for the other solvents, solvent consumption doubled for the same reason. The same reasoning can also be applied to productivity. Calculating productivity on the basis of the total yield (Eq. (6)) shows that it is approximately the same for all four solvents, as the total yield and the duration of the method are identical. However, if productivity is calculated excluding the fractions containing apamin and PLA-2, as shown in Table 1, it becomes evident that using DMC/IPA and IPA the productivity values almost doubled compared to ACN and EtOH. Furthermore, from Fig. 4 it can be seen that the pareto curve of DMC/IPA (red) lays above the other pareto curves, meaning that the purity obtained with DMC/IPA is the highest.

Nevertheless, despite differences in bee venom composition may occur depending on bee species, leading to a variation in melittin quantity and quality, the methods hereby proposed hold valid. Indeed, the use of CVs as reference unit instead of time provides more robustness to the method in terms of standardization. Therefore, this aspect allows to scale the method up to industrial level more easily.

It should be noted that the use of larger volumes of water in the DMC/IPA method may represent a limitation, particularly in the final stages of the purification process, due to the increased energy required for its removal. Nevertheless, the disposal of toxic and hazardous solvents is significantly more complex and expensive compared to greener alternatives. Currently, ICHQ3D does not set any restriction on the amount of DMC that can be contained in a pharmaceutical substance; however, based on its safety data sheet, the acceptable threshold should be higher than that for ACN (410 ppm) [33].

4.3. Greenness scores

Assessing the greenness of a chemical procedure is not trivial, since several factors and parameters can be considered, and also a different weight to each of them can be given too. However, up to now, a unique comprehensive metric applicable to all chemical procedures, from

sample preparation to sample purification, has not been developed yet. Indeed, most available green metrics are specifically designed for analytical methods, considering the number of replicates, the number of analytes identified, as well as the accuracy, precision, sensitivity, selectivity and reliability of the analytical method. This represents a significant limitation for the accurate evaluation and comparison of sustainability scores for preparative methods. Recently, some of the authors of this paper have proposed a modified AGREE metric for the application to preparative chromatography, which also considers performance parameters (purity, recovery, productivity). In this work, only the metrics able to discriminate the different types of organic modifiers and performance parameters were selected: AGREE, modified AGREE, AMGS, ChlorTox, and CHEMS-1 (see Section 2). The scores applied to the four purification methods are reported in Table 2 and the detailed explanation of the data and results are shown in supplementary information.

As predictable, the different metrics give different results. Unlike the other green metrics, AGREE scores converge too closely to offer strong differentiation among solvents. Indeed, AGREE approach keeps more into account the volume of the solvents utilized (which in the processes developed in this work are comparable) than their nature. It also considers other experimental conditions which were kept identical when changing the solvent (e.g. sample prep, energy consumption of the instrumentation, sample size, etc.). Therefore, this results in fairly close scores. ACN attained the worst score (0.5) followed by EtOH and DMC/IPA with the same score (0.58), whereas IPA attained the best score (0.61). Although the results suggest that IPA is marginally more advantageous, the narrow range of scores across the solvents highlights a limitation of the AGREE metric in differentiating their greenness levels. The absence of sensitivity to sharply distinguish among different solvents in this context suggests that the AGREE scores may have limited utility for greenness assessment in such cases. Better differentiation among scores is obtained using the modified AGREE metric. As for AGREE, ACN results in the worst score (0.34) closely followed by EtOH (0.43), while IPA and DMC/IPA reached almost double score compared to ACN (0.61, 0.62 respectively).

Concerning AMGS, DMC was not included in the available solvent input, therefore IPA was selected as a substitute. This choice is supported by the GSK solvent selection guide, which indicates that DMC and IPA share comparable green chemistry profiles [56]. Differently from AGREE, with AMGS the lower the score the greener the method, and as expected ACN led to the highest score (319.71). The other three solvents reached comparable scores, which can be listed in the following order: EtOH (209.52), IPA (205.17) and DMC/IPA mixture (202.49). In this regard, the score achieved by DMC/IPA reduced the value obtained for ACN by 36.6 %.

Employing data extracted from MSDSs, ChlorTox quantifies the hazard score of chemicals relative to chloroform. Given the variability in suppliers and consequently in the MSDS content for the same chemical reagent it is essential to use an equal number of MSDSs per chemical when calculating average hazard scores. This approach ensures consistent and unbiased evaluation of the toxicological profile of every substance. Among tested solvents, ACN represents the highest score (5.94) while as for AMGS the other solvents yielded a comparable score IPA (4.93), EtOH (4.74) and DMC/IPA records the lowest score (3.59), indicating that this mixture is the least toxic candidate and marking it as the most favorable option in this metric system reducing the value obtained with ACN by 39.6 %.

Finally, through CHEMS-1, all the hazard values pertinent to each organic solvent were calculated according to their SDS and the results multiplied by the volume of the organic modifier to assess their *pHV* values (CHEMS-1 score). As it can be observed by the scores reported in Table 2, this is the metric that shows the greatest difference between ACN and the other solvents, as it accounts not only for toxicity with exposure related parameters and the amount of the solvents used but also for the environmental impact factors. In this regard ACN scored the highest value (517.50), followed by EtOH (165.81), IPA (122.74) and DMC/IPA mixture (86.14). Compared to ACN, the use of DMC/IPA decreases the *pHV* value by 83.4 %, indicating it as the greenest approach.

5. Conclusions

Chromatography is constantly evolving towards greener approaches and methodologies. However, to date, most studies have been focused on evaluating the greenness or implementing the green transition at analytical scale, leaving a gap in preparative or industrial scale applications. In this context, the present study investigates the use of environmentally friendly solvents for the purification of melittin from crude bee venom and assesses the improvements achieved with these alternative solvents. Notably, it has been proven that the combination of IPA and DMC as organic modifiers can be a valuable choice for replacing ACN, given both the high elution strength of DMC that contributes to lowering the organic modifier consumption and its low toxicity profile, which grants environment and operator safety. To assess the greenness of the methods, different green metrics have been employed in this study (AGREE, AMGS, Cholortox and CHEMS-1), although not specifically designed for preparative applications. With the exception of AGREE, which showed similar results for all purification methods, the other metrics indicated an increase in the greenness scores when using alternative solvents to ACN, more specifically with the employment of DMC/IPA mixture. Among them, CHEMS-1, which corresponds to the total hazard value (pHV) and accounts for both the volume and toxicity of the chemicals involved, proved to be the most effective in highlighting the differences between the methods.

This study expands the application of green solvents by demonstrating their feasibility in purifying a bioactive peptide from a highly complex matrix. Chromatographic performance and sustainability aspects are closely correlated and must be therefore evaluated to support the transition to green analytical approaches. Moreover, the results reported in this study are readily scalable to industrial processes, enabling the production of high purity melittin in considerable quantities. The purified compound can be further employed to assess its therapeutic potential across different diseases and for the design of novel melittin-derived products with enhanced pharmacological properties.

Funding

The project was funded by Iran National Science Foundation (INSF) [Project No. 4002943].

The authors would like to thank the National Recovery and

Table 2
Volume and mass of organic modifiers used in the purification of the melittin, and estimated scores calculated as per AGREE and Modified AGREE, AMGS, ChlorTox, and CHEMS-1.

Modifier type	Volume of Organic Modifier (mL)	Mass of Organic Modifier (g)	AGREE Score	Modified AGREE Score	AMGS Score	ChlorTox Score	CHEMS-1 Score (*pHV*)
ACN	19.31	15.11	0.50	0.34	319.71	5.94	517.50
DMC/IPA	11.13	10.26	0.58	0.62	202.49	3.59	86.14
IPA	18.05	14.16	0.61	0.61	205.17	4.93	122.74
EtOH	23.03	18.19	0.58	0.43	209.52	4.74	165.81

Resilience Plan (NRRP), Mission 04 Component 2 Investment 1.5 - NextGenerationEU, Call for tender n. 3277 dated 30/12/2021; Award Number: 0001052 dated 23/06/2022. The authors would also like to thank the Italian University and Scientific Research Ministry (grant P2022PTYWP, title: "Design of highpRofit fostEring bioActive coMpounds through integral valorization of seaWEEDs infesting the MEditerranean sea (DreamWEEDme)" and grant P2022AJA7H8, title "High revenue-generating molecules from industrial hemp: cost-effective, chemical classification-driven solutions for their production (CHempion)") as well as the Regional Programme of the European Regional Development Fund (PR FESR 2021-2027 Priority 1) project title "Production of high-added-value ingredients from fruit supply chain by-products through a cascade biorefinery approach - Frurefinery" for financial support.

CRediT authorship contribution statement

Amin Tabesh: Writing – original draft, Formal analysis, Data curation. **Chiara De Luca:** Writing – original draft, Methodology, Formal analysis. **Amirmohammad Faraji Shovey:** Software, Formal analysis, Data curation. **Rachele Canton:** Software, Investigation. **Martina Catani:** Supervision, Funding acquisition, Conceptualization. **Alberto Cavazzini:** Supervision, Project administration, Funding acquisition. **Hassan Rezadoost:** Writing – review & editing, Supervision, Resources. **Chiara Nosengo:** Writing – review & editing, Validation, Supervision. **Simona Felletti:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2025.466460.

Data availability

Data will be made available on request.

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