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Characterisation and determination of indole alkaloids in frog-skin secretions by electrospray ionisation ion trap mass spectrometry

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The characterisation of selected indole alkaloids in a quadrupole ion trap mass spectrometer is presented. Fragmentation profiles for tryptamine, 5-hydroxytryptamine (5-HT), *N*′-methyl 5-hydroxytryptamine (*N*′-methyl 5-HT), *N*′,*N*′-dimethyl 5-hydroxytryptamine (bufotenine), *N*′,*N*′,*N*′-trimethyl 5-hydroxytryptamine (5-HTQ), and *N*′,*N*′-dimethyl 5-methoxytryptamine (5-MeODMT) are presented with proposed structures given for each product ion observed. Such MS^n experiments can be used to differentiate the isobaric molecular ions of the compounds 5-HTQ (M^+) and 5-MeODMT (MH^+). The quantitative determination of certain indole alkaloids in the skin secretions of the Australian Golden Bell frog, *Litoria aurea*, by LC/ESI-ion trap MS is also presented. The concentrations of 5-HT, *N*′-methyl 5-HT and 5-HTQ were found to be 2.68, 0.26 and 0.54 μg per mg of skin secretion, respectively. Copyright © 2002 John Wiley & Sons, Ltd.

Amphibian skin has been labelled as a ‘treasure trove’ of biologically active compounds including biogenic amines, peptides, proteins, steroidal bufadienolides and cardenolides.[1] Such a mixture constitutes a chemical irritant that serves as a defensive mechanism for the frogs. Early studies have demonstrated the potency of some of these compounds from various species of frog as shown in Table 1 (modified from Daly[2]). Some of the compounds may be found in mammals but at much lower concentrations. For example, it has been found that the amount of adrenaline stored in the paratoid glands of *Bufo gutturalis*, i.e., 10.72 mg per toad, is equivalent to that found in the adrenals of a sheep and greater than that found in man.[3] Humans have used frog-skin secretions in a number of ways, from dart poison for hunting to the more questionable practices of smoking dried toad skin and licking toads for their hallucinogenic effects.[2]

Since the realisation of the potential of frog-skin secretions for pharmacological applications, the past 30 years have seen the discovery of over 400 alkaloids from 20 structural classes.[4] Within that body of research, a number of important discoveries have been made. For example, the poison arrow frog, *Epipedobates tricolor*, was found to contain a painkilling agent which, in tests on mice, was seen to be 200 times more effective in blocking pain than morphine. This compound, named epibatidine, was considered too toxic to be used as a human painkiller but it is hoped that chemical modification of its structure may decrease its toxicity. Epibatidine was the first naturally occurring compound to contain a nitrogen-bridged, six-membered carbon ring (azabicycloheptane) and also a chlorpyridyl group that is extremely rare in mammals.[5]

Structural elucidation of these novel compounds is a long and complicated task and requires researchers to have at their disposal up-to-date analytical techniques. Daly[4] notes that throughout 30 years of work in this field techniques such as liquid chromatography (LC) and gas chromatography (GC) with MS detection (electron impact (EI) ionisation and chemical ionisation (CI)) have been used in the initial stages of compound identification. Powerful fingerprinting techniques such as Fourier transform infrared (FTIR) spectroscopy, nuclear magnetic resonance (NMR) and X-ray crystallography are used in conjunction with the fragmentation data from MS in structural identification. Spande *et al.*[6] used ^1H and ^{13}C NMR for the conformational analysis of decahydroquinolines from dendrobatid poison frogs and a myrmicine ant. The fact that the same compounds were found in the ant as in the frog strengthens the hypothesis that many of these frog-skin secretion constituents are of dietary origin.

The characterisation and determination of indolealkylamines by mass spectrometry has been carried out in a number of ways. LC coupled to electrospray ionisation (ESI) with tandem mass spectrometry has been used for the elucidation of indole-3-acetic acid metabolism in bacteria.[7] Combinations of techniques such as two-dimensional mass spectrometry and mass-analysed ion kinetic energy spectrometry have been used to establish fragmentation pathways in the gas-phase ion chemistry of indole, gramine, tryptamine and serotonin following production of molecular ions by EI.[8] The need to determine the presence of biogenic amines in complex matrices such as fermented foods has led

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Table 1. Examples of pharmacologically active compounds from amphibians (modified from Daly[2]). Reprinted with permission from Daly JW, Toxicity of Panamanian Frogs (Dendrobates): Some Biological and Chemical Aspects, *Science* 1967; **156**: 970 copyright 1967 American Association for the Advancement of Science http://www.sciencemag.org

Compound	Genus	Activity	Minimum lethal dose* (mg/kg mouse)
Nitrogenous bases			
Batrachotoxin	*Phyllobates*	Cardio- and neurotoxin	0.002
Samandarine	*Salamandra*	Centrally active	0.3
	Pseudophryne	convulsant	
Tetrodotoxin (tarichatoxin)	*Taricha*	Neurotoxin	0.008
Compound A ($C_{19}H_{33}NO_2$)	*Dendrobates*	Nerve-muscle activity	2.5
Compound B ($C_{19}H_{33}NO_3$)	*Dendrobates*	Nerve-muscle activity	1.5
Indole-alkylamines			
Serotonin	*Bufo*	Vasoconstrictor	300
	Leptodactylus		
Dehydrobufotenine	*Bufo*	Convulsant	6
O-Methylbufotenine	*Bufo*	Hallucinogen	75
Phenolic and catechol amines			
Norepinephrine	*Bufo*	Hypertensive agent	5
Candicine	*Leptodactylus*	Cholinergic agent	>10
Leptodactyline	*Leptodactylus*	Cholinergic agent	10
Imidazole-alkylamines			
Histamine	*Leptodactylus*	Local irritant	13,000
Spinaceamine	*Leptodactylus*		
Carnosine	*Eleutherodactylus*		
Bufogenins and bufotoxins			
Bufotalin	*Bufo*	Cardiotoxin	0.4
Bufotoxin	*Bufo*	Cardiotoxin	
Kinins			
Bradykinin	*Rana*	Local irritant	
Physalaemin	*Physalaemus*	Hypotensive agent	
Other kinins	*Ascaphus*		
	Phyllomedusa		
Proteins			
Hemolysins	*Trituris*	Hemolytic agents	0.002

* For comparison the minimum lethal dose of curare and strychnine is 0.5 mg/kg mouse; that of sodium cyanide is 10 mg/kg.

to the development of an LC method involving derivatisation with 3,5-dinitrobenzoyl chloride and detection by mass spectrometry.[9]

Frog venom is a similarly complex matrix. Biogenic amines and active peptides have been studied in the skin extracts of 32 European amphibian species.[10] Only indole-alkylamines were detected in the skin, i.e., 5-HT and its N′-methylated, cyclised and sulfo-conjugated derivatives. A similar study was carried out on the skin extracts of 140 American amphibian species other than bufonids. Again the most abundantly occurring amine category was the indole-alkylamines, *viz.*, 5-HT and its N′-methylated, cyclised and sulfo-conjugated derivatives.[11] The same results were found when the skin extracts of 52 African amphibian species other than bufonids were analysed.[12]

Of the alkaloids found in frog-skin secretions, bufotenine is considered to be the most psychedelic, ensuring the bufo toad a place in human mythologies and medicines worldwide since historic times.[13] Reports of fatalities caused by digoxin-like poisoning prompted the investigation of a West Indian aphrodisiac and a Chinese medication for the presence of bufotenine. Using GC/MS it was discovered that both products were derived from toads or highly similar sources and both contained bufotenine.[14] Takeda[15] notes that the production of bufotenine in *Bufo bufo japonicus* proceeds along the pathway 5-HT → N′-methyl 5-HT → bufotenine. In the same study bufotenine was not detected in *Rana nigromaculata*.

In this paper the skin secretions of *Litoria aurea*, the Australian Golden Bell frog, are investigated. To date, many literature references have been concerned with the peptide content of the skin secretions from this frog, in particular those which show anticancer activity[16,17] or which have an effect on blood pressure or smooth muscle contraction.[18] However, the presence of small molecules such as indole-alkylamines should not be overlooked.

The use of LC/ESI-ion trap MS has been investigated for the characterisation of some indole alkaloids found in frog-skin secretions. MS^n fragmentation of indole alkaloids has been carried out and compared with that of available standards for characterisation purposes. The use of ESI-ion trap fragmentation demonstrates the ability to distinguish two related compounds that yield molecular ions of identical *m/z* values, i.e., 5-HTQ (M^+) and 5-MeODMT (MH^+). Application of these investigations is found in the determination of some of these compounds in the skin

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Table 2. MS^n characterisation of indole alkaloids studied

Compound	MS Peak	MS/MS		MS^3		MS^4	
		Peak	CE	Peak	CE	Peak	CE
5-HT	177.0 160.3	160.2	16%	132.2	16%	117.1	18%
N′-methyl 5-HT	191.1 160.3	160.2	18%	132.2	18%	117.4 115.6	18%
N′,*N*′-dimethyl 5-HT	205.0 160.2	160.1	16%	132.1	22%	117.1 115.2	16%
Tryptamine	161.2 144.1	144.3	16%	117.1	20%	*	*
5-MeODMT	219.1 174.1	174.1	18%	159.2 144.1 117.1	18%	131.1	18%
5-HTQ	219.2 160.2	160.1	18%	132.1	16%	117.1 115.2	16%

* No peak observed; CE = collision energy and refers to arbitrary values set by the software.

secretion of *Litoria aurea* by LC/ESI-ion trap MS. Quantitative data are presented for the levels of 5-HT, *N*′-methyl 5-HT and 5-HTQ occurring in the secretion sample investigated.

EXPERIMENTAL

Instrumentation

MS^n characterisation and detection of the compounds present in frog-skin secretions was performed using an LCQ™ quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) utilising electrospray ionisation (ESI). Standard solutions of compounds under investigation were infused into the mass spectrometer by means of a syringe pump located on the instrument.

For HPLC analysis the system used was supplied by ThermoQuest (San Jose, CA, USA) and comprised a P4000 pump, AS 3000 autosampler, on-line UV 1000 UV detector and an SCM 1000 vacuum membrane degasser. The column used was a Luna C18(2) 5 μ, 150 mm × 4.6 mm i.d. obtained from Phenomenex (Macclesfield, Cheshire, UK). It was protected by a SecurityGuard™ cartridge system from the same supplier. For quantitative studies a Luna C8 5 μ, 150 mm × 4.6 mm i.d. (Phenomenex) column was used.

Reagents

All solvents were of LC grade while other chemicals used were of analytical reagent or LC quality. Methanol, acetonitrile and water were obtained from BDH (Poole Dorset, UK). Nitrogen gas for the LCQ™ was delivered from a Whatman nitrogen generator (Whatman Inc, Haverhill, MA, USA) while helium damping gas for the ion trap was obtained from BOC Medical Gases (Guildford, Surrey, UK). Tryptamine, 5-HT, *N*′-methyl 5-HT, bufotenine, 5-HTQ and 5-MeODMT standards were obtained from Sigma (Poole, Dorset, UK).

Frog-skin secretion samples

Frog-skin secretion samples were provided from in-house captive *Litoria aurea* frogs. Each frog specimen was 'milked' using a mild electrical charge to stimulate secretion and the skin then rinsed with distilled water to collect the sample. The collected aliquot was freeze-dried to produce a solid residue sample. A quantity of the secretion was dissolved in methanol for direct infusion MS at a concentration of 10 mg mL^{-1} for LC/ESI-ion trap MS determinations for which 0.05% trifluoroacetic acid (TFA) in water was used as the solvent. Prior to injection in either mode the sample was centrifuged in a MSE MicroCentaur centrifuge (Sanyo, Uxbridge, Middlesex, UK) at 13000 rpm for 10 min to remove particulate matter.

Instrumental procedures

MS^n characterisation

MS^n characterisation of the compounds present in frog-skin secretions was carried out by infusion of previously collected LC fractions from a conventional LC-UV/MS analysis at a typical flow rate of 10 μL min^{-1}. For relatively dilute samples in which weak signals were observed, the infusion rate was raised to between 20 and 50 μL min^{-1}. In the ESI source nitrogen sheath and auxiliary gas flows were maintained at 50 and 5 units, respectively, and refer to arbitrary values set by the software. The heated capillary temperature was 220 °C and the spray voltage set to 5 kV. Positive ion mode was used throughout. In order to establish the MS^n fragmentation pattern for selected compounds the precursor $[M + H]^+$ ion was chosen in the MS scan and its fragmentation initiated. The major peak observed in the resulting MS^2 scan was then chosen for the next stage of fragmentation and the process continued until no further ions were observed. An isolation width of 1 Th was used for the various MS^n stages.

LC/ESI-ion trap MS determinations

For initial qualitative investigations a binary gradient mobile phase was used comprising water with 0.05% TFA as solvent A and water/acetonitrile/TFA (20 + 80 + 0.05) as solvent B. Initially the proportions were 100% A 0% B and then altered in a linear gradient to 0% A and 100% B over a period of 240 min at a flow rate of 0.5 mL min^{-1}. This gradient allowed for the efficient separation of the multiple entities present in

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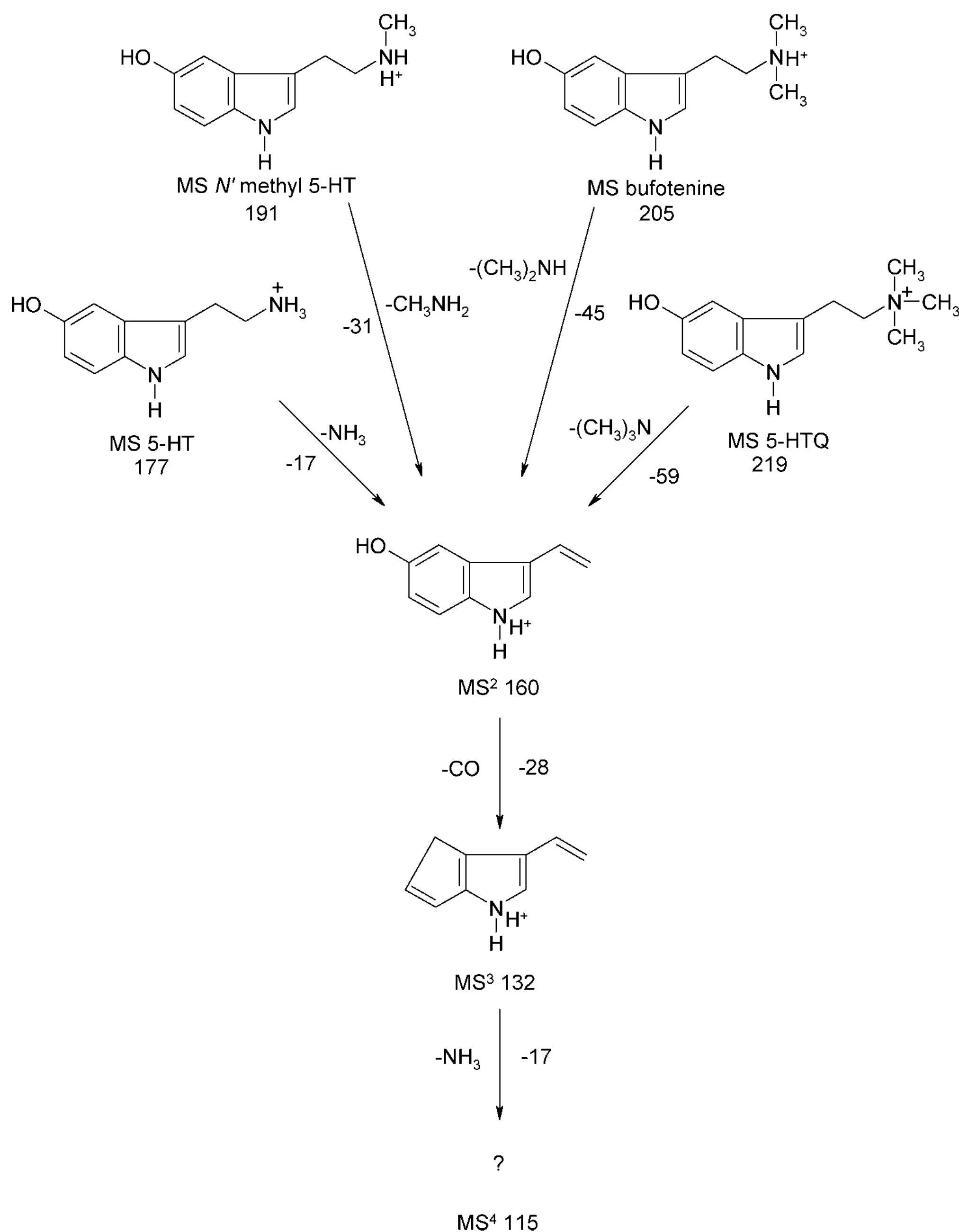

Figure 1. Proposed MS[1] to MS[4] fragmentation profiles of bufotenine, *N*′-methyl 5-HT, 5-HT and 5-HTQ.

the skin secretions. At the end of the 240-min analysis the column was washed with 100% acetonitrile and then conditioned with start-condition mobile phase for 10 min prior to the next injection being made. An injection volume of 200 µL was used throughout. An Upchurch Scientific microsplitter (Anachem, Luton, UK) was used to divert 90% of the post-UV detector flow to a Pharmacia LKB FRAC-100 fraction collector (Pharmacia-Biotech, Sweden) while the remainder of the flow entered the mass spectrometer. Fractions were collected at 1-min intervals over the analysis period. The LCQ™ parameters were similar to those outlined above for direct infusion analysis.

For quantitative determination of 5-HT, *N*′-methyl 5-HT and 5-HTQ a 200-µL injection of skin secretion at a concentration of 10 mg mL^{-1} was made with separation effected using an 80-min gradient system rather than the 240-min gradient. Flow splitting was not used and, to compensate for the increased solvent flow into the mass spectrometer, the sheath and auxiliary gas flows were raised to 65 and 30, respectively. Concentrations were calculated by peak area comparison to standards prepared in 0.05% TFA in water and analysed in a similar way.

RESULTS AND DISCUSSION

ESI-MS^n characterisation of 5-HT and related compounds

The neurotransmitter 5-HT is found in abundance in some frog-skin secretions. Infusion of 10 µg mL^{-1} standards of 5-HT, *N*′-methyl 5-HT, bufotenine, 5-HTQ and 5-MeODMT in

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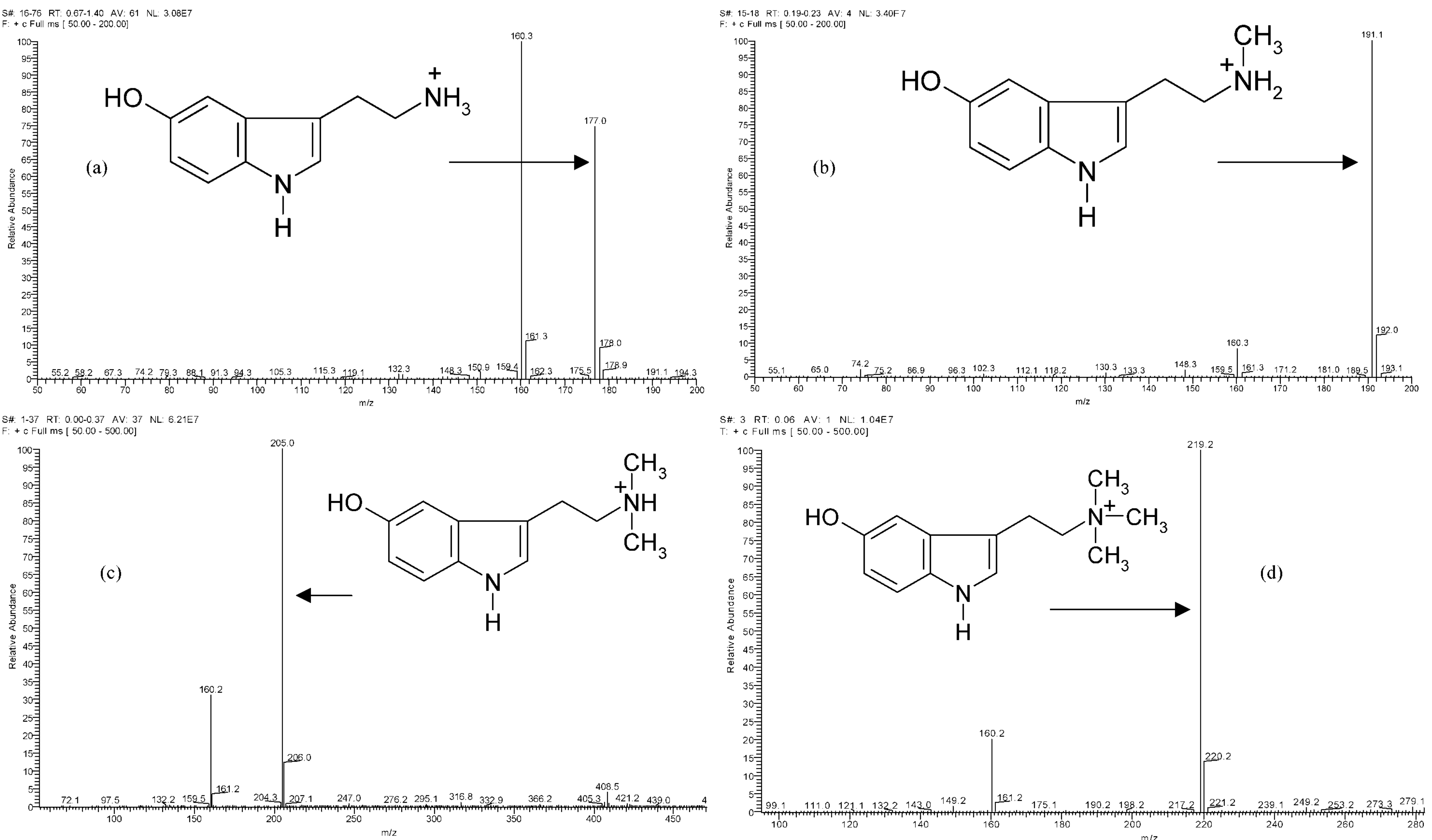

Figure 2. MS^1 spectra of (a) 5-HT (b) *N*′-methyl 5-HT, (c) bufotenine, and (d) 5-HTQ.

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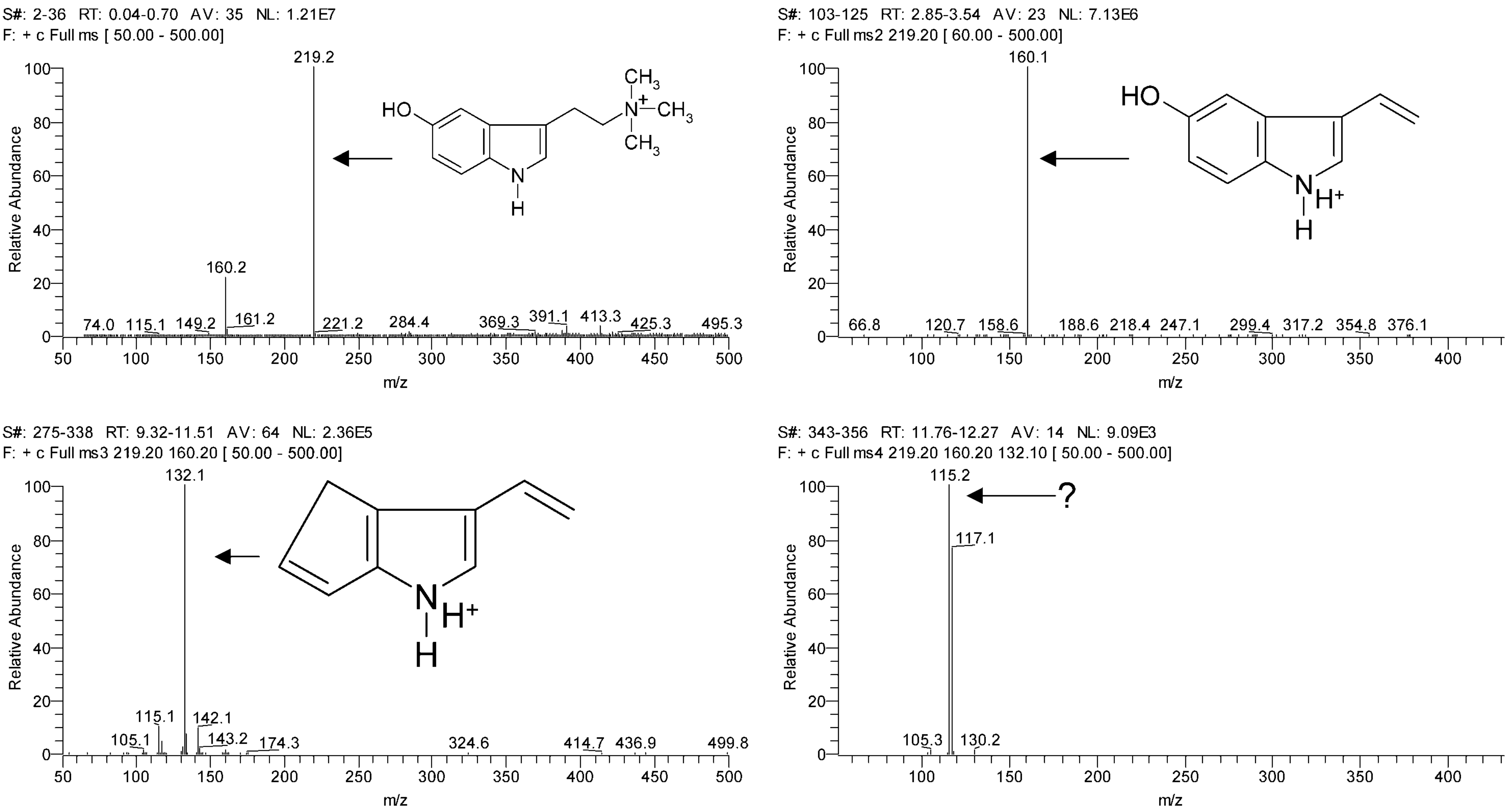

Figure 3. MS^{1} to MS^{4} profile of 5-HTQ and the proposed product ions formed at each stage.

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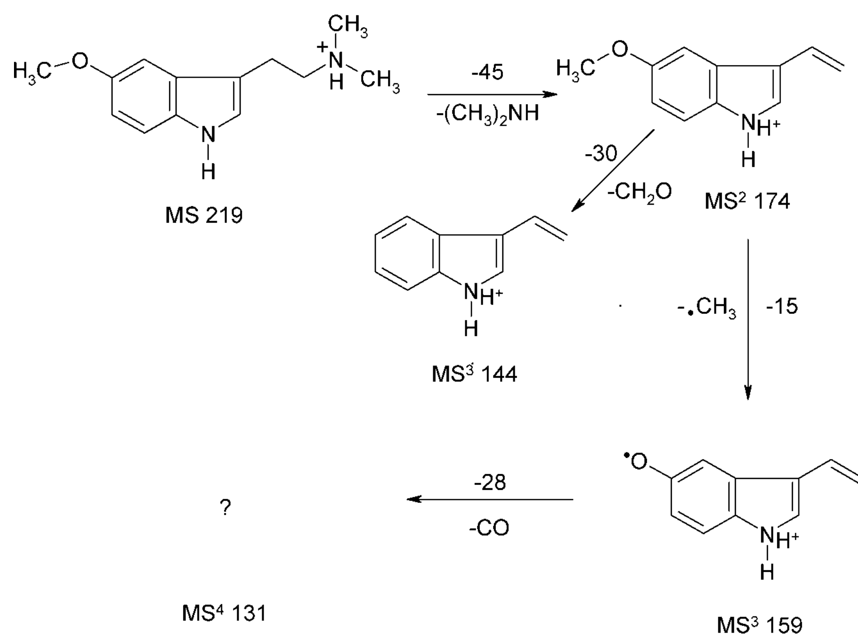

Figure 4. Proposed MS^1 to MS^4 fragmentation profile of *N′*,*N′*-dimethyl 5-methoxytryptamine (5-MeODMT).

methanol at a rate of 10 μL min^{-1} was used to study the MS^n fragmentation profiles while for tryptamine a concentration of 1.6 μg mL^{-1} was used. The data produced are reported in Table 2. Collision energy was optimised for each compound in order to maximise the signal of the product ion produced in each case. The proposed fragmentation patterns for 5-HT and related compounds are shown in Fig. 1. For all compounds apart from 5-HTQ an $[M+H]^+$ signal is observed in the MS^1 mode. Protonation is believed to take place on the amine nitrogen atom in the case of 5-HT, *N′*-methyl 5-HT and bufotenine. This is then followed by a charge-site-initiated α-fragmentation accompanied by hydrogen atom rearrangement to form a fragment ion at *m/z* 160, as shown in Fig. 1. This interpretation is supported by the fact that the quaternary ammonium variant 5-HTQ behaves in exactly the same way. This product ion was originally thought to be tryptamine, but the proposed fragmentation patterns show that this is not the case. The resulting fragment has charge on the indole nitrogen and shortening of the side chain to ethene occurs with the loss of neutral molecules such as NH_3, CH_3NH_2, etc. At the MS^3 stage it is proposed that loss of CO occurs with ring contraction to give a two five-membered ring ion still retaining charge on the indole nitrogen atom. Such CO losses are common in the fragmentation of organic cations using ion trap MS.[19,20] Finally, loss of 17 u at the MS^4 stage is due to NH_3. MS^4 also shows a loss of 15 u to form an *m/z* 117 species. Loss of a radical like CH_3 or NH from an even-electron ion in an ion trap operating with He seems unlikely and the *m/z* 117 species may be an artefact resulting from accidental resonances at higher harmonics. It should be noted that these are speculative structures in the absence of high-resolution mass spectrometry that was not available in the authors′ laboratory.

It is interesting to note that for all 5-HT-based compounds in-source fragmentation appears to take place in ESI as two peaks are observed, one due to the precursor ion and another at *m/z* 160 corresponding to the deaminated fragment that is formed in MS^2. For MS^1 of frog-skin secretions this is therefore a useful indicator for the presence of these compounds. MS^1 spectra for all 5-HT and related compounds and their corresponding structures are shown in Fig. 2.

5-HTQ has a somewhat anomalous behaviour under these conditions. It is a positively charged quaternary amine compound and thus yields a M^+ signal at *m/z* 219 in MS^1 mode. However, the *m/z* 160 ion is also observed in the MS^1 mode providing evidence for a 5-HT-based entity. 5-HTQ also follows the fragmentation pattern of the other 5-HT compounds in the scheme *m/z* $160 \rightarrow 132 \rightarrow 115$. The MS^1 to MS^4 spectra of 5-HTQ and the proposed product ions formed at each stage are shown in Fig. 3. The MS^2 to MS^4 spectra are common to all the 5-HT-based compounds studied.

A study of MS^n fragmentation has proven to be a useful tool in the differentiation of two very similar molecules. 5-HTQ (of mass 219 u) and 5-OMeDMT (of mass 218 u) both give ESI-MS signals at *m/z* 219. Initial observation may be sufficient to distinguish the two, in that the 5-HTQ signal has an accompanying *m/z* 160 peak in the MS^1 spectrum while 5-MeODMT has a 174 *m/z* ion present. Differentiation of the two is achieved by further fragmentation. 5-MeODMT has a pattern which proceeds as follows: *m/z* $219 \rightarrow 174 \rightarrow 159 \rightarrow 131$. It appears that 5-MeODMT is protonated, like the 5-HT-based compounds, on the amine nitrogen. Loss of 45 u in MS^2 corresponds to loss of the neutral molecule $(CH_3)_2NH$ and formation of a double bond on the ethyl side chain, as is also the case with the 5-HT-based compounds. MS^3 results in a major fragment at *m/z* 159. One possible explanation may be loss of the methyl group. A smaller

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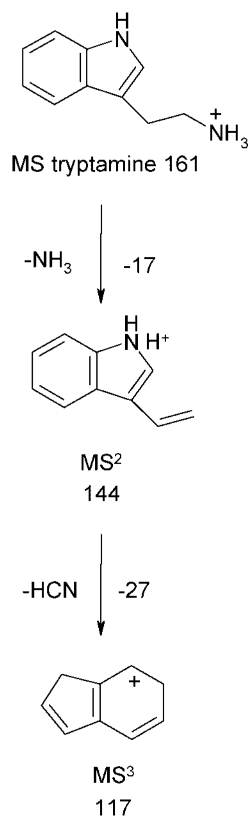

Figure 5. Proposed MS^1 to MS^4 fragmentation profile of tryptamine.

fragment is observed at *m/z* 144 and may be due to loss of formaldehyde, $H_2C{=}O$. It should be noted that the prevailing conditions (i.e., low-energy collisions) within the ion trap generally do not allow for homolytic bond cleavage with an even-electron positively charged ion generating an odd-electron ion. However, it is difficult to rationalise the loss of 15 u in any other way.[21] In both cases the indole nitrogen remains protonated. At the MS^4 stage loss of 28 u from the *m/z* 159 fragment is probably due to loss of neutral CO. However, no feasible structure of the remaining fragment can be proposed at this time. The proposed fragmentation pattern is shown in Fig. 4.

Tryptamine, like serotonin, appears to incur some in-source fragmentation. In MS^1 two peaks are observed at *m/z* 161 and 144. The former is due to $[M+H]^+$ while the latter is equivalent to the MS^2 product ion formed by loss of the neutral molecule NH_3 and formation of an ethene side chain. MS^3 provides an ion at *m/z* 117 following the loss of 27 u, presumably corresponding to a neutral molecule such as HCN (Fig. 5). Low collision energies of 16–20% show the ease with which fragmentation occurs in these compounds.

Determination of 5-HT and tryptamine-based compounds in *Litoria aurea* skin secretions

A 200-μL injection of 10 mg mL^{-1} *Litoria aurea* skin secretion was subjected to LC/ESI-ion trap MS analysis and separation took place over a 240-min gradient to qualitatively examine the components of the skin secretion. Small molecules such as neurotransmitters elute early in the analysis and constitute a relatively small proportion of the skin secretion when compared to the intensity of peptide signals and those of other large mass components that elute later. In this analysis, 5-HT-based compounds all have retention times between 24 and 30 min. 5-HT is the first to

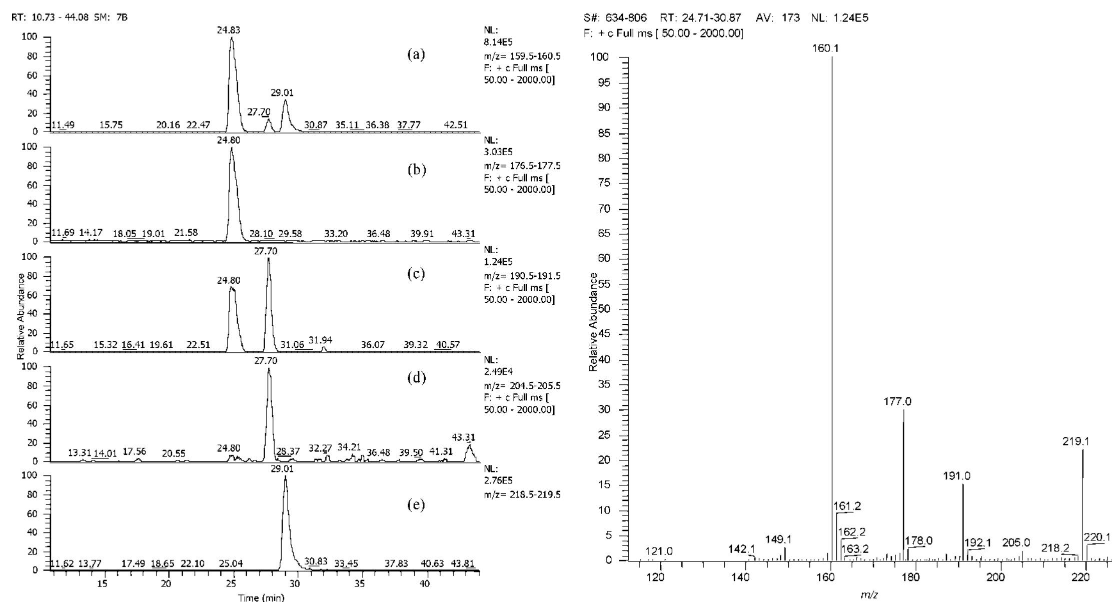

Figure 6. LC/ESI-ion trap MS analysis of skin secretion from *Litoria aurea*. A binary gradient mobile phase was used throughout comprising water with 0.05% TFA as solvent A and water/acetonitrile/TFA $(20+80+0.05)$ as solvent B. Initially the proportions were 100% A 0% B and then changed to 0% A and 100% B over a period of 240 min at a flow rate of 0.5 mL min^{-1}. Scans (a), (b) (c), (d) and (e) refer to mass scan filter of ions of *m/z* 160, 177, 191, 205 and 219, respectively. An ion at *m/z* 160 is associated with the retention times of the several ions studied and thus makes positive differentiation between 5-MeODMT and 5-HTQ. The MS spectrum to the right shows averaged data over the time range 24.71–38.70 min.

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elute with a retention time of 24.8 min, followed by *N*′-methyl 5-HT at 27.7 min and 5-HTQ at 29.0 min. Figure 6(c) shows a mass range scan for ions of *m/z* 190.5–191.5 and it exhibits a two-peak profile; the first eluted at the same retention time as 5-HT, and the second corresponded to *N*′-methyl 5-HT as confirmed by injection of standard compounds. The peak at 27.7 min was used as the analytical signal for *N*′-methyl 5-HT, while that at 24.8 min may be due to an unidentified isobaric ion, possibly originating from a compound of higher mass which yields a fragment ion at *m/z* 191 by in-source collision-induced dissociation (CID). Figure 6 shows mass range scans for these components and for the indicator ion at *m/z* 160 (Fig. 6(a)). It can be clearly seen in this latter figure how each 5-HT compound gives a signal when monitored at *m/z* 160. Therefore, in this MS analysis, 5-HT-based compounds can be readily identified.

For further confirmation collected fractions corresponding to each compound were infused by means of the syringe pump after LC/ESI-ion trap MS analysis had taken place and MS^n fragmentation were used to verify their identity. In this manner 5-HT (Fig. 6(b)), *N*′-methyl 5-HT (Fig. 6(c)) and 5-HTQ (Fig. 6(e)) were readily identified in the frog-skin secretion. A relatively low amount of the ion at *m/z* 205 (Fig. 6(d)) was also detected in the LC trace with retention time 27.7 min, possibly corresponding to bufotenine or another compound. However, this is also the retention time of *N*′-methyl 5-HT (see above), and the *m/z* 160 indicator may be due to it and not bufotenine. Since bufotenine has only ever been found in toads this suggests that the peak at *m/z* 205 is probably due to another yet unidentified compound and not bufotenine. As the concentration of this compound was very low, meaningful MS^2 analysis was not possible. The focus of further work will therefore be the identification of this compound as the present MS configuration lacks the sensitivity to do so.

The concentrations of 5-HT, *N*′-methyl 5-HT and 5-HTQ were determined by LC/ESI-ion trap MS analysis using an 80-min gradient system and were found to be 2.68, 0.26 and 0.54 μg per mg of skin secretion, respectively. It should be pointed out that such quantitation of components in frog-skin secretion is affected by variability from frog to frog and from secretion to secretion. However, these results provide some idea of the levels of indole alkaloids occurring in the skin secretions of *Litoria aurea*.

CONCLUSIONS

These studies have shown that ESI-ion trap MS is a useful tool in the characterisation and determination of indole alkaloids in frog-skin secretions. By establishing fragmentation profiles for each compound under investigation positive and unequivocal identification of compounds in a complex matrix such as frog-skin secretion is possible. Concentrations of indole alkaloids such as 5-HT, *N*′-methyl 5-HT and 5-HTQ were found to be 2.68, 0.26 and 0.54 μg per mg of skin secretion, respectively.

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