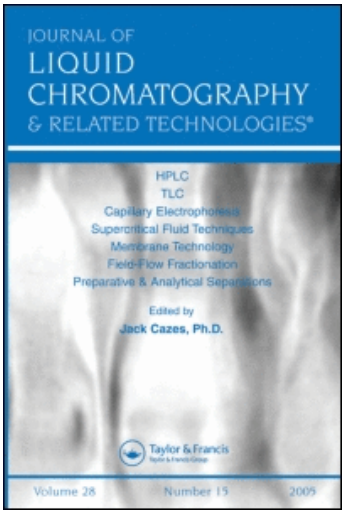

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LIQUID CHROMATOGRAPHIC ANALYSIS AND SEPARATION OF POLYPEPTIDE COMPONENTS FROM HONEY BEE VENOMS

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Abstract

Reversed-phase HPLC on different columns with acetonitrile-water-trifluoroacetic acid eluent system was used to characterize honey bee venoms and to separate and determine quantitatively its peptide components.

Introduction

Honey bee venom is a rich source of pharmacologically active peptides. It is a complex mixture of biogenic amines and peptides with both pharmacological and immunological activities [1-6]. It contains a wide range of proteins and peptide toxins in big quantity of water (80-85 %), only approximately 0.1μg of dry venom can be isolated from a single bee. Honey bee venom is collected by an electrical milking method or by extraction from bee venom glands or sticks. Its characterisation needs the quantitative determination of the components in it.

The main components of bee venom are the low-molecular weights proteins: melittin, apamine and MCD (mast cell degranulating-) peptide and the enzymes:

phospholipase A_2 and hyaluronidase beside small peptides, physiologically active amines, sugars, phospholipids, amino acids and pheromones.

For the characterization of this complicated biological matrix many methods have already been described. From chemical point of view the peptide components can be separated by means of electroforetic and chromatographic procedures [1,7-11]. Because of water-soluble biomacromolecules present in venom, gel filtration was at first the most important technique for their purification and separations [12-14]. In order to obtain pure components from the crude venom, a conventional preparative gel chromatographic method based on the work of Habermann and Reiz was used and further developed [1,6]. For its fractionation and analysis different gels have been used as various types of Sephadex and Sepharose [12-13]. In these cases the components elute according to their molecular size. The elution order was as expected, although the components could not be adequately separated. (See Fig. 1)

3 components: melittin, apamine and MCD peptide could be readily separated on column of Heparin-Sepharose CL-6B [15]. IEC gives only a limited separation, too. In principle, the preparative method found by Habermann, would be suitable for the assay of bee venom, but as the separation time is up to 24 hrs., it is impracticable for quality control purposes. Therefore the development of a rapid and efficient method for the qualitative and quantitative characterisation of bee venom was very desirable for both chemists and beekeepers. Unfortunately the conventional gel types lack mechanic stability, witch was a major limitation when operating with high mobile phase velocity. In order to achieve the high performance conditions for the separation, suitable rigid supports (as Shodex OH and ToyoSoda TSK SW) were developed for analysis of differents venoms [13].

Recently different high performance liquid chromatographic methods based upon FPLC media have been applied: at first gel filtration with Superose column and ion exchange with MonoS column. The elution profiles from the various separation techniques differed significantly [14]. Exclusion chromatography by Rader on an I-125 protein column gave good results: almost complete separation was possible within 12 min under isocratic conditions [17]. According to the experiences of many authors, separation trials with silica gel [8], amino-phase, ion-exchange resins [16] and reversed phase were unsatisfactory because of partial resolutions. Sometimes they gave good chance for final purification, but they were not enough for fingerprint analysis or quantitative characterisation of bee venom [17].

As RP-HPLC is one of the most powerful technique for separation of peptides and proteins, we examined the possibilities of reversed phase HPLC for the finger-print characterisation and assessment of purity of bee venoms originated from different sources.

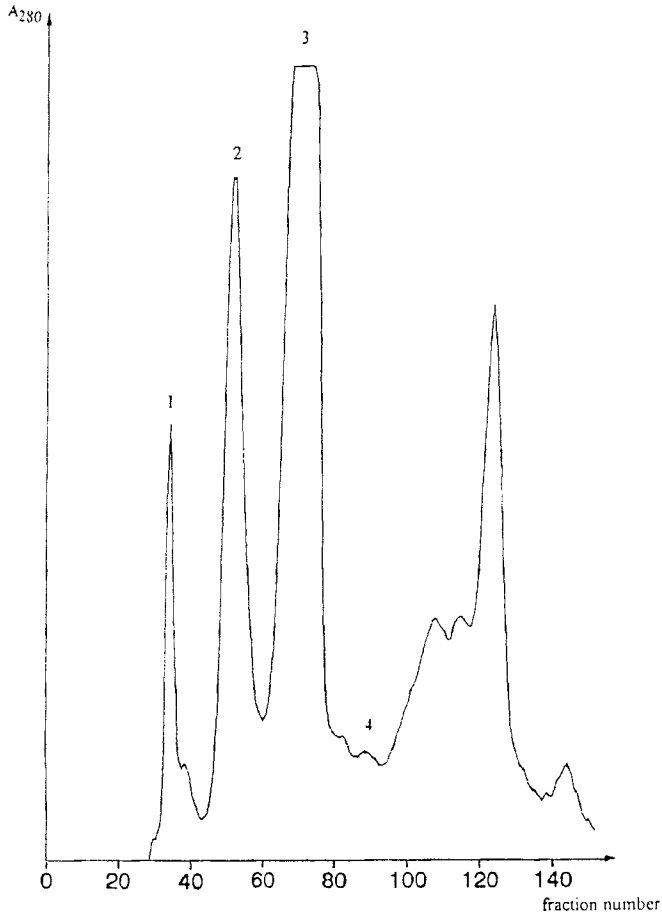


FIGURE 1. Conventional preparative chromatographic pattern of bee venom. Column: Sephadex G-50 (1400x200 mm). Eluent 0.1 M ammonium formiate buffer (pH 4.5). Flow rate: 0.4 ml/min. Fraction volume: 4ml. Identified peaks: hyaluronidase (1), phospholipase A_2 (2), melittin (3), apamine (4).

The structure of main bee venom components:

Ala-Leu-Cys-Asn-Cys-Asn-Arg-Ile-Ile-Ile-Pro-His-Met-Cys-Trp-Lys-Lys-Cys-Gly-Lys-Lys-NH2 Tertiapin

Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH2 Melittin-F

Tyr-Ile-ILe-Asp-Val-Pro-Pro-Arg-Cys-Pro-Pro-Gly-Ser-Lys-Phe-Ile-Lys-Asn-Arg-Cys-Arg-Val-Ile-Val-Pro Secapin

Ala-Gly-Pro-Gln-Histamine Procamine A

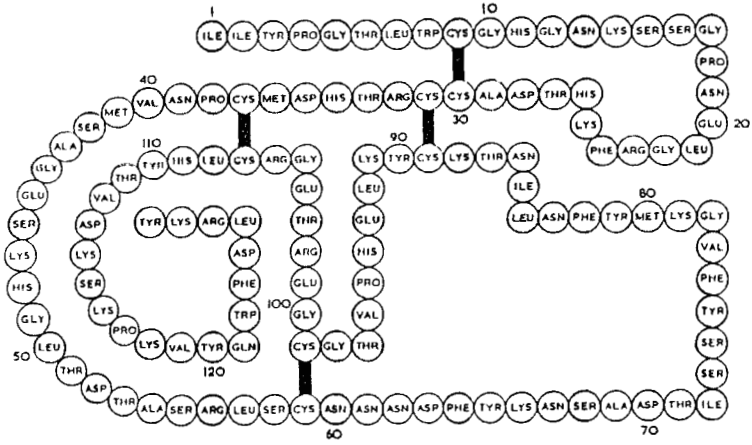
Ala-Gly-Gln-Gly-Histamine Procamine B

Ala-Gly-Pro-Ala-Gln-Histamine Nelson's peptide

H-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH2 Melittin

Cys-Asn-Cys-Lys-Ala-Pro-Glu-Thr-Ala-Leu-Cys-Ala-Arg-Arg-Cys-Gln-Gln-His-NH2 Apamine

Ile-Lys-Cys-Asn-Cys-Lys-Arg-His-Val-Ile-Lys-Pro-His-Ile-Cys-Arg-Lys-Ile-Cys-Gly-Lys-Asn-NH2 MCD-peptide



Phospholipase A_2

Experimental

Materials

Commercial-grade dried bee venoms were obtained from Hungarian beekeepers (Locations:Tököl, Szolnok, Hungary). Hyaluronidase and melittin were obtained from Fluka (F. R. G.), apamine, MCD peptide and phospholipase A_2 were all obtained from Sigma (St. Louis, MO, U.S.A.). Solvents were supplied from Reanal (Hungary).

Preparation of bee venom

Method A.

After electrical milking and collection the crude venom was dried or lyofilized from water.

Method B.

Extraction from powdered bee glands and sticks

a, with water, then lyofilization

b, with a mixture of n-butanol-pyridine-acetic acid-water (60:20:6:24 v/v), then evaporation, solvent change to water and lyofilization.

Apparatus and methods

Conventional preparative column chromatography

300 mg dissolved bee venom is placed on a Sephadex G-50 column (1400 mm x 20 mm I.D.). An aqueous 0.1 M ammonium formiate buffer of pH 4.5 is used as the eluent. The flow-rate was 0.4 ml/min. Each fraction was 4 ml. The separation is finished after 160 fractions and detection is carried out at 280 nm by Spektronom 203 (see Fig. 1). On the base of chromatographic pattern the fractions were pooled and lyophilized twice from water.

High-Performance Liquid Chromatography

Separations were performed on a KNAUER-HPLC instrument consisting of two pumps (Model 64) with analytical pumphead, a gradient programmer 50B, a variable-wavelength UV monitor fitted to an 8 µl flow-cell, an injection valve with 20 µl sample loop (Knauer GmbH, Bad Homburg, F.R.G.) and a sample injector (Knauer, F.R.G.). Peaks were recorded on a Model OH-314/1 chart recorder (Radelkis, Hungary).

Qualitative analysis

The bee venom was separated by a Knauer chromatograph using four reversed phase columns: Hypersil ODS, 125 mm x 4.6 mm, 6µ particle size, Hypersil WP-300 C18, 125 mm x 4.6 mm, 5µ particle size (Shandon), Delta-Pak C18 300, 300 mm x 3.9 mm, 10µ particle size (Waters) and Econosphere 300 C4, 250 mm x 4.6 mm, 7µ particle size (Alltech). The bee venom solution was prepared by dissolving 5mg of bee venom in 1ml distillated water or 1 ml A eluent. The mobile phases were: A: 0.1% TFA in water and B: 0.1% TFA in acetonitrile:water (80:20). The bee venom was separated by linear gradient 5% B - 80% B at 40 min. The flow-rate was maintained at 1.5 ml/min. The elution was monitored at 220 or 280 nm with recorder response set at 0.32 absorbance units full scale.

Quantitative analysis

The apamine, melittin and phospholipase A_2 standards were used for calibration. The apamine standard solution was prepared by dissolving 0.25 mg of apamine in 1 ml distillated water. The phospholipase A_2 standard solution was prepared by dissolving 1.00 mg of phospholipase A_2 in 1 ml distillated water. The melittin standard solution was prepared by dissolving 4.00 mg of melittin in 1 ml distillated water or eluent A. The mobile phases, flow-rate and monitor were the same as in case of qualitative analysis. For Hypersil WP-300 C18 column the mobile phase was 84% A eluent and 16% B eluent in case of apamine, 60% A and 40% B eluent at 5 min and after 5 min 45% A and 55% B eluent for phospholipase A_2 55 % A and 45% B eluent at 5 min and 45%A and 55% B eluent in case of melittin.

Results and discussion

We found, that RP-HPLC is the most efficient technique for separation of peptides, proteins and other components in bee venom. High resolution and efficiency were achieved using C_4, C_8 or C_{18} chemically bonded phases with acetonitrile-water or acetonitrile-water-methanol eluent mixtures containing ion-suppresser and ion-pairing reagent. Methanol-water eluent is not sufficient one, because methanol is not enough strong modifier, acetonitrile is better. The elution window was very narrow for the optimal resolution and it has different values for the different columns (Table I-II, Fig. 2-3).

The separation of all main components takes place satisfactorily because of their similar chemical structure and behaviour a very similar range of molecular weights. For chromatography of these biopolymers the columns with wide pore diameter (300 Å) gave better patterns, as the conventional ones (100 Å). The resolved components are readily detectable in the low UV-range (200-220 nm) with reproducible results on the base of peptide-amide chromophore units present in the molecules. Furthermore the absorption of these polypeptides is very similar. At higher absorbance wavelengths (280 nm) the proteins could be detected selectively, so the measurement of major melittin content (at 280 nm) can give important information for very rapid characterization of bee venoms. The elution profiles from the various RP-columns differed only very slightly (Table II). Similar pattern was obtained with FPLC on ProRPC (C8) column by Einarsson [14].

All polypeptide components of the bee venom contain more basic amino acid residues, therefore ion paring reagents were required to resolve them by RP-HPLC. Ragnarsson used different alkanesulphonic acids for determination single peptides, as apamine or MCD-peptide [18]. Bennett worked always with trifluoracetic acid as good ion pair forming agents in RP-HPLC of polypeptide hormones [20]. In our case the *RP-HPLC* was performed with it as especially favourable ion suppressor and ion-pairing reagent in the eluent. By this way the main components of bee venom could be easily analysed with reproducible results. Isocratically it was not possible to separate the all components, but the linear gradient resulted in good separation. By applying a gradient of 5-85% acetonitrile in 0.1% TFA a well-resolved pattern approximate 18-20 different peaks could be detected in honey bee venom when monitoring absorbance at 220 nm (Fig. 2). Optimising the gradient form it is was possible the improve further the resolution, e.g. on the first part of the chromatographic pattern: the minor components

Table I Solvent composition (%) for elution at different RP-columns. (Quantitative determinations).

Column type	Apamine		Melittin	
	A %	B %	A %	B %
Econosphere C4	85	15	50	50
DeltaPak C18	97	3	44	56
Hypersil C18	84	16	45	55

Table II Retention of bee venom components on different RP-columns.

Components	Retention time (t_R min)			
	Column type			
	Econosphere 300 Å C4	DeltaPak 300 Å C18	Hypersil 300 Å C18	Hypersil 100 Å C18
Apamine	8.4	13	3.7	-
MCD-peptide	9,4	15,2	9.4	-
Phospholipase A_2	23.6	25,0	19.3	-
Hyaluronidase	30,4	30,0	30.4	-
Melittin	31.2	32,4	26.6	15.2*

*:Isocratic value

surrounding the apamine could be separated very well (9 further peaks), applying very only soft gradient in the first 10 min of the chromatography (Fig. 4).

According to the chromatographic pattern the linear peptides (as melittin) have longer retention on C_{18} phase, then the cyclic peptides (the components containing one or more disulphide bridges e.g. apamine, MCD-peptide etc.). Even the enzymes with higher M_W (phospholipase A_2 and hyaluronidase) are eluated before melittin because of intermolecular cystine residues indicating that the hydrophobic side chains of the linear peptide (melittin) are more accessible for interaction with the hydrophobic stationary phase, then in the case of a more compact, cyclic compound as MCD-peptide or an enzyme. Here we observed a decreasing peptide retention due to the lower contact area between peptide and stationary phase. If a linear peptide is the

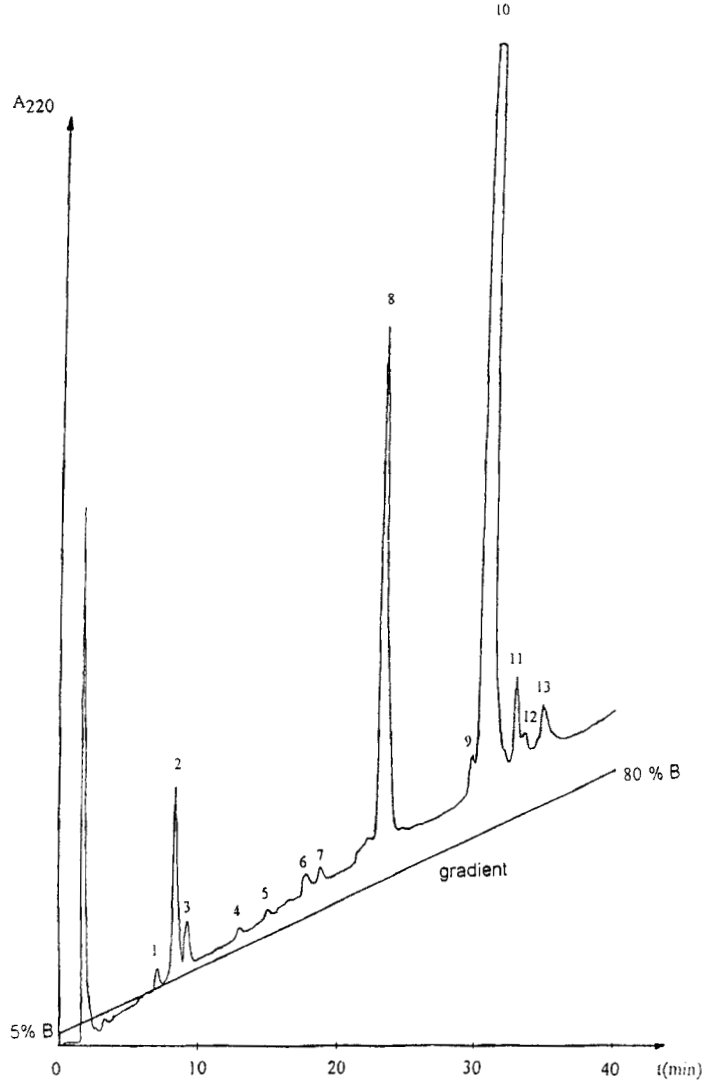


FIGURE 2. Chromatographic pattern of bee venom.
Column: Econosphere 300 A C4 (250x4 mm). Eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in acetonitrile-water (80-20). Linear gradient: 5% B - 80% B at 40 min. Injection volume: 20 µl. Detection 220 nm. Flow rate: 2.0 ml/min. Identified peaks: minor peptides (1, 4-7), apamine (2), MCD-peptide (3), phospholipase A_2 (8), hyaluronidase (9), melittin (10), melittin derivatives (11-13). A=0.32.

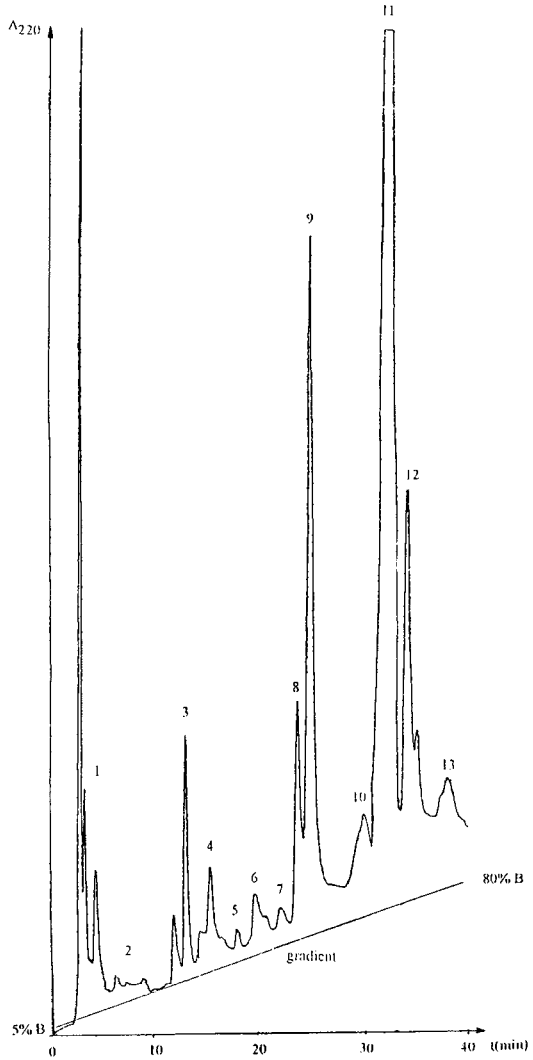


FIGURE 3 Chromatographic pattern of bee venom.

Column: Delta-Pak C18 300 (300x3.9 mm). Eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in acetonitrile-water (80-20). Linear gradient: 5% B - 80% B at 40 min. Injection volume: 20 µl. Detection 220 nm. Flow rate: 2.0 ml/min. Identified peaks: amino acids, biogenic amines (1), procamines (2), apamine (3), MCD-peptide (4), tertiapin (5), melittin-F (6), secapin (7), lysophospholipase (8), phospholipase A_2 (9), hyaluronidase (10), melittin (11), melittin derivatives (12), acid monophosphoesterase (13). A=0.32.

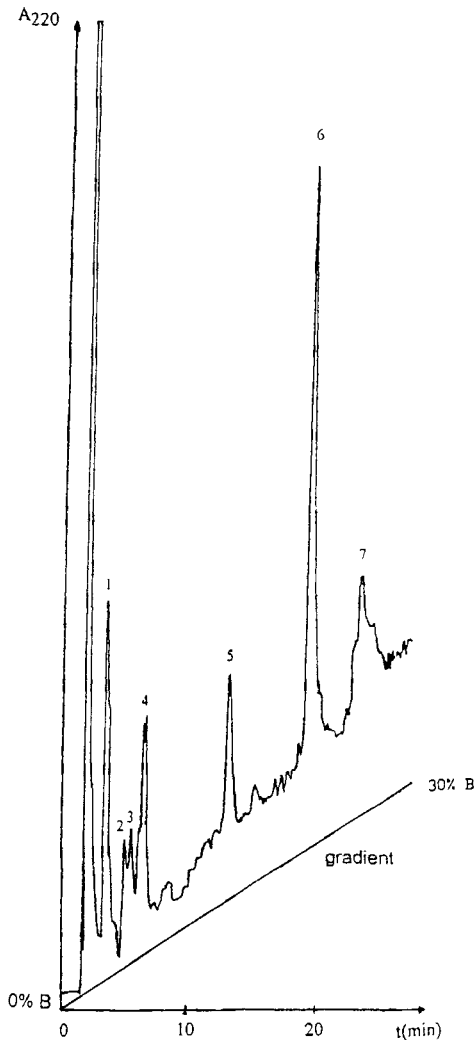


FIGURE 4. Chromatographic pattern of the minor components of bee venom surrounding the apamine.
Column: Delta Pak C18 300 A (300x3.9 mm). Eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in acetonitrile-water (80-20). Linear gradient: 0% B - 30% B at 30 min. Injection volume: 20 µl. Detection 220 nm. Flow rate: 1.5 ml/min. Identified peaks: minor peptides (1-5), apamine (6) MCD-peptide (7). A=0.04.

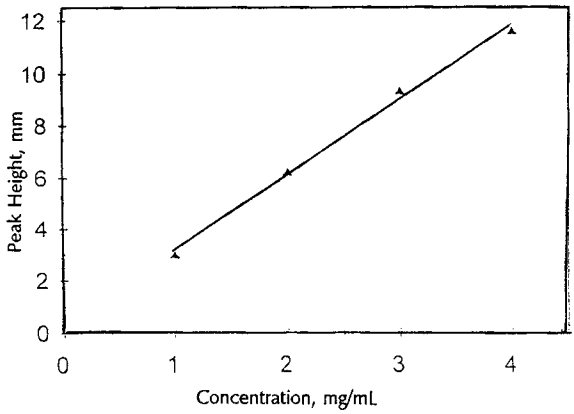


FIGURE 5. Calibration curve of melittin

analyte, there is a larger contact area between the analyte and the retentive site, which results in a strong retention (see retention data of melittin).

On the base of their cyclic structure, the small quantities (below 1%) of tertiapin and secapin could be identified on the chromatographic pattern (prediction: peak 7-tertiapin, peak 8-secapin).

The different procamines are very hydrophilic peptides because of C-terminal histamine and Gln residue, so they are coeluted at the very beginning of the gradient. Amino acids and biogenic amines are together with the eluent peaks in our system.

A more minor, partly degraded form of melittin, called melittin-F locks the first seven residues of the native molecule, and its retention time is much more lower, than that of the parents compound.

The peaks of the chromatographic pattern (Fig. 2-4) are quite well identified, and so different honey bee venoms could be characterised very precisely by this finger-print (and quantitative composition, of course).

Under these conditions a reproducible, about complete separation of bee venom into most of its components was possible within 40 min. Under isocratic condition (see experimental parts) quantitative determination of melittin, apamine, phospholipase A_2 was possible using standards of the corresponding components. The plots of the peak height of the determined component versus the known concentration were used as calibration curves to calculate the quantity of bee venom components (Fig. 5). The calibration graph of standard melittin, apamine and phospholipase A_2 was linear and

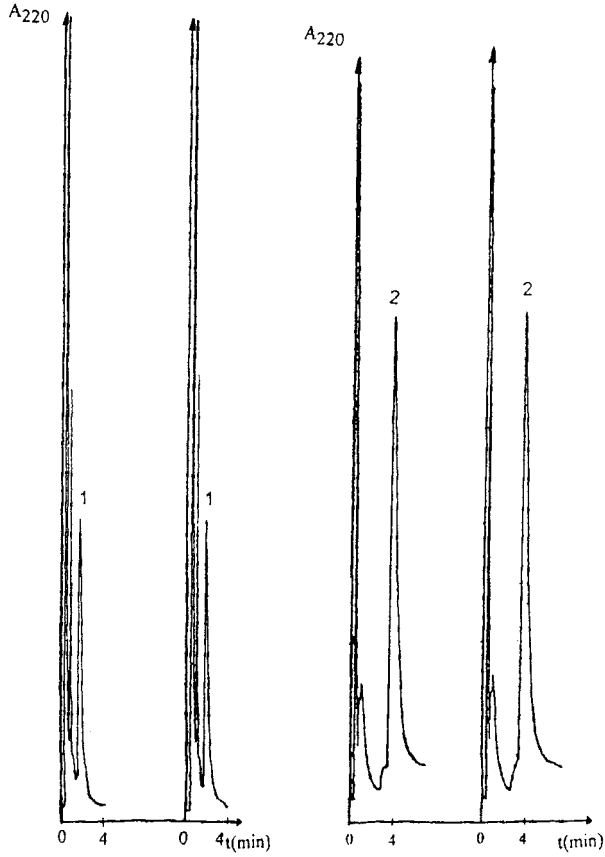


FIGURE 6. Reproducibility of quantitative measurements.
Column: Hypersil WP-300 C18 (125x4.6 mm). Flow rate: 1.5 ml/min. Detection: 220 nm. Eluents: apamine (1): 84% A and 16% B, melittin (2): 45 % A and 55 % B. A=0.32.

Table III Distribution of the components apamine, melittin and phospholipase A_2 in bee venom and in GPC fractions.

Sample	Location or isolation method	Apamine %	Melittin %	Phospholipase A_2 %
bee venom	SERVA	3.2	51.5	-
bee venom	Latvia	4.4	52.0	12.6
bee venom	Tököl	3.4	51.0	-
bee venom	Tököl by extraction	4.3	45.8	-
bee venom	Kengyel	4.5	61.0	13.8
dried bee venom	Szolnok*	3.5	57.1	12.5
lyophilized bee venom	Szolnok	3.4	57.0	12.6
frozen bee venom	Szolnok	3.5	57.0	12.5
bee venom, after 1 year	Szolnok	3.4	56.9	10.4
Melittin	via picrate	-	87.3	-
Melittin	GPC	-	85.8	-
Phospholipase A_2	GPC	-	-	95.5

* Hyaluronidase 2.8%, MCD peptide 1.4% (measured by similar procedure using standards for calibration)

passes through zero. Reproducibility of quantitative measurements was excellent with 0.2% error for apamine and 0.4-0.5% for melittin (Fig. 6). Optimising the method the different peaks do not disturb the quantitative determination of each other, isocratically base line separation could be achieved for the main components (see experimental part).

During measurements the melittin is pure monomer polypeptide, although it is known to exist in a tetrameric form (in concentrated aqueous solution at high ionic strength).

It was found, that in pure batches of bee venom the amount of main component melittin was between 50-60% (see Table III), the minor component, apamine was less,

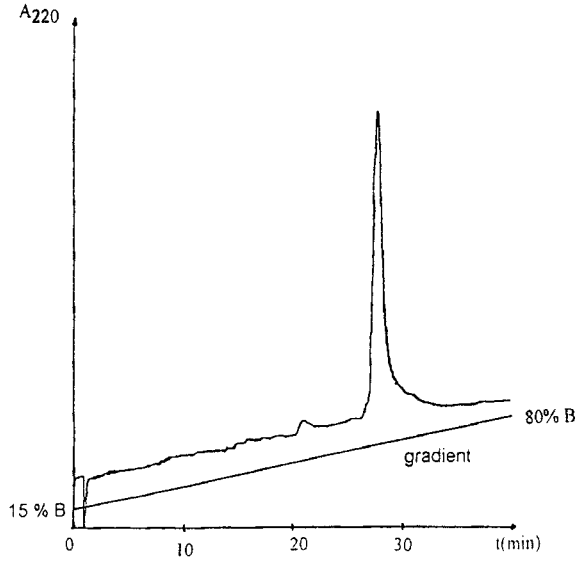


FIGURE 7. Chromatographic pattern of pure melittin(1) from GPC. Column: Hypersil WP-300 A C18 (125x4.6 mm). Eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in acetonitrile-water (80-20). Linear gradient: 15% B - 80% B at 40 min. Injection volume: 20 µl. Detection 220 nm. Flow rate: 1.5 ml/min. A=0.32.

approximate 2-6%. Our RP-HPLC could be used directly to finger-print analyse the bee venom in a single run derived from different sources as well as for competing various manufacturer's materials, too. The system described above was excellent for quantitative determination of bee venom's enzymes e.g. phospholipase A_2, which usually is determined on the base of its biological activity. Comparing the values of composition, the stability of lyophilized and frozen bee venom has not changed: it seems, that the compositions do not depend on the year of production (Table III). The preparative isolation methods of pure components could be controlled very adequately (Fig. 7, Table III). At GPC fractionation the purity of isolated components in the fractions could be checked very precisely (Table III).

If the isolations took place by means of extraction from bee glands and sticks, we found, that n-butanol-pyridine-acetic acid-water (60:20:6:24 v/v) mixture is not only the best one, but the chromatographic pattern of obtained substance is very similar to

that of original bee venom and the ratio of the component in different batches was practically the same. Melittin could be prepared from solution via its picrate salt [7] as pure substance. Its purity checked by HPLC was higher, than 85 %. Applying the optimised gradient profile for separation, in some cases the finger-print analysis showed different melittin peaks (on great quantity) according to N-formil-melittin, melittin-F and desamido-melittin substances.

The RP-columns can be used with displacement chromatographic technique, too. A method elaborated recently by Fellegvári [19] helps chiefly the preparative work in isolation of main components from honey bee venom; e.g. melittin [21].

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