Identification and Quantitative Determination of Biogenic Amines and Their Metabolites by Gas Chromatography Negative Ion Chemical Ionisation Mass Spectrometry (GC-NICIMS)

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Summary: Biogenic amines in the brain tissue of the American Cockroach have been identified and quantified by an extraction derivatisation procedure using ditrifluoromethyl benzoyl chloride (DTFMBCL) and isopropyldimethyl silylchloride (IPDMS CL). The molecular ion of these *N*-DTFMB-XIPDMS derivatives carried most of the ion current under NICI conditions, with potential limit of detection below the picogram level. 5-Hydroxytryptamine and *N*-acetyl-5-hydroxytryptamine have been quantified by converting their *O*- and *O*-, *N*-acetylates with perfluoroacyl anhydrides. The limit of detection of these spirocyclic products on-column was less than 15 pg.

Introduction

The comparative studies of the metabolism of amines in insects have suffered from the lack of a sensitive and specific method for measuring the very small quantities of amines and their metabolites in biological systems. *p*-octopamine has been extensively studied in the CNS of insects, and measurements have been made almost exclusively using modifications of the radioenzymatic assay first introduced by Molinoff *et al.* in 1969 [1] which is sensitive but lacks specificity [2,3].

High performance liquid chromatography (HPLC) with electrochemical detection (ECD) is a relatively new procedure for estimation of catecholamines [4] and biogenic amines [5-9]. The combination of gas chromatography with mass spectrometry (GC-MS) has resulted in a technique of considerable applications, in that compounds may be identified unequivocally by high resolution capillary GC combined with monitoring of significant ions in the mass spectrum of a compound: these may afford characteristic ratios of ion intensities or their *m/z* values may be changed in a predictable manner to provide additional proof of identity, by the preparation of a different derivatives of the same chemical class.

In our earlier work [10] we have reported an extraction derivatisation method suitable for the analysis of picogram amounts of biogenic amines by gas chromatography Negative ion chemical ionisation mass spectrometry (GC-NICIMS). In this report the silylating reagent has been changed in order to shift both the *m/z* value of the molecular ion and the retention time of the derivative to ensure that its identification is unequivocal in particular, *p*-synephrine and adrenaline in the central nervous system of the cockroach. This technique is specific and allows for the first time the unambiguous measurement of such substances in the brains of individual insects.

Results and Discussion

Gas chromatographic and mass spectrometric properties were determined for a variety of biogenic amines and their corresponding isotopomers as their DTFMB-x IPDMS derivatives. DTFMB- amides of a variety of biogenic amines were reacted with *N*-isopropyl-dimethylsilyl-*N*-methyl-trifluoroacetamide (IPDMS-MTFA) and the retention times and base peaks of the resultant derivatives are summarized in Table-1.

In each case the principal ion in the mass spectrum was the molecular ion (M^{+}), which carried almost all of the ion current under NICI conditions and the sensitivity of this method of derivatization for a given biogenic amine was comparable to that of the corresponding DTFMB-xTMS ether derivative *i.e.* 1 pg on column [10]. The silylating reagent was changed in order to shift

Table-1: Retention times and base peaks (in each case the M^+) in the NICI mass spectra of DTFMB-xIPDMS derivatives of biogenic amines and their corresponding isotopomers.

	Compound	tR(Min)	Base peak (m/z)
1.	[$^2H_0$] *p*-TY	14.68	477
2.	[$^2H_2$] *p*-TY	14.67	479
3.	[$^2H_0$] *p*-SYN	16.56	607
4.	DHB	16.76	579
5.	[$^2H_0$] *p*-OA	16.75	593
6	[$^2H_5$] *p*-OA	16.73	598
7.	[$^2H_0$] DA	17.06	593
8.	[$^2H_3$] DA	17.05	596
9.	[$^2H_0$] 5-HT	18.03	616
10.	[$^2H_0$] Ad	18.47	723
11.	[$^2H_0$] Norad	18.50	709

both the *m/z* value of the molecular ion and the retention time (tR) of the derivative, to identify and quantify the biogenic amines, particularly, *p*-synephrine and adrenaline from an extract of a single cerebral ganglion of the cockroach. A NICI SIM trace of the M^- ions of the DTFMB-xIPDMS derivatives of a standard mixture of the deuteriated and undeuteriated biogenic amines (20 ng each), monitoring ions of [$^2H_2$] *p*-tyramine (*m/z* 479), [$^2H_0$] *p*-synephrine (*m/z* 607), [$^2H_5$] *p*-octopamine (*m/z* 598), 3,4-dihydroxybenzylamine (*m/z* 579), [$^2H_3$] dopamine (*m/z* 596), [$^2H_0$] adrenaline (*m/z* 723) and [$^2H_0$] noradrenaline (*m/z* 709 is shown in the Fig. 1(a). The selected ion trace of the DTFMB-xIPDMS derivatives of endogenous *p*-tyramine (*m/z* 477), *p*-synephrine (*m/z* 607), *p*-octopamine (*m/z* 593), dopamine (*m/z* 593), 5-hydroxy-tryptamine (*m/z* 616) and adrenaline (*m/z* 723) from an extract of a single brain of the cockroach, is shown in the Fig. 1(b). 5-HT and adrenaline were quantified using DHB as internal standard while *p*-synephrine was quantified relative to [$^2H_5$] *p*-octopamine as internal standard. The average amounts/brain of most of the amines determined by this method show good agreement with the estimates for such compounds in cockroach brain previously obtained (Table-2).

Table-2: Concentration of biogenic amines in individual brains of the cockroach

Biogenic amine	Concentration (ng/brain)	
	(a)	(b)
p-Tyramine	2.0 ± 0.6 (n=4)	3.5 ±1.2 (n=14)
p-Octopamine	2.2± 0.4 (n=4)	2.4 ±0.6 (n=14)
p-Synephrine	0.26± 0.1 (n=4)	0.08± 0.06 (n=12)
Dopamine	2.1 ±0.7 (n=4)	2.7± 0.5 (n=14)
Noradrenaline	0.85 ±0.15 (n=2)	0.7 ±0.3 (n=13)
5-Hydroxy-tryptamine	7.2 ±3.9 (n=4)	2.9 ±2.3 (n=14)
Adrenaline		0.09 ±0.07 (n=10)

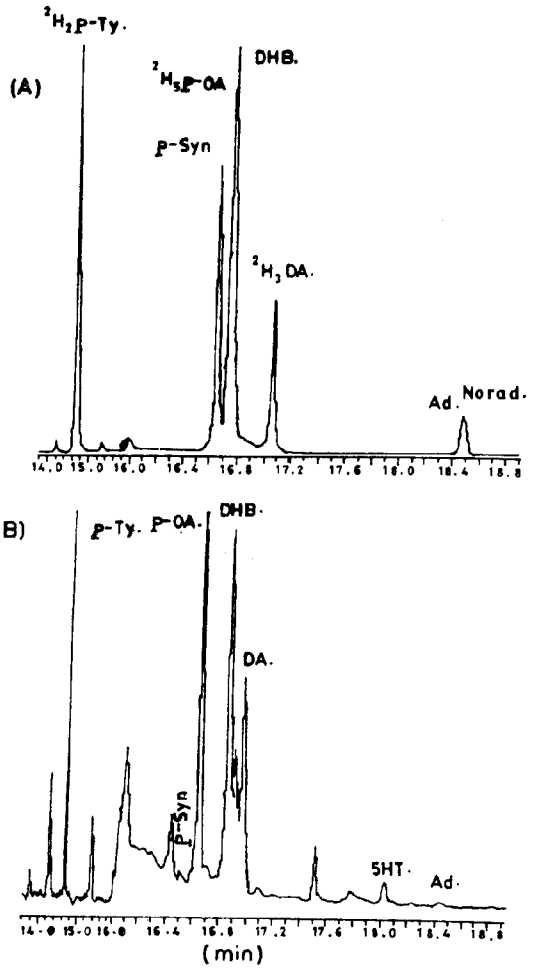

Fig.1: (A) NICI SIM trace of DTFMB-IPDMS derivatives of deuteriated and undeuteriated biogenic amines (each 20 ng) and (B) the corresponding endogenous biogenic amines from a single brain of American cockroach after addition of deuteriated internal standards (20 ng).

The figures are the average obtained by different methods of derivatizations (a) Concentration of biogenic amines (ng, as DTFMB-x IPDMS derivatives) (b) Concentration of biogenic amines (ng, as DTFMB-xTMS derivatives) extracted with HCl (0.1 N) [10].

The peak corresponding to the M^- ion of *p*-synephrine was observed in the analysis of almost every sample and also, in some cases, a clearly defined peak was observed for the ion of *m/z* 723 at the retention time corresponding to that of the

derivative of adrenaline. The results of the study using different methods of derivatization showed that *p*-tyramine, *p*-octopamine, dopamine, 5-hydroxytryptamine were widely distributed and occurred in higher amounts whilst, noradrenaline also present in the central nervous system of each insect but in half the amount of that of dopamine. The identification of subnanogram concentrations of the *N*-methyl derivatives of both *p*-octopamine and noradrenaline suggested that it was not possible to rule out the possibility that *N*-methyltransferase activity was present in the central nervous system of the insect, a minor route of biosynthesis. There has been no previous evidence in the literature for the presence of *p*-synephrine and adrenaline in any biological tissue or fluid of the cockroach. This was not surprising since both amines occurred in such low concentrations that it required the use of a highly sensitive and specific method of analysis to identify and quantify them.

It was possible to quantify 5-hydroxytryptamine, together with the other biogenic amines satisfactorily, from extracts of single brain by both methods of derivatization *i.e.* DTFMB-xTMS [10] and DTFMB-xIPDMS. However, the coefficients of variance of these determinations were generally higher for 5-HT than for those of other biogenic amines (Phenylethanol amines and catecholamines) described previously (Table-2). The reason for this could reside in the physiological behaviour of the insect itself or inefficient extraction of 5-HT into organic solvents from the aqueous homogenates of brain.

Therefore, 5-hydroxytryptamine and *N*-acetyl-5-hydroxytryptamine were quantitated using the modified and highly sensitive assay of Markey *et al.* [12], where perfluoroacylanhydrides further react with *O*-acylated 5-HT and *O,N*-diacylated 5-HT to produce stable spirocyclic products. The derivatized compound gave mass spectra where the (M-HF) carried > 60% of the ion current under NICI conditions. The retention times and intensities of the characteristic ions in the mass spectra of the *O*, *N*-diacyl-PFP derivatives of 5-HT [$^2H_0$] and [$^2H_3$] and *N*-acetyl-5-HT have been summarized in Table-3.

Table-3: Retention times and base peaks in the NICI mass spectra of Pr-PFP derivatives of indolealkylamines.

	Compound	tR (min)	Base peak (*m/z*)	Other significant ion *m/z* (% relative intensity)	
1.	[$^2H_0$] NA-5-HT	13.58	362	382(90):	326(50):
2.	[$^2H_0$] 5-HT	14.07	396	376(80):	340 (50):
3.	[$^2H_3$] 5-HT	14.06	399	379 (80):	343 (50):

The base peak (*m/z* 396 and *m/z* 399 respectively) was due to the loss of HF from the molecular ion ($M^{+\cdot}$) of derivatized 5-HT and its isotopomer. (Fig. 2). In the mass spectrum of derivatized *N*-acetyl-5-HT the base peak (*m/z* 362) was due to ion of [M-2HF] and there was another ion of *m/z* 382 (Corresponding to [M -HF] which occurred with a relative abundance of 90% (Fig. 3).

The limit of detection of these indolealkylamines on column was less than 15 pg. It was decided to prepare the propionyl-PFP (rather than the acetyl - PFP) derivative of 5-HT and its *N*-

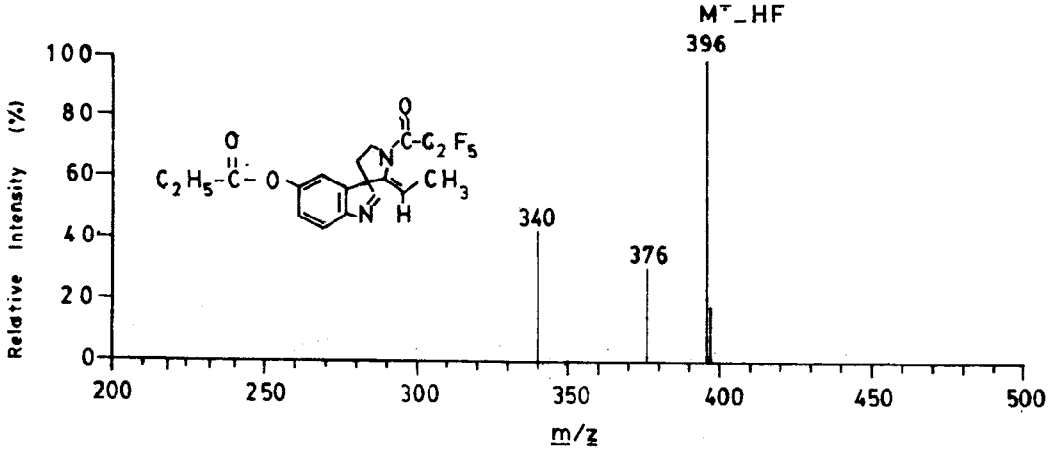

Fig.2: NICI Mass spectrum of Pr-PFP derivative of 5-hdyroxytryptamine.

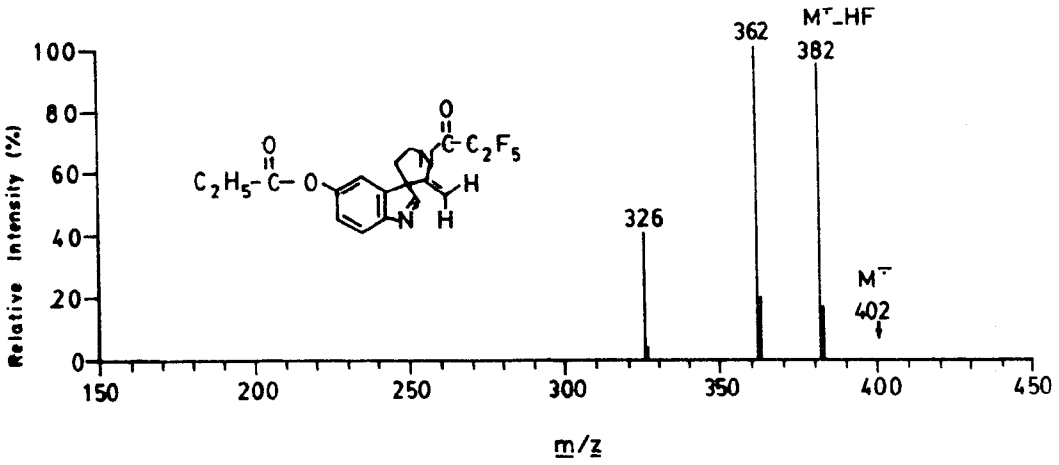

Fig. 3: NICI Mass spectrum of Pr-PFP derivative of *N*-acetyl 5-hydroxytryptamine.

acetylated-5-HT so that they could be assayed unambiguously and simultaneously by SIM in extracts from the cerebral ganglion of the cockroach. Both derivatized compounds contained the two major ions which were monitored in the trace obtained from the derivatized biological samples and there was little interference in the region of the chromatogram of interest. The use of [$^2H_3$] 5-HT as the internal standard increased the sensitivity and specificity (and thus the accuracy) of determination of the subnanogram concentrations of endogenous 5-HT and its *N*-acetylated metabolite in the biological tissues. The selected ion trace obtained by monitoring the characteristic base peak ions of the *O,N*-dipropionyl-PFP spirocyclic derivatives (of *m/z* value 362, 396 and 399 respectively) of *N*-acetyl-5-hydroxytryptamine, [^2H_O)5-HT and [$^2H_3$] 5-HT and that of the appropriate ions for the same derivatives of endogenous *N*-acetyl-5-hydroxytryptamine and 5-HT (with [$^2H_3$] 5-HT as internal standard) extracted from cockroach brain are shown in the Fig. 4(a) and (b) respectively. The average amounts/brain of 5-HT and *N*-acetyl 5-HT were 3.8 $\pm$ 2.4 (n=8) and 0.4 $\pm$ 0.1 (n=7) respectively.

The concentration of these compounds in the cerebral ganglia of the insect by this method of derivatization were similar to those reported previously by different methods of analysis: Particularly by HPLC (ECD) [13,14], where the average concentration of 5-HT and *N*-acetyl -5HT were 4-7 ng and 0.3 - 0.34 ng per sample respectively. Thus the stability of the ion [M-HF]$^-$ of PFP-Spirocyclic derivatives allows for high sensitivity in the detection of compounds and make quantitation more reliable when an analogue rather than an isotopomer is used as the internal standard, since the fragmentation pattern is effected by change in ion source conditions.

Experimental

Gas chromatography mass spectrometry (GC-MS) in the NICI mode was carried out using a Hewlett-packard 5988A gas chromatograph mass spectrometer interfaced with a HP RTE-6/VM data system. The following mass spectrometric conditions were used.

The instrument was tuned in the NICI mode to the ions at *m/z* 452, 595 and 633 from the perfluorotributylamine (PFTBA) calibrant, source temperature was 140°C, electron energy 200 ev and methane reagent gas was introduced to give source pressure ~0.9 Torr. The gas chromatograph was fitted with a HP-1 fused silica column of 0.2 μm layer thickness, (either 12.5 m x 0.2 mm, i.d. or 25 m x 0.2 mm i.d); helium carrier gas was used with a head pressure of 8 p.s.i. for the 12.5 m column or 25 p.s.i. when the 25 m column was installed, with flow rate of 40 ml/min. The GC conditions were as follows: injector temperature 250°C, transfer line temperature 280°C, the oven temperature was

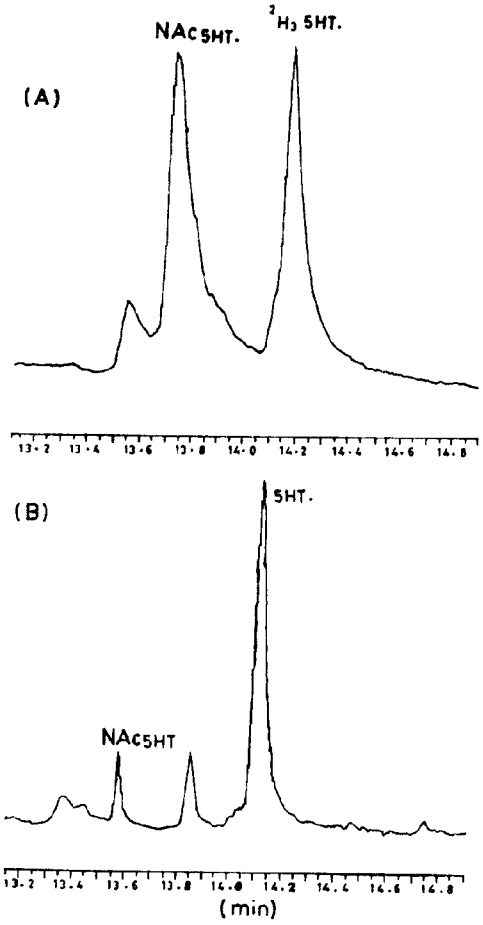

Fig. 4: (A) NICI SIM trace of Pr-PFP derivatives of deuteriated and undeuteriated biogenic amines (each 20ng) and (B) the corresponding endogenous biogenic amines from a single brain of American cockroach after addition of deuteriated internal staandards (20 ng).

maintained at 100°C for 1 min, then programmed at 10°C min^{-1} to 300°C. Injections were made using a Grob splitless injection system.

Material and reagents

All the solvents used in extraction and derivatization were HPLC grade (Rathburn chemicals, peebleshire, U.K). Chemicals were obtained from the following sources: DTFMBCL; 3, 5-Ditrifluoromethylbenzoyl chloride (Fluorochem Ltd); IPDMS: Isopropyldimethylsilyl chloride, TA: Tyramine, DA: Dopamine hydrochloride, OA: Octopamine hydrochloride, AD: Adrenaline, Norad: Noradrenaline hydrochloride, Syn: Synephrine hydrochloride, 5-HT: 5-Hydroxytryptamine hydrochloride, [$^2H_1$] Cl: Deuterium chloride [$^2H_2$] O: Deuterium oxide, C [$^2H_3$] COO [$^2H_1$], Deuterioacetic acid, were obtained from the Aldrich chemical Co. Ltd: DHB : 3,4-Dihydroxybenzylamine hydrochloride (Sigma chemical Co. Ltd); PFPA: Pentafluoropropionic anhydride (Pierce Chemical Co. Ltd).

Synthesis of deuterium labelled internal standards

[2,5,6-$^2H_3$] Dopamine deuteriochloride, [3, 5-$^2H_2$] tyramine deuteriochloride and [α, α, β, 3, 5-$^2H_5$] *p*-octopamine deuteriobromide were prepared as described in our previous work [10]. [2,5,6-$^2H_3$] Adrenaline deuteriochloride and [2,5,6-$^2H_3$] noradrenaline deuteriochloride were synthesized as their crystalline deuteriochloride salts by heating the corresponding undeuteriated compound with 20% [$^2H_1$] Cl in sealed tube at 80°C for 40 h. [$^2H_3$] Adrenaline deuteriochloride was obtained in 85% yield, m.p. 155-157°C decomp (Lit. [11] mp. 157°C decomp)[a]. The ^{1}H-NMR spectrum showed that signals due to the protons at position C_2, C_5 and C_6 of the aromatic ring were absent and GC-NICIMS with selected ion monitoring (SIM) of the M cluster of DTFMB-TMS derivative gave the isotopic composition as : [$^2H_3$] 52.7% and [$^2H_0$] 1.7%. [$^2H_3$] Noradrenaline deuteriochloride was also obtained in 85% yield, m.p. 189-190°C (Lit. [11] m.p. 191°C)[a]. The ^{1}H-NMR spectrum showed that signals for the aromatic protons were absent and GC-NICI MS with SIM of the M cluster of the DTFMB-TMS derivative gave the isotope composition as : [$^2H_3$] 66.9% and [$^2H_0$] 1.9%.

[2,4,6,7-$^2H_4$] 5-Hydroxytryptamine deuteriochloride was obtained by heating the unlabelled compound with aqueous [$^2H_1$] Cl (20% w/w) at 75-80°C for 40 h. The dark yellow microcrystalline solid thus obtained was applied to precoated silica gel plates to afford pure colourless microcrystalline needles in 20% yield, m.p. 160-163°C (Lit. [11] m.p. 167-168°C)[a]. The ^{1}H-NMR showed that the signals due to C_2, C_4, C_6 and C_7 protons were absent. GC-NICI MS analysis using [M^+ -HF] of the compound as the *O, N*-dipropionyl- PFP Spirocyclic derivative gave [$^2H_4$] 32.7%, [$^2H_3$] 65.9% and [$^2H_0$] 1.4%.

a. m.p. of the non deuteriated compound.

Extraction and derivatisation

Adult cockroaches (*P. americana*) of both sexes were left undisturbed at room temperature for 1-2 h after removal from the main colony. The insects were anaesthetized and the brain (Cerebral ganglion) was removed directly from the anaesthetized insect and either processed immediately or frozen by placing it on a piece of dry ice followed by storage at -20°C until required. The tissue was homogenised in 1 ml of 0.1 M hydrochloric acid. The extract was centrifuged for 30 min at 4500 g and the internal standards were added to the extraction medium prior to homogenization. The pH of the supernatant liquid was adjusted to 7.2 with an equal volume of 1M potassium phosphate buffer. The solution was then shaken with DTFMBCL (2 µl) for 10 min, extracted with ethylacetate (2 x 1.5 ml) and the combined extract was shaken with 10 M aqueous ammonium hydroxide (0.5 ml, 5 min) to hydrolyze phenolic ester groups. The organic solvent removed to dryness and the residue was then reacted (60 min., 65°C) with IPDMS-MTFA (20 µl). Most of the reagent was removed and the derivatised compound was dissolved in ethyl acetate for GC-MS analysis. For the quantitation of 5-hydroxytryptamine and *N*-acetyl-5-hydroxytryptamine extract of the brain tissue was added to 0.3M perchloric acid (0.5 ml). Acylation was carried out by the addition of saturated aqueous sodium carbonate (0.2 ml), pyridine (30 µl) and propionic anhydride (0.2 ml), followed by vigorous vortexing. The *O-N*-dipropionylated product was extracted into ethyl acetate and solvent removed to dryness. The residue was reacted (15 min. 60°C) with PFPA (200 µl) and excess of the reagent was removed. The residue was dissolved in benzene (0.5 ml) and the resultant solution was thoroughly vortexed with 0.05 M aqueous potassium phosphate buffer (0.3 ml, pH 8.0) until it was completely clear. The organic layer was separated and then removed by evaporation with a stream of nitrogen. The derivatised product thus obtained was dissolved in ethyl acetate for GC-MS analysis.

Standard mixtures (20 ng) of deuteriated and undeuteriated amines (1:1) were derivatised and analysed when each batch of samples was processed and blanks were also performed on the reagents on each occasion.

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