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Determination of the natural abundance δ^{15}N of nicotine and related alkaloids by gas chromatography/isotope ratio mass spectrometry

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A method is described by which the natural abundance δ^{15}N values of nicotine, analogues, and metabolites can be determined. The alkaloids are extracted from their biological matrix by solid-phase extraction and analysis is conducted using isotope ratio mass spectrometry interfaced to gas chromatography. Repeatability and precision are sufficient to allow differences in the δ^{15}N values of less than 1.0‰ to be satisfactorily measured, with a standard deviation routinely less than 0.5‰. The methodology has been tested by determining the changes in the δ^{15}N values of nicotine, *N*-methyl-2-phenylpyrrolidine and their respective demethylation products, nornicotine and 2-phenylpyrrolidine, during biotransformation by cell suspension cultures of *Nicotiana* species. Sufficient precision and reproducibility were obtained to allow the kinetic isotope effects associated with the demethylation reaction to be calculated.

Nicotine is a naturally occurring alkaloid found primarily in members of the Solanaceae.[1] It is well known for its stimulatory effects on human physiology, due to its interaction with the nicotinamide receptor.[2] Despite its high toxicity—only about 5-fold less than hydrocyanic acid—nicotine remains widely used and abused throughout the world. Indeed, until relatively recently, it was used as an insecticide in Japan and the USA.[3] It is only due to the efficient and effective detoxification of nicotine by liver metabolism that nicotine poisoning in humans is not more commonly encountered. Toxicity is reduced in the body by the efficient metabolism of nicotine by liver enzymes—mostly cytochrome P450 oxidases—to less toxic metabolites.[4] These include principally nornicotine—produced by *N*-demethylation, cotinine—the product of oxidation in the 5′ position, and various hydroxylated derivatives, which are excreted in the urine. In mammalian systems, these oxidative reactions appear to be initiated by the activation of the pyrrolinium ring at the 5′ position.[5]

In contrast, over the last decade, it has been increasingly evident that nicotine can have beneficial effects in treating neurodegenerative disorders, notably Altzheimer's and Tourette's syndromes.[2,6] A number of promising derivatives are now undergoing clinical trials.[7] Hence, the need to follow the metabolism of nicotine or analogues at low concentrations in biological systems is of importance. In such cases, the use of stable isotopes for *in vivo* pharmacokinetic studies is increasingly preferred.

Isotope ratio mass spectrometry (IRMS) permits isotopic determination at natural abundance or at low levels of enrichment, making it an excellent choice for studies in which enrichment is difficult, expensive or prohibited. It can also be used to follow the metabolism of more than one pool of metabolite simultaneously.[8] An advantage of using δ^{15}N values rather than δ^{13}C values is that, in some molecular species, including nicotine[9] and 3,4-methylenedioxymethamphetamine (ecstasy),[10] the δ^{15}N has a wider range of values than the δ^{13}C, thus increasing the ease of discrimination between different sources. It is also better adapted to following the metabolism of nitrogenous molecules. The drawback of IRMS is that when direct oxidation of the sample is used (typically by coupling an elemental analyser (EA) to an IRMS system), the sample must be pure. Due, at least in part, to the intrinsic difficulties of quantitatively and reproducibly isolating mg quantities of alkaloids from dilute solution, we turned our attention to the potential of IRMS interfaced to gas chromatography (GC) as a means of obtaining accurate δ^{15}N values. This technique has been proved to be precise and reproducible for the measurement of δ^{15}N values in 3,4-methylenedioxymethamphetamine extracted from ecstasy tablets, a study that used nicotine for column calibration.[10] However, the analysis of extracts from biological samples has not been frequently reported. In this paper we show that satisfactory δ^{15}N values for nicotine, the related non-natural alkaloid, *N*-methyl-2-phenylpyrrolidine, and their respective demethylation

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products, nornicotine and 2-phenylpyrrolidine, can be obtained. The technique has potential for the non-invasive examination of the turnover of nicotine and analogues in human subjects.

To test the methodology in a biological system, we have examined the demethylation of nicotine and *N*-methyl-2-phenylpyrrolidine in plant cell suspension cultures of *Nicotiana plumbaginifolia* and *N. glutinosa*. These cultures, while incapable of *de novo* synthesis of nicotine, are efficient at converting exogenous nicotine into nornicotine.[11,12] Our objective was to assess whether the method was sufficiently precise to determine the kinetic isotope effects (KIEs) associated with the demethylation reaction, as a means to further understand the mechanism of nicotine demethylation in plants.[13,14] In order to achieve this, it is necessary to measure with high precision the natural abundance δ^{15}N values, preferably in both the substrate consumed and the product accumulated. The results of this analysis will be reported elsewhere.

EXPERIMENTAL

Chemicals

(*S*)-Nicotine (purity > 98%) was obtained from Sigma Chemical Co. (www.sigmaaldrich.com) and (*R,S*)-nornicotine (purity > 96%) from Lancaster Synthesis (www.lancaster-synthesis.com). (*R,S*)-2-Phenylpyrrolidine (purity > 97%) was prepared[12,15] by condensing allylzinc with benzaldehyde to form 1-phenylbut-3-en-1-ol,[16] which was converted via the 1-mesylate into 1-azido-1-phenylbut-3-en-1-ol. Treatment of this compound with dicyclohexylborane led to ring closure and the formation of (*R,S*)-2-phenylpyrrolidine.[17] (*R,S*)-*N*-Methyl-2-phenylpyrrolidine (purity > 97%) was prepared by the methylation of (*R,S*)-2-phenylpyrrolidine with formic acid/formaldehyde.[12,18]

Plant cell cultures

Plant cell cultures of *Nicotiana plumbaginifolia* and *N. glutinosa* were grown as previously described.[12,13] Both these cultures have the ability to carry out the demethylation of nicotine and analogues of nicotine and to accumulate the demethylation product in the extracellular medium, but they do not synthesise nicotine.[11,13] The cell cultures were cultivated in a defined growth medium containing sugar, mineral salts and vitamins, designated medium 'FMD',[13] and were maintained in an incubator at 25°C, on a gyratory shaker at 100 rpm, under constant illumination. The cells were subcultured into a new medium every 10 to 14 days.

Biotransformation was conducted over various periods between 0 and 8 days. Cells harvested in growth phase (day 8 of culture) were resuspended in a non-growth medium 'FMS',[13] and substrate added as a filter-sterilised aqueous solution. At harvest, cultures were frozen at −20°C until required.

Sample preparation

The culture stored at −20°C was rapidly thawed, H_2SO_4 (2 mL, 1 M) was added and the cellular material disintegrated with an Ultra-Turrax® tissue disintegrator (IKA Labortechnik, Janke & Kunkel, Staufen, Germany) at 13500 rpm (1 min). The entire volume was basified to pH > 10 with NaOH solution (1–3 mL, 1 M) and filtered under suction through a glass fibre paper (grade GF/A) using a Büchner filter.

This filtrate was applied directly to a preconditioned (washed with 5 mL methanol and 5 mL distilled water) solid-phase extraction (SPE) cartridge (500 mg Discovery C18, Supelco, www.sigmaaldrich.com) fitted to a Visiprep vacuum manifold (Supelco). Sample was introduced by low suction, the cartridge rinsed with water/methanol (5 mL, 95:5 v/v) and dried with a flow of N_2 gas for 30 min. Compounds were recovered in methanol (10 mL).

Quantification of compounds

Quantification was carried out using a gas chromatograph calibrated with pure standards. Standard conditions were: chromatograph, Hewlett Packard 6510 (www.hp.com); carrier gas, helium at 1.2 mL/min (constant flow); split ratio, 1:40; column, fused poly(5% diphenyl/95% dimethylsiloxane) deactivated for non-specific interaction with basic compounds (PTA-5, Supelco, 30 m, i.d. 0.32 mm, film thickness, 0.52 μm), injector temperature, 220°C, detection by FID at 280°C; injected volume (manual), 1 μL.

The gas chromatogram was developed using a thermal gradient. For nicotine and its metabolites the thermal elution conditions were: 100°C for 1 min; 10°C/min to 200°C, 20°C/min to 260°C, 3 min at 260°C. Calibration was by reference to external standards (range 0.05–0.5 mg/mL; nicotine $r^2 = 0.9979$, nornicotine $r^2 = 0.9994$) and 2,6-di-*tert*-butyl-4-methylphenol (BHT) (0.5 mg/mL) was used as internal correction reference. For *N*-methyl-2-phenylpyrrolidine and its metabolites the thermal elution conditions were: 100°C for 1 min; 5°C/min to 160°C, 15°C/min to 240°C, 1 min at 240°C. Calibration was by reference to external standards (range 0.05–0.5 mg/mL; *N*-methyl-2-phenylpyrrolidine $r^2 = 0.9999$, 2-phenylpyrrolidine $r^2 = 0.9996$) and BHT (0.5 mg/mL) was used as an internal correction reference.

For GC analysis, the samples were diluted as appropriate (2- to 4-fold in methanol) with the internal correction reference of BHT added to a final concentration of 0.5 mg/mL. Each sample was analysed at least three times.

Determination of the δ^{15}N ratio

Reference compounds

The δ^{15}N (‰) values of pure compounds were determined by encapsulation and analysis using a Finnigan MAT Delta-S isotope ratio mass spectrometer (www.thermo.com) coupled to an elemental analyser (EA; NA2100 Fisons Instruments, www.thermo.com). Between 1 and 2.5 mg of compound (giving approx. 0.2 mg nitrogen) were sealed in a tin capsule and the δ^{15}N determined by reference to a working standard of glutamic acid standardised against a calibrated international reference material (IAEA-N1 or IAEA-N2; IAEA, Vienna, Austria).[19]

Cellular samples

The δ^{15}N (‰) values of samples were determined using a Finnigan MAT Delta-S isotope ratio mass spectrometer coupled to a Hewlett Packard 5890 series II gas chromatograph via a combustion interface (GC/C/IRMS).

The following chromatographic conditions were used: carrier gas, helium at constant pressure of 70 kPa (initial flow 1.2 mL/min); injection, splitless; column, PTA-5 (Supelco, 30 m, i.d. 0.32 mm, film thickness, 0.52 μm), injector temperature, 220°C; injected volume (autosampler), 1 μL. Elution was by thermal gradient. For nicotine and metabolites the thermal gradient conditions were: 90°C for 1 min; 10°C/min to 200°C, 40°C/min to 240°C, 1 min at 240°C. For *N*-methyl-2-phenylpyrrolidine and metabolites the thermal gradient conditions were: 90°C for 1 min; 7°C/min to 160°C, 40°C/min to 240°C, 1 min at 240°C.

Eluted compounds were combusted at 940°C into NO_x, CO_2 and H_2O in a combustion furnace composed of a nonporous alumina tube (Al_2O_3, 0.5 mm i.d., 1.55 mm o.d., 320 mm length) containing three wires (Cu/Ni/Pt, 0.125 mm diameter, 240 mm identical length) braided and centered end-to-end within the tube. Nitrogen oxides were reduced to N_2 in a reduction furnace at 600°C. This furnace is of the same configuration as the oxidation furnace except that the reactor filling is pure Cu (three twisted Cu wires of 0.125 mm diameter). Water vapour was removed using a water semi-permeable trap (Nafion membrane) and CO_2 was removed using a liquid nitrogen trap. Only N_2 was admitted into the ion source.

The GC/C/IRMS system was calibrated using a solution of nicotine of $\delta^{15}N = -5.01 \pm 0.19$‰ (n = 18), determined by encapsulation as described above. For each series of acquisitions, this value was used to correct for any drift or fluctuations during the analysis of an experimental series. Each reference and unknown was analysed three times and the maximum correction applied was 0.5‰. In order to minimise the influence of drift, series were run in the following sequence: solvent only (1×); nicotine (3×); solvent only (1×); sample 1 (3×); solvent only (1×); sample 2 (3×); solvent only (1×); nicotine (3×); solvent only (1×); sample 3 (3×), and so on.

Prior to analysis by GC/C/IRMS, samples were concentrated under a gentle stream of N_2 gas without heating to bring the concentration of the compounds of interest into the range suitable for measurement by IRMS. The concentration was ~2.5-fold for nicotine samples and ~5-fold for *N*-methyl-2-phenylpyrrolidine samples.

RESULTS AND DISCUSSION

Validation of the extraction protocol

The analysis of isotopic values requires *a priori* that the sample preparation protocol does not itself introduce fractionation in the sample, or that any such fractionation is consistent. Thus, while there are numerous methods available for the extraction of nicotine and related metabolites from biological samples,[20] it was necessary to verify that the method to be employed did not cause undue fractionation. Solid-phase extraction (SPE) offers the advantages both of concentrating the analyte in the aqueous sample and of recovering the target compounds in a relatively small volume of organic phase.[21]

Eight samples of nicotine, treated in three separate series, were extracted by SPE from cell culture media and their δ^{15}N (‰) values measured by GC/C/IRMS. As can be seen (Table 1), the protocol gave a high and reproducible recovery of nicotine, with a mean recovery of 94 ± 3%. A mean δ^{15}N value of −5.17 ± 0.29‰ (n = 8) was obtained from these samples, which is not significantly different (t-test, $p < 0.001$) from the mean δ^{15}N value of −5.01 ± 0.19‰ (n = 18) for the reference nicotine obtained by EA-IRMS. Therefore, it could be concluded that the extraction protocol did not introduce isotopic fractionation for nicotine.

Table 1. Test for reproducibility of recovery and δ^{15}N (‰) of nicotine from *N. plumbaginifolia* cultures

		Quantity			δ^{15}N (‰)[c]	
Sample N°	N°	Total[a] (μmol)	SD[b]	Recovery (%) (%)	Mean[a]	SD[b]
1	T01	57.8	0.4	99.0	−5.26	0.34
3	T03	56.6	1.3	96.9	−5.43	0.35
4	T04	54.2	0.9	92.8	−4.82	0.11
5	T05	55.4	0.6	94.8	−4.98	0.15
6	T06	67.3	0.1	92.6	−4.81	0.76
7	T07	67.9	1.9	93.4	−5.62	0.46
8	T08	63.8	4.0	87.7	−5.32	0.15
9	T09	69.9	0.2	96.2	−5.15	0.34
Overall mean					**−5.17**	**0.29**

[a] Initial values: T01–T05, 58.4 μmol; T06–T09, 72.7 μmol.
[b] Each sample determined in triplicate.
[c] Initial δ^{15}N value: −5.01 ± 0.19‰.

Similarly, ten samples of *N*-methyl-2-phenylpyrrolidine were subjected to the extraction protocol from cell culture media and their δ^{15}N values measured by GC/C/IRMS (Table 2). These samples came from two separate syntheses (M and T) of this compound and had different initial δ^{15}N values, as determined by EA-IRMS: M series, −9.09 ± 0.28‰ (n = 5); T series, −20.00 ± 0.46‰ (n = 5). The protocol gave a slightly lower recovery than with nicotine, with a mean of 81 ± 5%, presumably due to some non-specific interactions with the column. The reproducibility of the determination of the δ^{15}N (‰) values by GC/C/IRMS was slightly less precise than for nicotine, giving mean δ^{15}N values of −9.25 ± 0.37‰ (n = 4) and −20.65 ± 0.90‰ (n = 6) for the M and T series, respectively. However, as for nicotine, the values of δ^{15}N for the recovered *N*-methyl-2-phenylpyrrolidine are not significantly different (t-test, $p < 0.001$) from the mean δ^{15}N values obtained by EA-IRMS. Therefore, it can be concluded that the extraction protocol does not introduce fractionation for *N*-methyl-2-phenylpyrrolidine.

It was not possible to carry out as rigorous an analysis of the fractionation of the demethylation products, nornicotine and 2-phenylpyrrolidine, due to an insufficiency of material. A limited experiment to test the recovery of 2-phenylpyrrolidine from the SPE columns using 15.1 μmol of the 'M' series gave a mean recovery of 71 ± 3% (n = 4). In addition, recoveries in excess of 75% were always achieved from biological samples, as assessed by GC.

Application of the protocol to biotransformation by *Nicotiana* cell cultures

The experimental model in which the analysis of the δ^{15}N values was applied consisted of plant cell cultures of

Table 2. Test for reproducibility of recovery and δ^{15}N (‰) of *N*-methyl-2-phenylpyrrolidine and 2-phenylpyrrolidine from *N. plumbaginifolia* cultures

		N-Methyl-2-phenylpyrrolidine					2-Phenylpyrrolidine		
		Quantity			δ^{15}N (‰)[c]		Quantity		
Sample N°	N°	Total[a] (μmol)	SD[b]	Recovery (%) (%)	Mean[b]	SD[b]	Total[d] (μmol)	SD[b]	Recovery (%) (%)
1	M1	30.3	0.6	83.3	−9.39	0.13	11.1	0.2	73.5
2	M2	30.4	0.4	83.7	−9.31	0.56	11.0	0.2	73.0
3	M3	29.9	0.3	82.5	−9.25	0.22	10.7	0.1	70.8
	M4	31.8	1.4	87.6	[e]				
4	M5	54.2	3.8	84.9	−9.06	0.37	11.0	0.1	72.6
Mean series M					**−9.25**	**0.37**			**71.0**
5	T01	44.3	0.5	73.4	−21.80	1.40	[e]		
6	T02	44.8	1.0	74.2	−20.61	1.39			
7	T04	46.5	2.0	77.1	−20.17	0.45			
8	T06	47.8	0.8	77.9	−19.65	0.41			
9	T07	53.0	3.0	86.5	−20.46	0.62			
10	T08	47.9	2.5	78.2	−21.22	1.12			
Mean series T					**−20.65**	**0.90**			
Overall mean				**81.0**					

[a] Initial values were: M1–M3, 36.3 μmol; M4, 63.83 μmol; T01–T04, 60.3 μmol; T06–T08, 61.3 μmol.
[b] Each sample determined in triplicate.
[c] Initial values: M series, -9.09 ± 0.28‰; T series, -20.00 ± 0.46‰.
[d] Initial values were: M1–M4, 15.1 μmol.
[e] Grey areas indicate that values were not determined.

Nicotiana plumbaginifolia and *N. glutinosa*. Both these cultures have the ability to carry out the demethylation of nicotine or *N*-methyl-2-phenylpyrrolidine and to accumulate the demethylation products, nornicotine or 2-phenylpyrrolidine, respectively, in the extracellular medium.[12,13,15]

The biotransformation of nicotine into nornicotine by *N. plumbaginifolia* cell cultures was conducted in two series of experiments, 4 months apart, using nicotine of initial $\delta^{15}N = -5.01 \pm 0.19$‰ ($n = 18$). In the first series, all cultures were incubated for 6 days while, in series 2, cultures were incubated with substrate for 4, 6 or 8 days. The biotransformation of nicotine into nornicotine by *N. glutinosa* cell cultures was conducted in one experimental series (3), conducted 1 month after series 2, with a trial experiment conducted with the series 2 *N. plumbaginifolia* samples (Table 3).

Each series of biotransformations showed a slightly different rate of demethylation, reflecting small differences both in the physiological status of the cells at the start of the

Table 3. Recovery of nicotine and nornicotine and the respective δ^{15}N values

				Compound recovery[a]						δ^{15}N (‰)[b]			
				Nicotine		Nornicotine		Total		Nicotine		Nornicotine	
Species	Series	Time (days)	Sample	(μmol)	SD	(μmol)	SD	(μmol)	(%)	Mean	SD	Mean	SD
N. plumbaginifolia	1	6	N1	19.1	0.3	30.9	0.5	50.0	85.5	−2.66	0.53	−6.63	0.57
		6	N2	1.8	0.0	45.5	2.4	47.3	80.9	0.11	0.43	−5.58	0.06
		6	N3	10.4	0.1	38.8	1.3	49.2	84.2	−2.08	0.25	−6.76	0.11
		6	N4	9.6	0.1	43.9	1.6	53.4	91.5	−1.82	0.09	−6.83	0.02
		6	N5	13.4	0.2	37.3	0.8	50.7	86.8	−1.81	0.21	−5.90	0.30
N. plumbaginifolia	2	4	N6	36.3	1.5	26.5	4.1	62.8	86.4	−3.53	0.47	−11.11	0.60
		4	N7	37.7	0.7	20.7	0.9	58.5	80.4	−3.70	0.31	−9.27	0.37
		6	N8	35.3	0.5	23.3	2.4	58.6	80.6	−3.67	0.42	−8.10	0.07
		8	N10	27.3	0.3	30.8	0.5	58.2	80.0	−2.92	0.55	−6.38	0.36
		8	N11	27.1	0.2	27.5	1.5	54.6	75.1	−2.95	0.53	−7.75	0.43
N. glutinosa	1	6	G1	39.4	0.4	7.4	0.7	46.8	64.4	−3.88	0.51	−7.73	0.63
N. glutinosa	3	6	GN1	4.8	0.1	34.1	1.9	38.9	53.5	−1.02	0.73	−3.57	0.45
		6	GN2	2.9	0.1	38.2	2.8	41.1	56.6	0.10	0.38	−6.11	0.21
		6	GN3	15.2	0.5	20.0	0.3	35.2	48.5	−2.20	0.40	−6.91	0.26
		6	GN4	10.6	0.1	27.4	0.9	38.0	52.3	−1.44	0.25	−4.68	0.33
		6	GN5	9.4	0.0	31.5	0.4	40.9	56.2	−1.42	0.17	−5.61	0.22
		6	GN6	14.1	0.4	37.4	1.5	51.4	70.8	−1.60	0.36	−6.25	0.48

[a] Initial values were: N1–N5, G1, 58.4 μmol; N6–N11, GN1–GN6, 72.7 μmol.
[b] Initial δ^{15}N value for nicotine: -5.01 ± 0.19‰.

experiment and in the species used. Notably, the *N. glutinosa* culture showed a higher rate of demethylation than the *N. plumbaginifolia* culture.[12] For *N. plumbaginifolia*, an overall mean recovery of residual total alkaloid of $83 \pm 5\%$ was obtained. Taking into account the mean losses in extraction (see Table 1), this indicates that about 12% of the initial nicotine was apparently metabolised to minor products, of which myosmine[12] and *N*-formylnornicotine[22] have been identified. This level is compatible with results given in the literature.[13] For *N. glutinosa*, the more rapid kinetics resulted in smaller amounts of residual total alkaloid of $58 \pm 8\%$, and a greater overall loss from the system (37%) was observed.

However, despite the range of recoveries and the extents of biotransformation present in the samples examined, δ^{15}N values of both the nicotine and the nornicotine recovered could be determined by GC/C/IRMS for all samples (Table 3). For both *Nicotiana* species, the δ^{15}N values in the residual substrate and in the product are significantly different. Taking together all the δ^{15}N values determined on nicotine and nornicotine extracted from cell cultures, a standard deviation (SD) of 0.36‰ (n = 34) is obtained, with only a few values >0.5‰. This indicates that a satisfactory precision is obtained by the methodology for the determination of small fluctuations in ^{14}N/^{15}N ratios, such as need to be measured in the context of a study of KIEs or pharmacokinetics. In general, the precision was less for the lower concentrations of metabolites, due to the difficulty in obtaining an accurate integration of the area under the peak in the chromatogram.

The biotransformation of *N*-methyl-2-phenylpyrrolidine into 2-phenylpyrrolidine by *N. plumbaginifolia* cell cultures was conducted in three series of experiments, 8 months apart. Series 1 used *N*-methyl-2-phenylpyrrolidine of initial $\delta^{15}N = -9.09 \pm 0.28$‰ (n = 4) and series 2 and 3 used *N*-methyl-2-phenylpyrrolidine of initial $\delta^{15}N = -20.00 \pm 0.46$‰ (n = 6). For series 1 and 2, all cultures were incubated with substrate for 6 days, while, in series 3, the cultures were incubated with substrate for 8 days. The biotransformation of *N*-methyl-2-phenylpyrrolidine (initial $\delta^{15}N = -20.00 \pm 0.46$‰ (n = 6)) by *N. glutinosa* cell cultures was conducted in one experimental series (4), conducted 1 month after series 3, with a trial experiment conducted with the series 2 *N. plumbaginifolia* samples (Table 4).

As found for nicotine, each series of biotransformations shows slightly different rates of demethylation. For *N. plumbaginifolia*, the series 1 and 2 show slower product accumulation than does series 3. Series 4, using the *N. glutinosa* culture, showed a lower rate of demethylation than was found for nicotine, closer to that obtained for *N. plumbaginifolia*. An overall mean recovery of $75 \pm 2\%$ was obtained for the samples from *N. plumbaginifolia*, indicating a loss due to metabolism of only 4%. For *N. glutinosa*, the recovery was $82 \pm 9\%$, indicating that there was no loss from the system.

Once again, as for nicotine, the δ^{15}N values for *N*-methyl-2-phenylpyrrolidine and 2-phenylpyrrolidine could be determined by GC/C/IRMS in all samples, despite the range of recoveries and extents of biotransformation present (Table 4). Again, for both species, the δ^{15}N values in the residual substrate and in the product are significantly different. Taking together all the δ^{15}N values determined on *N*-methyl-2-phenylpyrrolidine and 2-phenylpyrrolidine extracted from

Table 4. Recovery of *N*-methyl-2-phenylpyrrolidine and 2-phenylpyrrolidine and the respective δ^{15}N values

				Compound recovery[a]						δ^{15}N (‰)[b]			
				N-Methyl-2-phenylpyrrolidine		2-Phenylpyrrolidine		Total		*N*-Methyl-2-phenylpyrrolidine		2-Phenylpyrrolidine	
Species	Series	Time (days)	Sample	(μmol)	SD	(μmol)	SD	(μmol)	(%)	Mean	SD	Mean	SD
N. plumbaginifolia	1	6	B2	27.3	0.3	19.3	0.3	46.6	73.0	−8.08	0.10	−12.00	0.55
		6	B3	38.8	1.6	11.1	0.4	49.9	78.2	−9.23	0.48	−10.71	0.94
		6	B4	39.5	2.1	9.0	0.1	48.5	76.0	−8.59	0.29	−11.97	1.01
		6	B5	40.9	1.4	8.7	0.1	49.6	77.7	−8.52	0.19	−11.20	0.46
N. plumbaginifolia	2	6	P1	38.6	1.8	7.1	0.1	45.7	75.9	−21.12	0.41	−26.10	0.84
		6	P2	38.6	1.2	6.2	0.1	44.7	74.2	−20.48	0.37	−25.91	1.18
		6	P3	40.4	1.3	5.6	0.1	46.0	76.3	−20.55	0.30	−25.65	0.43
		6	P5	39.7	1.4	6.4	0.2	46.1	76.4	−20.65	0.72	−26.86	1.34
N. plumbaginifolia	3	8	P6	38.4	1.9	6.0	0.3	44.4	73.6	−20.24	0.55	−28.02	0.41
		8	P7	39.3	2.0	7.1	0.1	46.4	76.9	−20.12	0.44	−26.91	0.66
		8	P8	36.8	2.1	6.2	0.1	43.0	71.3	−19.29	0.41	−27.97	0.37
		8	P9	37.6	0.7	6.5	0.1	44.1	73.1	−19.89	0.24	−25.70	0.53
		8	P10	38.1	0.9	6.0	0.1	44.1	73.1	−19.97	0.54	−27.64	0.47
N. glutinosa	2	6	G2	12.4	0.3	32.9	0.4	45.3	75.0	−18.57	0.26	−22.79	0.23
N. glutinosa	4	6	GP1	42.4	1.1	11.5	1.3	53.9	88.0	−20.36	0.18	−24.00	1.52
		6	GP2	44.4	1.1	10.4	0.4	54.8	89.5	−20.71	0.53	−23.72	0.53
		6	GP3	42.5	0.2	11.6	0.5	54.1	88.3	−19.43	0.91	−21.46	0.44
		6	GP4	38.9	0.5	11.6	0.3	50.5	82.4	−20.05	0.94	−22.30	0.76
		6	GP5	31.3	0.6	9.0	0.5	40.3	65.7	−20.43	0.15	−24.84	0.43
		6	GP6	40.3	1.2	10.5	0.9	50.9	83.0	−20.79	0.82	−22.40	0.65

[a] Initial values were: B2–B5, 63.8 μmol; P1–P10, G2, 60.3 μmol; GP1–GP6, 61.3 μmol.
[b] Initial δ^{15}N values (‰)were: B2–B5, -9.09 ± 0.28‰; P1–P10, G2; GP1–GP6 -20.00 ± 0.46‰.

cell cultures, an SD of 0.57‰ (n = 40) is obtained, with a few values >1.0‰. Thus, with this substrate and product, the mean precision of the measured δ^{15}N values was slightly less than for nicotine, but, once again, the less precise values were obtained with the lower concentrations of metabolite.

CONCLUSIONS

The protocol described herein has proved sufficiently precise to determine changes in the $^{14}N/^{15}N$ ratio of nicotine or of *N*-methyl-2-phenylpyrrolidine within ±1.00 δ^{15}N units during their partial demethylation to nornicotine and 2-phenylpyrrolidine, respectively. Furthermore, it has proved possible to determine these values over wide ranges of quantity: from 1.8 to 69.9 μmol for nicotine; from 7.4 to 45.5 μmol for nornicotine; from 12.4 to 54.2 μmol for *N*-methyl-2-phenylpyrrolidine; and from 6.0 to 32.9 μmol for 2-phenylpyrrolidine. In addition, the protocol has proved robust over a range of δ^{15}N values from +0.11 to −28.02‰ in the various compounds examined.

The application of this method to the determination of the KIEs of demethylation will be described elsewhere.

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