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RESEARCH PAPER

An LCMS method for the assay of melittin in cosmetic formulations containing bee venom

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Abstract There is a growing interest in the potential of bee venom in cosmetics as a rejuvenating agent. Products currently on the market do not specify exactly their content of bee venom (BV). Therefore, we developed a method for the detection and quantification of melittin, as a marker of bee venom content, in selected commercial creams which contained BV according to their marketing claims, in order to gauge the relative quality of such formulations. A quantitative method was achieved following a rigorous extraction procedure involving sonication, liquid-liquid extraction and solid phase extraction since carryover of excipients was found to cause a rapid deterioration in the chromatographic performance. The method employed a standard additions approach using, as spiking standard, purified melittin isolated from bee venom and standardised by quantitative NMR. The aqueous extracts of the spiked creams were analysed by reversed phase LCMS on an LTQ Orbitrap mass spectrometer. The purity of the melittin spiking standard was determined to be 96.0 %. The lowest measured mean melittin content in the creams was 3.19 ppm (±1.58 ppm 95 % CI) while the highest was 37.21 ppm (±2.01 ppm 95 % CI). The method showed adequate linearity ($R^2 \geq 0.98$) and a recovery of 87.7–102.2 % from a spiked blank cream. An assay precision of <20 % RSD was achieved for all but one sample where the RSD value was 27.5 %. The method was sensitive enough for use in routine assay of BV-containing cosmetic creams. Differences in the melittin content of the commercial products assayed were nearly tenfold.

Keywords Melittin · Bee venom · Extraction · LCMS · Creams · Cosmetics · Quantitative NMR

Introduction

The venom of *Apis meliffera* (honey bee) and its components are increasingly being used as primary ingredients in various cosmetic formulations including skin creams, balms, face masks and serums. Cosmetics are some of the most widely used consumer goods [1], with the market annually generating billions of pounds worldwide [2], and so their testing must be thorough in view of their widespread usage. Although the separate testing of constituents may not necessarily indicate properties of the final formulation, appropriate methods are needed for the routine assay, stability monitoring and quality control of primary ingredients in order to set a quality standard for a particular product even though there is no prescribed content for BV in such creams [3].

A. meliffera venom contains various ingredients ranging from relatively low molecular weight (MW) amines, such as histamine (MW ~111), to relatively large-sized proteins such as phospholipase (MW ~16,000) and hyaluronidase (MW ~53,000) enzymes. Melittin (MW ~2,800) is the main constituent of the venom, constituting approximately 45–60 % of the bulk venom material and is a 26-amino acid peptide [4]. The other components are the peptides apamine, mast cell

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degranulating peptide (MCDP), secapin, adolapin and apidaecin [3, 5, 6]. Both phospholipase (api m1) and hyaluronidase (api m3) are classified as major allergens according to the International Union of Immunological Societies (IUIS) [7]. The other bee venom allergens include dipeptidyl dipeptidase IV (api m5), serine carboxypeptidase (api m9), CUB serine protease (api m7) and vitellogenin (api m12) among others. A host of other ingredients including amino acids, carbohydrates, amines and lipids have also been described [6, 8, 9]. The presence of ionisable free primary amino and the highly basic guanidino groups (on lysine and arginine, respectively), in addition to polar amido (on N-terminal glycine and both C-terminal glutamines) and hydroxyl (on threonine and serine residues) groups in melittin reduces its retention on a hydrophobic C18-type column, particularly when ionised in an acidic medium (0.1 % *v*/*v* formic acid). However, due to the presence of valine (2), leucine (4) and isoleucine (3) residues (all with non-polar side chains) in its amino acid sequence, the molecule is retained long enough for analysis by a reversed phase method.

Recent advances in cosmetic analysis have focused on developing new methods for determining cosmetic preservatives, fragrance allergens and plasticizers such as phthalates, using chromatographic and mass spectrometry techniques [10]. New methods become more necessary when new ingredients are used in formulations for general use. Thus, although bee venom-based products have been on the European markets for quite some time (such as Forapin in Germany, Virapin in Slovakia, Apiven in France, Melivenon in Bulgaria and Apifor in Russia) [11], these have been available more as topical medications rather than as general use consumer products in the wider international market. Products designed to fit the latter category (e.g. the Manuka cosmetics range) have only recently appeared on the EU markets. These products are being marketed as containing "purified bee venom" or "bee venom extracts" (e.g. 10 Natural Effects Bee Venom Essence by Laboratorios DIET Esthetic S.A.) without further specification. Despite this growing use of bee venom, the current literature does not report a sufficient number of studies showing how to assess the composition of cosmetics in general [2], let alone the bee venom content in these products.

Analysis of cosmetic products may be considered as relating to that of non-oral semi-solid dosage forms (which include ointments, gels, creams and pastes) in pharmaceutical formulations and whose sample preparation methods for analysis have been previously reviewed [12]. It would appear from The Cosmetic, Toiletry and Perfumery Association (CTPA) [13] that the main difference, at least in practical terms, between a cosmetic product and its corresponding pharmaceutical counterpart is the purpose of application and composition. Both cosmetics and topical pharmaceutical products have common sites of application although the former tend to be more complex in composition.

Directive 93/35/EEC, the Sixth Amendment to the original Cosmetic Directive of 1976, incorporates the following definition of a cosmetic product:

> A "cosmetic product" shall mean any substance or mixture intended to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odours and/or protecting them or keeping them in good condition.

Cosmetics are not expected to contain substances with therapeutic action, but are instead only formulated for topical applications exerting local effects. Thus, the level of emphasis placed on exact proportions of their chemical constituents (i.e. content uniformity) is lower than for pharmaceutical products. In addition, the control of how many constituents there are in a single formulation (complexity), component compatibility and susceptibility to degradation, and the general requirement for standardisation is also lower than for pharmaceuticals. Even the labelling requirement does not expect the manufacturer to specify quantities of ingredients while "top secret" ones are not even included on the label [14]. A cosmetic product tends to be considered acceptable as long as it does not contain banned substances (or restricted substances beyond allowed limits), is nontoxic, does not make unjustified marketing claims and generally satisfies the customer's needs.

Although current legislation does not require full profiling of all constituents in a cosmetic product, but only focuses on controlling restricted or banned ingredients [15], it is likely that such requirements will be invoked in the future as technologies advance, and new molecules or formulations, such as nanoparticles, become more commonly available.

Thus in order to meet current and anticipated formulation and regulatory requirements for bee venom formulated skin products, it is important to be able to detect, quantify and control the amount of active bee venom material. Since bee venom is only present in small amounts in creams, the current work focussed on developing a reliable sample preparation and clean-up procedure in order to isolate melittin, the most abundant marker compound for the presence of bee venom, from the formulation excipients with subsequent analysis of the extracts by LCMS using a standard additions technique. For an analytical method based on an efficient separation technique such as reversed phase HPLC and highly selective and sensitive detection systems such as Orbitrap mass spectrometry [16], one would ideally not have to worry much about coeluting compounds in the extract solution [17]. However, because cosmetic products are generally very complex in composition [12], with most of the ingredient structures

unknown but likely to be comprised of varying proportions of lipophilic and hydrophilic materials, the whole process of analytical method development may become unpredictable even when employing some of the latest highly selective analytical devices. At the very least, in order to protect the analytical column and detection system, there is a need to attempt to selectively extract and concentrate the analyte of interest from the matrix of the complex product. This paper reports a method based on liquid-liquid extraction in a ternary solvent system followed by solid phase extraction (SPE) on a reversed phase (C18) cartridges to obtain relatively clean samples for analysis of melittin, which gives an indication of bee venom content, in bee venom-containing cosmetics by LCMS.

Materials and methods

Study samples

Six commercial cosmetics which were stated to contain bee venom were analysed. Throughout the experiments, the samples were stored in a cool, dry environment and away from direct sunlight as recommended by the manufacturers. Prepared solutions for analysis were run immediately to avoid any sample degradation.

Solvents and chemicals

HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific, UK, while chloroform was from Sigma-Aldrich Ltd, UK. Deionised purified water was produced in the lab using a Direct-Q 3 Millipore Ultrapure water purification system (Millipore, UK). AnalaR-grade formic acid (BDH-Merck, UK) was used as a pH modifier. D_2O was obtained from Sigma-Aldrich, Dorset, UK. Crude bee venom from which melittin was purified was supplied by Wissen Co., Seoul, S. Korea.

Instrumentation and consumables

The syringes and filters were obtained from Fisher Scientific, UK. The following equipment were also used: a micro-centrifuge, a vortex mixer, an ultrasonic bath (Fisher Scientific, UK) and automatic pipettes (Gilson, Anachem, UK). The Reveleris® Flash Chromatography was supplied by Alltech, UK. The Reveleris system uses two variable wavelengths and evaporative light scattering (ELSD) detectors to detect both chromophoric and non-chromophoric compounds in a single run. The LCMS system consisted of a Surveyor pump connected to a LTQ Orbitrap (Thermo Fisher, Hemel Hempstead, UK). The HPLC column used was a reversed phase ACE 3 C18 column; 150×3.0 mm, 3 μm, supplied from Hichrom, Reading, UK. For sample purification of melittin, a reversed phase semi-prep HPLC column (250 mm length×10 mm I.D., 5 μm particle size), supplied by HiChrom Ltd, UK, was used.

LCMS

Final diluted and filtered sample solutions were run on the LCMS under these conditions. Mobile phases consisted of 0.1 % *w*/*v* formic acid in water (A) and 0.1 % formic acid in acetonitrile (B). The solvent gradient used was 20–70 % B (from 0 to 10 min), 70 % (10–16 min), 70–20 % (16–20 min) and finally 20 % (20–25 min) at a flow rate 0.3 mL/min. Injection volume was 10 μL. The ESI interface was employed in positive ionisation mode for detection of $[M+H]^+$ ions, with a spray voltage of capillary and cone at 4.5 and 35 kV, respectively. The sheath and auxiliary gas flow rates were 50 and 15 arbitrary units, ion transfer capillary temperature was set at 275 °C and full scan data were collected between *m*/*z* 100–2,000. The data was collected and processed using Xcalibur 2.1.0 software (Thermo Fisher Scientific, UK).

Melittin isolation from bee venom

The melittin used in the spiking standard solution was prepared by medium pressure liquid chromatographic (MPLC) fractionation of a bee venom sample on a Reveleris Flash chromatography system. Approximately 800 mg of bee venom sample was mixed with 3 g of purified silica (Celite) in a dry-loading cartridge prior to the fractionation. The column used was prepared by packing an empty 20 g Easyvarioflash D24 cartridge (VWR International, UK) with ca 13 g of Polymeric Retain PEP for SPE (Thermo Scientific, UK). The mobile phases used were water (solvent A) and acetonitrile (solvent B) under the following gradient conditions: 0–10 min (0 % B), 10–20 min (20 % B), 20–30 min (50 % B), 30–60 min (60 % B) and 60–70 min (100 % B) at a flow rate of 12 mL/min. The melittin peak eluted between 22 and 28 min. Following LCMS analysis on the Orbitrap, similar fractions were pooled together and further purification of the melittin fraction was achieved by semi-preparative HLPC using a Thermo Separations P2000 pump and ACE C18 column (250 mm length×10 mm I.D., 5 μm particle size; HiChrom Ltd, UK). To this end, aliquots of the pooled melittin fractions from MPLC (100 μL of a 0.1 g/mL aqueous solution) were injected onto the HPLC column. The injected samples were eluted with water/acetonitrile (60:40) at a flow rate of 5 mL/min. The dual UV detector was set at wavelengths 220 and 295 nm, and data was collected using the ChromQuest software. The melittin peak was collected and lyophilised.

Melittin purity measurement by NMR

A solution of the melittin spiking standard was made by dissolving 14.23 mg of the sample in 1 mL of D_2O to give a final

concentration of 5.0 mM. A portion of exactly 600 μL of this solution was then taken for NMR analysis. After this, 30 μL of a 50 mM solution of MeOH in D_2O was then spiked into the melittin sample to give a final methanol concentration of ca 2.5 mM. The samples were each run in triplicate at 310 K for 16 scans with a pre-saturation pulse programme which had a long recovery time (D1=58 s) and a short pre-saturation time (D2=2 s). This long recovery time allowed for all the resonances to be fully detected. One dimensional ^{1}H NMR data were acquired under Topspin (version 2.1, Bruker Biospin, Karlsruhe) using a method analogous to that previously described by Evstigneev et al. [18]. A Bruker AVANCE III 600 NMR spectrometer operated at a proton resonance frequency of 600.13 MHz was equipped with a TBI [^{1}H, ^{13}C, ^{31}P–^{15}N]-z triple resonance probe head fitted with an actively shielded gradient coil for delivery of pulsed-field gradients.

Assay method

A sample of cream (ca 1 g) was weighed into a 20-mL glass vial and dispersed in 10 mL of methanol-chloroform (1:2) mixture. The mixture was then sonicated, with intermittent shaking, for 30 min in an ultrasonic bath until homogenous. A portion (1 mL) of the extraction solvent was pipetted and transferred into a 2-mL centrifuge tube and spiked with a known quantity of melittin standard reconstituted in 0.1 % *v*/*v* formic acid, and then made up to 2 mL with 0.1 % *v*/*v* formic acid. The mixture was then shaken on a vortex mixer for about 2–3 min to allow complete mixing. Thereafter, it was centrifuged at 6,000 rpm for 15 min upon which the supernatant was transferred into a separate vial for SPE. SPE was performed on Strata C18, 100 mg/mL packed columns (Phenomenex, UK). The sample was loaded onto the SPE column and washed with 1 mL of 30/70 (acetonitrile/water) and then eluted with 1 mL of 50/50 (acetonitrile/water). Final eluted solutions were run on the LCMS according to the method described earlier. Peak area was obtained by integrating extracted ion chromatograms of the abundant +4 (*m*/*z* 712.45±0.01) and +5 (570.16±0.01) melittin ions [17] using the XCalibur software.

Calibration with standard additions

Sample extracts were spiked with aliquots of 0.1 mg/mL of a freshly prepared standard spike solution of melittin. Spike weights of 0.0, 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 μg of melittin corresponded to 0, 25, 50, 75, 100, 125 and 150 μL of the spiking standard solution. Following the spiking, samples were gently vortexed and mixed thoroughly before liquid-liquid extraction by centrifugation and later solid phase extraction. A calibration curve of analyte response versus amount of spiked standard melittin was then constructed using the unspiked sample as the lowest point on the curve. From this curve, the content of melittin in each cream sample was then determined by extrapolating the line to meet the concentration (horizontal) axis.

Results

Melittin purity determination

The NMR spectra obtained showed clear distinction at σ3.4 ppm chemical shift between melittin spiking standard and that of its solution after spiking with pure methanol internal standard (Fig. 1). The methyl protons in methanol overlapped with some unidentified protons in melittin and thus they could not be integrated independently. However, the aromatic region of melittin clearly showed the 5 protons corresponding to the single tryptophan residue in melittin. Integrating this reference region to 5 protons, the rest of the standard melittin spectrum integrated to 174 protons—representing the other non-exchangeable protons in melittin. In addition, the methanol-spiked standard solution integrated to 174 protons plus the contribution from the extra 3 non-exchangeable protons from methanol.

The purity of the standard melittin was calculated by using the formula proposed by Malz and Jancke [19] given as:

$$p_x = \frac{I_X\, N_{\mathrm{std}}\, M_x\, m_{\mathrm{std}}}{I_{\mathrm{std}}\, N_x\, M_{\mathrm{std}}\, m_x} P_{\mathrm{std}} \qquad (1)$$

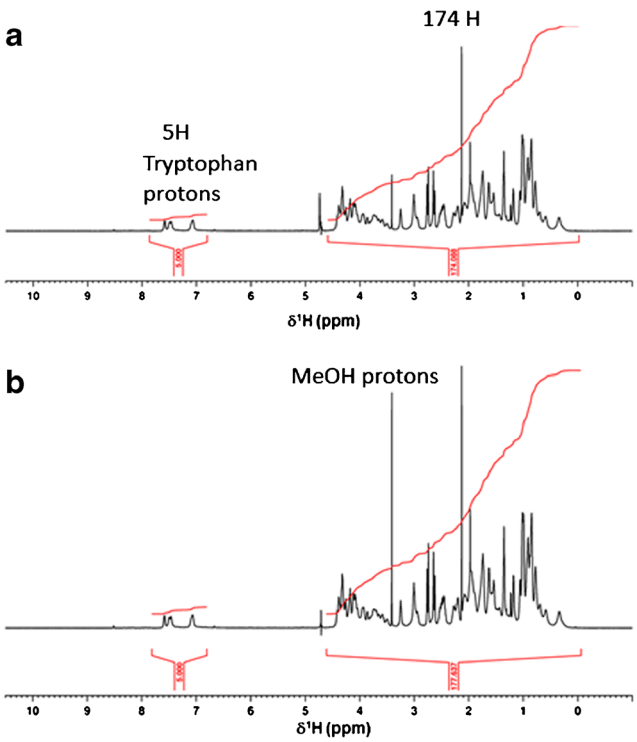

Fig. 1 Proton NMR spectra of melittin (**A**) and melittin+methanol (**B**)

where I, N, M, m and P represent magnitude of signal response (area integral), number of resonant nuclei (protons), molar mass, sample weight and purity of the unknown and standard compounds, respectively.

For this experiment, the following values apply for the unknown sample (melittin) and internal standard (methanol) (Table 1). Although melittin has 229 protons in total, 50 of them are exchangeable with deuterium atoms from the D_2O used as solvent so that the resonant ones are only 179.

Substituting the values in Table 1 into Eq. 1 above gives P_x, the purity of melittin, as 96.0 %. Since the mass spectrum of the melittin spiking solution showed only a single peak representing melittin, it is likely that any impurity present might be due to water or counter anions associated with the basic side chains in melittin. Figure 2 shows a chromatogram obtained for 10 μg/mL of melittin standard.

Mean melittin content in creams

The Table 2 shows a summary of results obtained after assay of six samples of each cream on three separate occasions. Each analysis was conducted by running seven spiked 1 mL aliquots of extract, each prepared from 1 g of cream, on the LCMS and using the melittin peak areas obtained to plot a straight line from which the content in the un-spiked extract was estimated by extrapolation of the standard additions plot.

Assay precision

Analytical precision was determined both between and within runs. Inter-assay precision was checked by testing, on three separate occasions, each of the cream samples using the developed method and then calculating the relative standard deviation (Table 2). The calculated between-run precisions were found to be less than 20 % except for product F where the RSD was 27.5 % perhaps due to the melittin content being close to the limit of quantification of the method. The between sample variations could also be due to variation in the uniformity of content within the creams rather than the analytical precision of the method.

Table 1 Values used in the calculation of melittin purity

Parameter	Internal standard (methanol)	Unknown (melittin)
Signal response (I)	3 (177 less 174)	174
Number of resonant nuclei (N)	3	174 (179 less 5 of Trp)
Molecular weight (M)	32.04	2,846.46
Sample weight in mg (m)	0.09649	8.9649
Purity % (P)	99.9	P_x

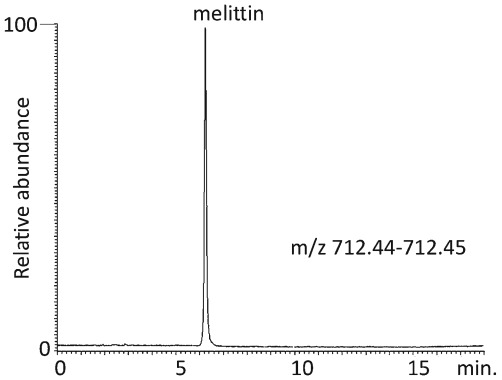

Fig. 2 The chromatogram of the melittin spiking standard

Intra-assay precision was calculated using Eq. 2 below, as previously described by Bruce and Gill [20], to calculate the standard error (s_c) in the concentration (c_x) of the assay mixture obtained by extrapolation of the linear regression standard additions plot.

$$s_c^2 = \frac{s_y^2}{m^2}\left(\frac{1}{N} + \frac{\bar{y}^2}{m^2 S_{xx}}\right) \quad (2)$$

where s_y is the standard deviation around the regression line (standard deviation of the residuals), m is the slope of the regression line, N is the number of samples for each plot, $\bar{y}$ is the response mean and S_{xx} is the corrected sum of squares of the independent variable (spiked concentration).

Table 3 shows the margin of error (precision) estimates calculated for each assay using the equation above and the 95 % confidence intervals for the determined melittin content in the cosmetic products assayed.

From Table 3, it can be seen that the margin of error was, unsurprisingly, high for products whose melittin content was below 10 ppm (A, B and F). However, for the rest of the products, the margin of error was well below the 10 % threshold set for mass fraction of ≥1,000 μg kg^{-1} in accordance with CD2002/657/EC [21], although the direct application of this standard in a standard additions technique does not seem feasible given the complexity of the sample, nor has it been reported previously. The observed degradation of precision is expected in standard additions procedures as described by Ellison and Thompson [22], although such variations are also expected to arise from the detailed extraction procedures required for this type of formulation [12, 23]. Table 4 summarises the equations of the lines obtained for the standard addition curves prepared for each of the samples analysed.

Recovery, specificity and linearity

Since no appropriately matched matrix samples were available, extraction recovery of the method was determined using

Table 2 Triplicate assays of commercial facial creams claimed to contain unstated amounts of bee venom. The values represent complete assays performed on three separate occasions using a seven-point standard additions technique

Samples	Assay of melittin content (in ppm)				Retention time	Linearity
	I	II	III	Mean (RSD, %)	Mean (RSD, %)	Mean R^2 (RSD, %)
A	3.90	4.21	5.42	4.51 (17.8)	6.29 (0.56)	0.989 (0.35)
B	4.37	3.98	3.51	3.95 (10.8)	6.26 (0.88)	0.985 (0.60)
C	36.60	37.21	32.55	35.45 (7.1)	6.36 (0.55)	0.979 (0.18)
D	15.53	17.03	14.45	15.61 (8.9)	6.35 (0.24)	0.992 (0.35)
E	32.59	35.06	34.55	34.07 (3.8)	6.37 (0.24)	0.990 (0.54)
F	3.19	5.62	4.45	4.42 (27.5)	6.69 (0.09)	0.981 (0.82)

a spiked base cream (Nivea) because it was expected to offer comparable extraction challenges to those exhibited by the samples assayed. The blank cream samples were fortified at 5.0, 10.0 and 15.0 μg per 100 mg with melittin and assayed in triplicate. The peak areas obtained were compared to those of external standards prepared in triplicate at the mid-point of the expected concentration range (10.0 μg/mL). The mean recovery obtained was 94.0 % (range, 87.7–102.2 %) with a coefficient of variation (RSD) of 4.6 % (Tables 5 and 6).

Specificity of detection was accomplished by using extracted ion chromatograms for the melittin molecule using two of its abundant ions its mass spectrum (Fig. 3), filtering within a tight range of *m*/*z* 712.445±0.005 and 570.165±0.005. This was further confirmed by looking at the ion spectrum of the peak within the retention time range of the melittin standard. No other compounds from the samples appeared in the chromatogram at the retention time of melittin under these conditions. This was further confirmed by running blank samples.

Linearity of the response was evaluated by using a spiked blank matrix at six calibration points i.e. 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 μg/mL and then carrying out a linear regression analysis of the analyte peak areas obtained versus concentration. The method showed good linearity in the analytical range with correlation coefficient (R^2)≥0.99. Standard melittin peaks were stable with a retention time mean 6.26 min (RSD≤1.0 %). The mean chromatographic efficiency for the melittin was calculated at ~75,000 plates/m. The limits of detection (LOD) and quantification (LOQ) of melittin in the spiking solution, determined according to ICH guidelines [24], were 50 and 150 ng/mL, respectively.

Table 3 Intra-assay precision estimates at 95 % confidence level (p=0.05, df=5). The t value for a two-tailed t test is ±2.5706 from the Student t table. The 95 % confidence interval was calculated as (95 % CI=tsc) as described in [20]

Product	Run	Assay (ppm)	Standard error (s_c)	Margin of error (%)	±95 % CI (±ts$_c$) (ppm)	Lower CI (ppm)	Upper CI (ppm)
A	1	3.90	0.3952	26.05	1.0159	2.88	4.92
	2	4.21	0.4173	25.48	1.0727	3.14	5.28
	3	5.42	0.5270	25.00	1.3548	4.07	6.77
B	1	4.37	0.4683	27.54	1.2037	3.17	5.57
	2	3.98	0.4447	28.72	1.1431	2.84	5.12
	3	3.51	0.6230	45.63	1.6015	1.91	5.11
C	1	36.60	0.7662	5.38	1.9697	34.63	38.57
	2	37.21	0.7817	5.40	2.0094	35.20	39.22
	3	32.55	0.8060	6.37	2.0720	30.48	34.62
D	1	15.53	0.3310	5.48	0.8509	14.68	16.38
	2	17.03	0.3656	5.52	0.9398	16.09	17.97
	3	14.25	0.4927	8.89	1.2664	12.98	15.52
E	1	32.59	0.6143	4.85	1.5790	31.01	34.17
	2	35.06	0.4388	3.22	1.1281	33.93	36.19
	3	34.55	0.4624	3.44	1.1885	33.36	35.74
F	1	3.19	0.6138	49.46	1.5778	1.61	4.77
	2	5.62	0.4211	19.26	1.0826	4.54	6.70
	3	4.45	0.6801	39.28	1.7481	2.70	6.20

Table 4 Summary of the equations of calibration curves (in the form of y=mx+c) obtained during the assay of the six creams (A–F)

Samples	Slope, m (μg^{-1})	y-intercept, c	|x-intercept| (μg)	Linearity, R^2
A	82,462.4629	32,135.2843	0.3897	0.9911
	82,367.8220	34,708.6236	0.4214	0.9902
	79,832.8949	43,257.5386	0.5419	0.9847
B	85,601.1771	37,387.7843	0.4368	0.9877
	83,035.3569	33,012.0036	0.3976	0.9888
	85,052.6091	29,889.6814	0.3514	0.9781
C	6,567.3226	24,035.9279	3.6599	0.9807
	6,513.4514	24,235.4514	3.7208	0.9802
	6,970.9136	22,688.1725	3.2547	0.9774
D	89,737.9669	139,404.2871	1.5535	0.9949
	86,782.1707	147,767.1025	1.7027	0.9929
	76,890.2507	109,605.3996	1.4255	0.9885
E	65,242.3570	212,602.9011	3.2587	0.9868
	62,561.6680	219,322.2243	3.5057	0.9935
	63,100.6986	218,032.7721	3.4553	0.9927
F	339,024.0249	108,079.3764	0.3188	0.9786
	322,348.3874	181,137.1043	0.5619	0.9902
	359,450.2041	159,968.0232	0.4450	0.9744

Table 5 Recovery data obtained using a blank cream fortified at three different levels of 5, 10 and 15 μg per mL of extract containing 100 mg cream

Replicates	Fortification level (μg per 100 mg)	Peak area	RT (min)	Recovery (%)	Mean area (%)	RSD (%)
1	5.0	425,882	6.22	87.7	95.1	7.7
2	5.0	463,353	6.24	95.4		
3	5.0	496,710	6.25	102.2		
1	10.0	918,056	6.24	94.5	92.4	2.2
2	10.0	896,992	6.25	92.3		
3	10.0	877,902	6.24	90.3		
1	15.0	1,413,491	6.24	97.0	94.5	3.7
2	15.0	1,398,745	6.26	96.0		
3	15.0	1,318,737	6.27	90.5		
Overall mean			6.25	94.0		4.6

Discussion

The standard additions method is used to eliminate matrix effects in samples that would lead to biased results during analysis [25]. This is particularly likely to occur with trace amounts of a chemically complex moiety such as melittin in the complex cream matrix. It has been proposed that the method of standard additions solves a particular type of matrix effect (called the rotational effect), but not translational effects—which must be separately dealt with [22]. In the method of standard additions, known quantities of the analyte being assayed are spiked into a sample containing the analyte at increasing concentrations, starting from zero, and then extracted. Final solutions are subsequently analysed and the peak areas obtained are plotted against the spiked volumes or concentration of spiked samples. Provided that the area response is directly proportional to concentration, a straight line is obtained. This straight line crosses the response (vertical) axis at the response value of the un-spiked sample; extrapolating this straight line to the concentration (horizontal) axis gives, numerically, the weight of analyte in the un-spiked sample (Fig. 4). The main advantage of using standard additions is that there is no need for complete extraction of analyte from matrix provided all the samples have been subjected to exactly the same extraction process. It is important that the samples

Table 6 Peak areas of standard melittin assayed at 10 μg/mL

Replicates	Concentration (μg/mL)	Peak area	RT (min)	Mean area	Precision (RSD, %)
1	10.0	978,938	6.24	971,682.3	0.79
2	10.0	963,628	6.24		
3	10.0	972,481	6.25		

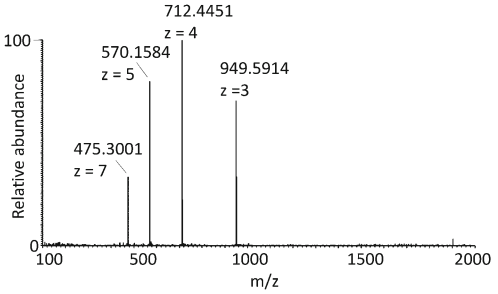

Fig. 3 The mass spectrum of melittin. Melittin molecules can acquire up to six positive charges during electrospray ionisation (ESI) in the mass spectrometer. The most abundant ions in the spectrum are *m/z*: 712.45 (+4) and 570.16 (+5) species

are not spiked to levels exceeding linearity limits of the analyte response. The amount of standard added depends on the approximate concentration of the un-spiked samples. Ideally, samples should be spiked at any evenly spaced concentrations of standard solutions within the linear range, although it has been recommended that spiked concentrations should be at least four times the concentration of analyte [22].

In this experiment, initial analyses had given us varying composition of melittin in the creams ranging from approximately 5 to 100 ppm (μg/g). This translates to a melittin content of about 0.5–10 μg per 100 mg of the cream. Thus in this work, the samples were spiked with the melittin standard at 0, 25, 50, 75, 100, 125 and 150 μg per 100 mg. These spiking levels conform to the sequence $x_1 \approx x_0$, $x_2 = 2x_1$,.... $x_p = px_1$ (x being analyte amount) which is generally acceptable [25]. The levels are nevertheless slightly below those recommended by Ellison and Thompson (2008) where at least five times the expected concentration of analyte should be used with repetitive measurements, if necessary, to improve precision [22]. Clearly, the latter approach also reduces the total amount of sample preparation time required. Based on results obtained and observations made during this assay, it is quite clear that the extraction and determination of melittin in the creams is a complex and laborious process which might introduce some errors depending on the degree of control of extraction conditions. The liquid layers formed after solvent extraction

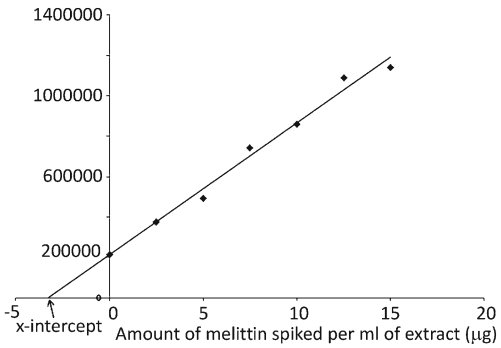

Fig. 4 Sample calibration plot representing cream sample E. The plot represents data obtained with replicate 1. The values of m, c and R^2 for this assay are 65,242.4, 212,602.9 and 0.9868, respectively

centrifugation were quite distinctly separated, but with a white precipitate forming at the liquid-liquid interface for three of the samples analysed (C, E and F). This was thought to be possibly due to lignin from the herbal components stated to be present in these products. Our preliminary liquid-liquid extraction (LLE) extracts without SPE had exhibited poor compatibility with the reversed phase analytical column leading to significant peak distortions. This was understood to arise from matrix interferents that probably suppressed melittin ionisation or its ability to chromatograph properly leading to poor chromatographic efficiency as peaks became broader and noisier, especially at spiked analyte concentrations $\leq$2.5 μg/mL. Extraction with either aqueous or organic solvent alone proved inadequate as this led to incomplete dispersion of the creams. Ideally, a good SPE method should achieve strong enough retention of an analyte on the column during sample loading and washing steps so that it can be concentrated and eluted in a more controlled manner to obtain relatively clean fractions [26]. The initial lack of adequate retention of melittin on the SPE cartridge was found to be associated with fast loading under vacuum. Allowing the sample to load slowly, freely under gravity, overcame this problem. Attempts at preventing early breakthroughs by using high pH (at which the melittin was less ionised) during LLE proved fruitless due to low extraction recovery. This observation concerning the role of low pH (with formic acid) in the extraction of melittin from the cream might have been expected since a basic amphiphilic peptide such as melittin needs to be ionised in order to effectively partition into the aqueous layer that was analysed in this assay. Complete elimination of formic acid thus prevents such ionisation leading to insufficient extraction.

Aqueous solutions of whole bee venom demonstrated gradual degradation of melittin within the ^{21}Lysine-^{22}Arginine-^{23}Lysine-^{24}Arginine region of the amino acid sequence under sterile conditions at room temperature (data not shown). Zhou et al. have previously reported a similar behaviour in pure melittin and apamin aqueous samples as well as in aqueous crude bee venom extracts [17]. We have observed here that purified melittin degrades comparatively much more slowly than when it is in the crude venom. The observed activity appears to be enzyme catalysed and the enzyme involved seems to be trypsin-like. It is probable that the observed activity is due to a serine carboxypeptidase already identified in bee venom as an allergen through genome analysis. That would mean that our method of purification of melittin either removes or denatures this peptidase activity. Currently, we cannot confirm if such degradation does occur in the formulated products and, if it does, the extent of such degradation. Thus, the results of this assay can only confirm the melittin content of the creams at the time of analysis which may differ from the original amount incorporated.

Conclusion

A reversed phase LCMS method was developed for the assay of melittin in six commercially available creams containing unspecified amounts of purified bee venom. Given that the proportions of bee venom in the products were not specified, it is not possible to comment on how well the products conform to a label claim. Extraction recovery suggests the accuracy of our assay method to be acceptable, although the blank matrix was an entirely different cream altogether, but with no bee venom in it. This might have biased the results of extraction recovery since this can vary across different blank matrices. Nevertheless there, certainly, is significant variation in the amount of melittin measured in the creams which ranged between 3.2 and 37.2 ppm, which is more than tenfold but with satisfactory intra and inter- assay precisions. Production of a good quality product requires adequate quality control for the finished product. The chemical and physical stability of bee venom in cream matrices would require careful assessment and this is something which we are now able to do.

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