The metabolic fate of nectar nicotine in worker honey bees

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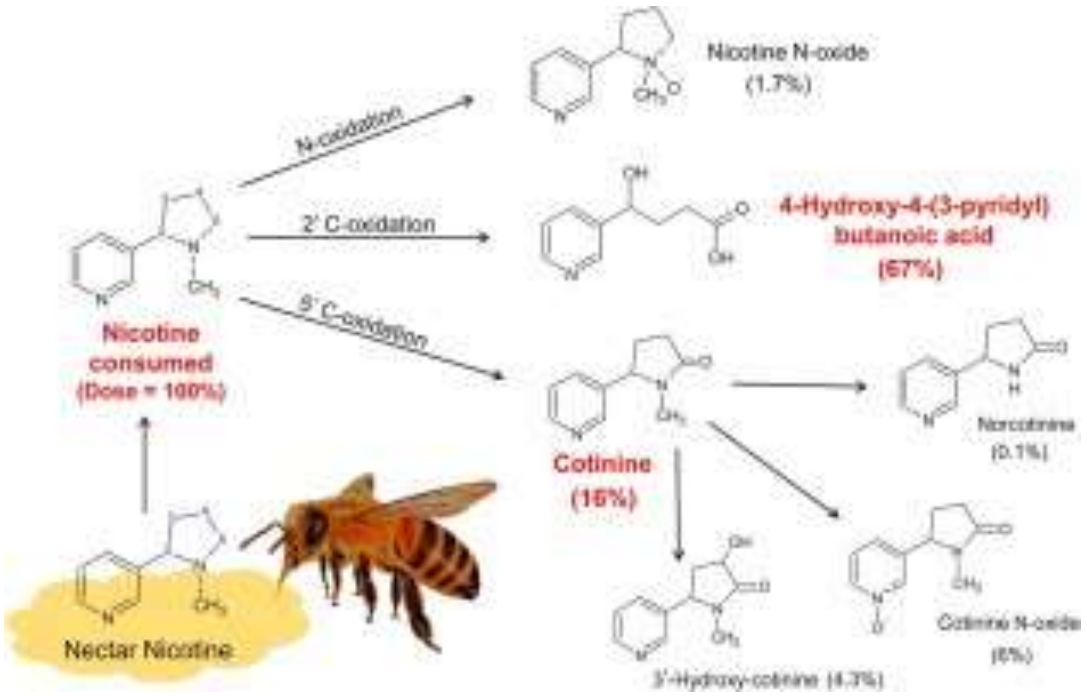
Graphical abstract



Abstract

Honey bees (*Apis mellifera*) are generalist pollinators that forage for nectar and pollen of a very large variety of plant species, exposing them to a diverse range of secondary metabolites produced as chemical defences against herbivory. Honey bees can tolerate high levels of many of these toxic compounds, including the alkaloid nicotine, in their diet without incurring apparent fitness costs. Very little is known about the underlying detoxification processes mediating this tolerance. We examined the metabolic fate of nicotine in newly emerged worker bees using radiolabeled nicotine and LC-MS/MS analysis to determine the kinetic distribution profile of nicotine as well as the absence or presence and identity of any nicotine-derived metabolites. Nicotine metabolism was extensive; virtually no unmetabolised nicotine were recovered from the rectum. The major metabolite found was 4-hydroxy-4-(3-pyridyl) butanoic acid, the end product of 2'C-oxidation of nicotine. It is the first time that 4-hydroxy-4-(3-pyridyl) butanoic acid has been identified in an insect as a catabolite of nicotine. Lower levels of cotinine, cotinine N-oxide, 3'hydroxy-cotinine, nicotine N-oxide and norcotinine were also detected. Our results demonstrated that formation of 4-hydroxy-4-(3-pyridyl) butanoic acid is quantitatively the most significant pathway of nicotine metabolism in honey bees and that the rapid excretion of unmetabolised nicotine does not contribute significantly to nicotine tolerance in honey bees. In nicotine-tolerant insects that do not rely on the rapid excretion of nicotine like the Lepidoptera, it is possible that the 2'C-oxidation of nicotine is the conserved metabolic pathway instead of the generally assumed 5'C-oxidation pathway.

Key words: *Apis mellifera scutellata*, secondary metabolites, nicotine metabolism, detoxification, 4-hydroxy-4-(3-pyridyl) butanoic acid, radiotracer

1. Introduction

Honey bees (*Apis mellifera*) are generalist pollinators that forage for nectar and pollen of a very large array of plant species (Free, 1970; Hepburn and Radloff, 1998). This polylectic foraging behaviour exposes honey bees to a diverse range of secondary metabolites produced by plants as defences against herbivory. These compounds are not only present in vegetative tissues, such as the leaves, stems and roots, but are also commonly found in flowers and floral rewards, with up to 55% of surveyed plant species exhibiting secondary metabolites in floral nectar and pollen (Adler, 2000; Adler and Irwin, 2005; Baker, 1977; London-Shafir et al., 2003; Nicolson and Thornburg, 2007; Sedivy et al., 2012; Serra Bonvehí et al., 2001). Secondary metabolites such as alkaloids, phenolics and non-protein amino acids are not only bitter tasting, but most display toxic pharmacological activity as well. Like foliar herbivores, bees can experience both beneficial and adverse effects from consuming these compounds (Stevenson et al., 2017). Although it has been shown that secondary metabolites can reduce parasitic infections (Baracchi et al., 2015; Manson et al., 2010; Richardson et al., 2015; Simone-Finstrom and Spivak, 2012; Thorburn et al., 2015) and enhance memory and foraging efficiency (Wright et al., 2013), most defence compounds are repellent to bees at low concentrations and toxic at high concentrations (Detzel and Wink, 1993; Singaravelan et al., 2005). As well as reducing survival, consumption of secondary metabolites can also negatively impact colony fitness parameters such as the production and size of offspring (Arnold et al., 2014).

Despite their bitter taste and toxic biological activity, secondary metabolites at ecologically relevant concentrations do not necessarily deter honey bees and bumblebees (Köhler et al., 2012; Richardson et al., 2015; Singaravelan et al., 2005; Tiedeken et al., 2014; Wright et al., 2013). Deterrence threshold values for secondary metabolites like caffeine, amygdalin and grayanotoxin may be up to 60 times higher than concentrations in nectar (London-Shafir et al., 2003; Singaravelan et al., 2005; Tiedeken et al., 2014; Wright et al., 2010). This, together with the observation that honey bees have fewer genes encoding gustatory receptors relative to other insect genomes (Robertson and Wanner, 2006), led to the suggestion that honey bees have diminished ability to detect nectar toxins through taste and largely rely on post-ingestive detection to modulate appetite (London-Shafir et al., 2003; Tiedeken et al., 2014; Wright et al., 2010). Nonetheless, whether honey

bees detect toxins pre- or post-ingestively, several studies have demonstrated that they are able to tolerate high levels of toxic secondary metabolites in their diet (Human et al., 2014; Köhler et al., 2012; London-Shafir et al., 2003; Mao et al., 2009; Sedivy et al., 2012; Singaravelan et al., 2006; Wright et al., 2010).

The toxic defence metabolite nicotine is an example of such a compound. Nicotine is a pyridine alkaloid primarily found in the Solanaceae and is used as a natural insecticide in the form of tobacco tea in organic farming methods, especially against sucking insects (reviewed in Isman, 2006). Nicotine acts on the insect central nervous system as an agonist of the post-synaptic nicotinic acetylcholine receptors, similar to the mode of action of the synthetic neonicotinoid insecticides (Casida and Durkin, 2013). Nicotine is present in the nectar and pollen of *Nicotiana* species at levels between 0.1-5 ppm in nectar and up to 23 ppm in pollen (Adler et al., 2012; Detzel and Wink, 1993; Tadmor-Melamed et al., 2004). Nicotine in artificial nectar at concentrations up to 50 ppm (300 µM) had no notable adverse effects on honey bee worker survival, hatching success or larval survival (Human et al., 2014; Köhler et al., 2012; Singaravelan et al., 2006, 2005). Only a few other insect species are known to tolerate nicotine in their diet: these include peach-potato aphids (*Myzus persicae*), whiteflies (*Bemisia tabaci*) and the tobacco hornworm (*Manduca sexta*) (Bass et al., 2013; Kliot et al., 2014; Snyder et al., 1994). There is also evidence that bees can metabolise, filter or remove nicotine from collected nectar, as honey produced from artificial nectar containing 50 ppm contained less than 10% of the expected nicotine (Singaravelan et al., 2006).

Detoxification, or the enzymatic conversion of toxic compounds to less toxic forms that can then be eliminated, is a common mechanism employed by insects to cope with a variety of xenobiotics (reviewed in Li et al., 2007). The most prominent enzyme superfamilies that are responsible, and frequently associated with tolerance and evolved resistance to both natural and synthetic toxins in insects, are the cytochrome P450 monooxygenases (P450s), glutathione transferases (GSTs) and carboxylesterases (Li et al., 2007). In nicotine-resistant lineages of *M. persicae,* nicotine tolerance is associated with the overexpression of CYP6CY3, the P450 responsible for the oxidation of nicotine to pharmacologically less active metabolites, aminoketone and cotinine (Bass et al., 2013). Likewise, in *B. tabaci* nicotine tolerance is associated with the overexpression of CYP6CM1 (Kliot et al., 2014).

In the tobacco specialist *M. sexta*, CYP6B46 has been linked to this insect's ability to feed on its nicotine-producing hostplant (Kumar et al., 2014). The detection of known nicotine metabolites such as cotinine and the N-oxides of nicotine and cotinine in the excretions of *M. sexta* fed a nicotine-rich diet suggests that oxidative detoxification is likely one of the mechanisms employed by *M. sexta* and other Sphingidae to cope with dietary nicotine (Kumar et al., 2014; Snyder et al., 1994; Wink and Theile, 2002). In addition, the Malpighian tubules of *M. sexta* remove nicotine from the haemolymph at high rates via non-specific alkaloid pumps. These alkaloid pumps are also present at the blood-brain barrier, preventing the delivery of nicotine to its biological target, the nicotinic acetylcholine receptor (Gaertner et al., 1998; Murray et al., 1994). Recently it has been suggested that *M. sexta* can also excrete nicotine via its spiracles in a form of defensive halitosis (Kumar et al., 2014).

In honey bees nicotine tolerance has been linked to oxidative detoxification: two oxidation catabolites of nicotine, cotinine and cotinine N-oxide, were present in nicotine exposed bees, and there is evidence that phase II nicotine metabolites may also be present (Du Rand et al., 2015). However, the possibility that rapid excretion of unmetabolised nicotine might also contribute substantially to the ability of honey bees to tolerate high levels of dietary nicotine was not excluded. In order to better understand the processes underlying nicotine tolerance in honey bees, the aim of the present study is to investigate the metabolic fate of nicotine in honey bees using radiolabelled nicotine and LC-MS/MS analysis to determine the kinetic distribution profile of nicotine as well as the absence or presence and identity of any nicotine-derived metabolites.

2. Materials and Methods

2.1 Chemicals

All reagents were of analytical grade unless otherwise indicated. (-)-Nicotine, (±)-nornicotine, (±)-anabasine, (-)-cotinine and methanol CHROMASOLV® for HPLC were purchased from Sigma-Aldrich (St. Louis, MO, USA). Radiolabeled nicotine, DL-[N-methyl-^{14}C] nicotine (50-60 mCi/mmol or 1.85-2.22 Gbq/mmol), was obtained from

American Radiolabeled Chemicals, Inc. (MO, USA). All solutions and dilutions were prepared with double distilled de-ionised water, produced by an ELGA PURELAB Ultra water purification system from Veolia Water Systems Ltd. (High Wycombe, UK) unless otherwise stated.

2.2 Sample collection and caged bees

Frames with capped worker brood were collected during summer (January – April 2014) from each of six colonies of *Apis mellifera scutellata* maintained at the University of Pretoria apiary. Newly emerged workers were collected from the frames within 24 h of emergence and placed in disposable clear PVC hoarding cages (5.9 x 5.9 x 5.9 cm; 20 or 40 bees per cage) as shown in Supplementary Fig. A.1. Cages were kept in an incubator (HCP108, Memmert GmbH & Co. KG; Bavaria, Germany) at 34 ± 1 °C and 45% relative humidity in darkness, to simulate conditions within the hive. Plastic feeding vials (2 ml Eppendorf tubes) with a feeding aperture (0.5 x 0.5 cm) were inserted horizontally into the cages, one with water and one with the experimental or control diet, both provided fresh daily. No protein was provided as caged *Apis mellifera scutellata* have been shown to survive longest on a sugar-only diet (Archer et al., 2014; Pirk et al., 2010).

2.3 Determining tissue distribution of ^{14}C nicotine and its metabolites after ingestion

Newly emerged worker bees (20 per cage) from six colonies were placed on a diet of 1 M sucrose containing 300 µM (50 ppm) nicotine. The bees were kept on the diet for 48 h and then switched to a 1 M sucrose and 300 µM nicotine diet containing 0.02 µCi DL-[N-methyl-^{14}C] nicotine per microliter for an additional 24 h before haemolymph and tissue samples were collected from five randomly selected bees from each group. Haemolymph (2 µl per bee), the honey crop, midgut, hindgut (ileum and Malpighian tubules), rectum, head, thorax (including legs) and the remaining carcass were collected. The Malpighian tubules connect to and empty into the ileum at the end of the midgut and were grouped with the ileum. Haemolymph was collected via an incision made on the thorax beneath the wing. Two microliters of haemolymph per bee was removed by pressing a micro-capillary lightly on the incision and added to 50 % methanol (200 µl). Three control diets were used: 1 M sucrose, 1 M sucrose containing 300 µM nicotine and 1 M sucrose containing 20% ethanol (20% ethanol was the final concentration of ethanol in the experimental diet after adding 0.02 µCi DL-[N-methyl-^{14}C] nicotine per microliter diet). The control groups

were fed the control diets for a total of 72 h before haemolymph and tissue samples were collected as above from five randomly selected bees from each group.

The collected tissue samples were homogenised in 200 µl of 50% methanol before addition of 2 ml Soluene®-350 (PerkinElmer, Waltham, MA, USA) followed by incubation for 48 h at room temperature. Soluene®-350 (2 ml) was added directly to the haemolymph samples which already contained 200 µl 50% methanol. Total radioactivity was counted in each sample using a Tri-Carb 2800 TR liquid scintillation counter (Packard, Downeres Grove, IL, USA) and 10 ml of ULTIMA Gold™ XR scintillation fluid (PerkinElmer, Waltham, MA, USA) per sample. The total volume of haemolymph per bee was corrected for by applying a factor of 10 to the total radioactivity counted in the haemolymph samples; the average total volume of haemolymph of fed hive bees is 20 µl (Crailsheim, 1985). Recovery rates were calculated using the total amount of radioactivity ingested per bee, estimated from the amount of diet consumed per 20 bees over 24 h. Food and water uptake were recorded by weighing the feeding vials (±0.01 mg, Mettler Toledo AG-64, Microsep Ltd., Johannesburg, South Africa) before and after the 24 h feeding period. Radioactivity levels are reported as a percentage of the total radioactivity detected in all the body compartments.

2.4 Identification of nicotine metabolites

Newly emerged worker bees (40 per cage) from the same six colonies used for the radioisotope study, were placed on a diet of 0.63 M sucrose containing 300 µM nicotine (50 ppm). The bees were kept on the diet for 72 h (the estimated total body load was 3 µg nicotine per bee over 72 h) and then switched to a nicotine free diet. At 0, 2, 6, and 24 h after switching to a nicotine free diet, haemolymph (2 µl per bee), the crop, midgut, hindgut (ileum and Malpighian tubules) and rectum were collected from ten randomly selected bees. Control groups received the standard diet of 0.63 M sucrose and the same tissues were collected. The collected samples were homogenised in 500 µl of double distilled de-ionised water before being freeze-dried and stored at 4°C until further analysis.

2.4.1 UPLC-MS/MS sample preparation.

The freeze-dried samples were reconstituted in 500 µl of 50% methanol and centrifuged at 12 000 x g for 10 min. The supernatant was collected and filtered through 0.2 µm syringe filters to remove particulate matter.

2.4.2 UPLC-MS/MS metabolite identification and quantification.

Analyses were performed on a Waters® ACQUITY UPLC™ system (Milford, MA, USA) coupled to a Waters® Xevo™ TQ tandem quadrupole mass spectrometer (Milford, MA, USA) with MassLynx™ software. Reverse phase chromatographic separations were carried out on an ACQUITY UPLC™ HSS T3 column (2.1 x 150 mm, 1.8 µm) obtained from Waters (Milford, MA, USA). The column temperature was maintained at 50 °C and the flow rate was set to 0.35 ml/min. Mobile phase A consisted of 10 mM ammonium acetate (adjusted to pH 3 with acetic acid) and mobile phase B consisted of 10 mM ammonium acetate (adjusted to pH 3 with acetic acid) in acetonitrile (25:475; v:v). The following gradient was used: 0 – 0.5 minutes, 5% B; 0.5 – 8 minutes, 5 – 60% B; 8 – 12.9 minutes, 60 - 100% B; 12.9 – 13 minutes, 100 – 5% B; 13 – 15 minutes, 5% B. The injection volume was 2 µl. The mass spectrometer was operated in electrospray positive mode using multiple reaction monitoring (MRM) data acquisition. The following electrospray ionisation (ESI) conditions were applied: capillary voltage 3.53 kV; desolvation temperature 400 °C; desolvation gas (nitrogen) 788 L/h; cone gas (nitrogen) 51 L/h; and collision gas flow rate 0.2 mL/min. Analyte-specific cone voltages, collision energies and MRM transitions are provided in Table 1.

Table 1. MRM transitions and ion optic parameters for nicotine and nicotine metabolites.

Compound	Cone voltage (V)	Collision Energy (V)	ES^+ MS transition $(m/z)^+$
Nicotine	25	20	163.0 → 130.1
Nicotine	25	26	163.0 → 117.0
Nicotine	25	15	163.0 → 132.1
Nornicotine	25	18	149.2 → 132.1
Nicotine N-oxide	25	22	179.3 → 132.1
Cotinine	15	20	177.2 → 79.8
Cotinine	15	15	177.2 → 146.0
Norcotinine	25	15	149.2 → 146.1
Cotinine N-oxide	25	15	193.2 → 162.0
3'-Hydroxy-cotinine	25	15	193.3 → 134.0
4-Hydroxy-4-(3-pyridyl) butanoic acid	25	18	182.2 → 164.0
Nicotine-glutathione	25	12	339.2 → 163.1
Cotinine-glutathione	25	12	353.1 → 177.1
3'-Hydroxy-cotinine-glutathione	25	12	369.0 → 193.0

3. Results

3.1 The distribution of total radioactivity within the body compartments

The estimated average amount of radioactivity ingested per bee was 0.44 µCi with an average recovery rate of 90%. Radioactivity was present in all the body compartments after 24 h, with the highest levels in the honey crop (45% of the total) and rectum (19% of the total) as shown in Fig. 1. The level of radioactivity in the midgut was 9% while the hindgut (ileum and the Malpighian tubules) contained only 2% of the total radioactivity counted in all body compartments. Notably, the body compartments not part of the digestive tract all contained less than 10% of the total radioactivity detected. Haemolymph (corrected for total volume) accounted for only 5% of the total radioactivity.

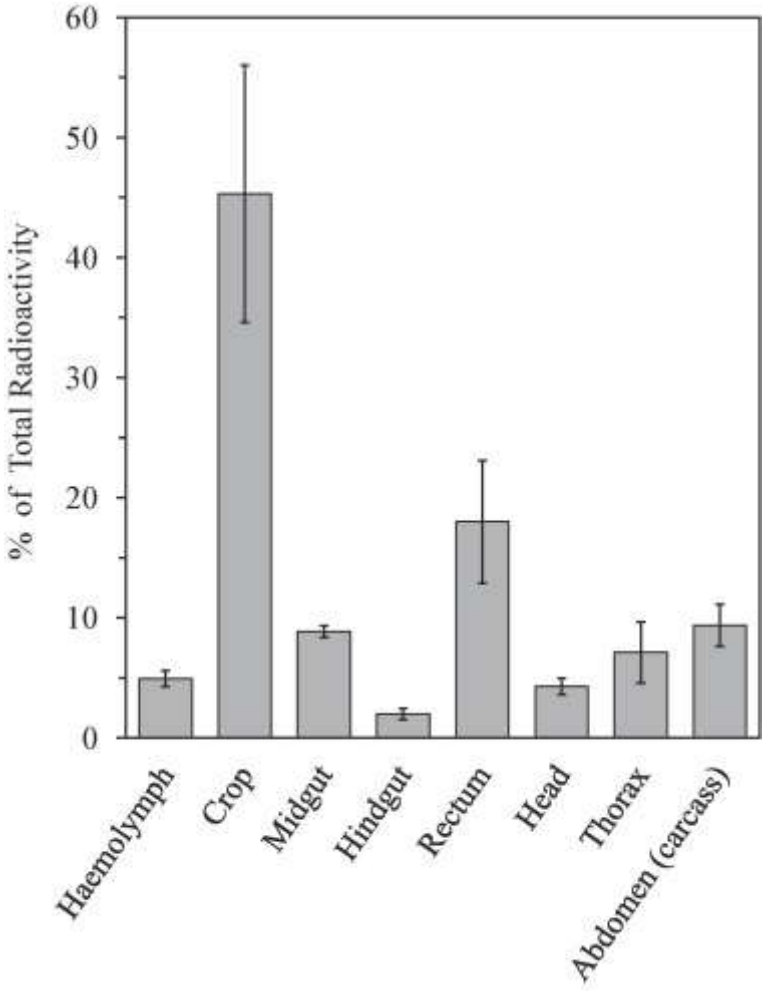


Figure 1. The distribution of total radioactivity in the body of the honey bee after 24 h on a 300 µM nicotine diet containing 0.02 µCi DL-[N-methyl-14C] nicotine/µl diet.

Data represent the mean of six experiments ± SE and are expressed as percentage of the total radioactivity counted (n=6; averaged values of 5 bees were used for each colony).

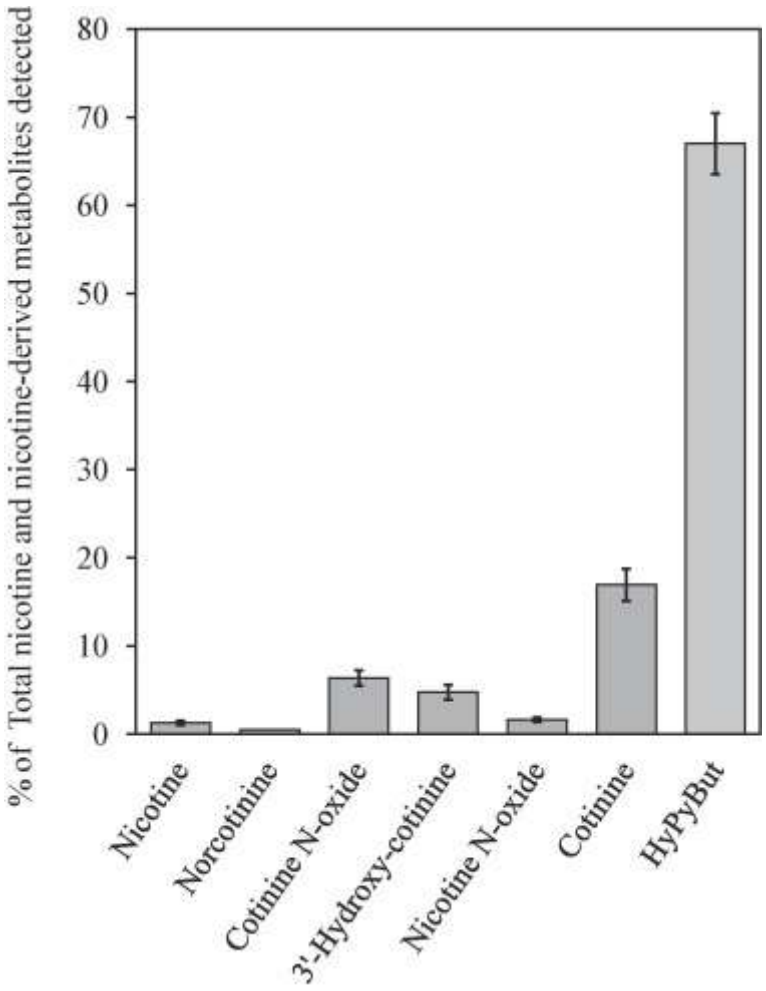


Figure 2. Nicotine and its metabolites detected in the rectum of the honey bee 24 h after being switched to a nicotine-free diet.

Caged honey bees do not defecate, consequently waste products accumulate in the rectum over time, representing the final metabolites destined for excretion. No glutathione or mercapturic acid derivatives were detected. Data represent the mean of six experiments ± SE and are expressed as percentage of the total nicotine and nicotine metabolites detected (n=6; averaged values of 10 bees were used for each colony).

3.2 Metabolic fate of ingested nicotine

Chemical analysis of haemolymph and regions of the digestive tract collected from newly emerged workers was performed in search of nicotine and its likely metabolites. In the rectum (where waste products accumulate because caged bees do not defecate), the end product of 2'C-oxidation of nicotine, 4-hydroxy-4-(3-pyridyl) butanoic acid, was present at levels 56 times higher than nicotine and at least four times higher than any other metabolite detected (Fig. 2 and Supplementary Table A.1). This is the first time that 4-hydroxy-4-(3-pyridyl) butanoic acid has been identified in an insect as a catabolite of nicotine. Apart from 4-hydroxy-4-(3-pyridyl) butanoic acid, which represented 67% of the

total nicotine and nicotine-derived compounds detected in the rectum, lower levels of cotinine (16%) and smaller amounts of cotinine N-oxide (6%), 3'-hydroxy-cotinine (4.7%), nicotine N-oxide (1.6%), and very small amounts of nicotine (1.28 %) and norcotinine (0.1%) were also present (Fig. 2).

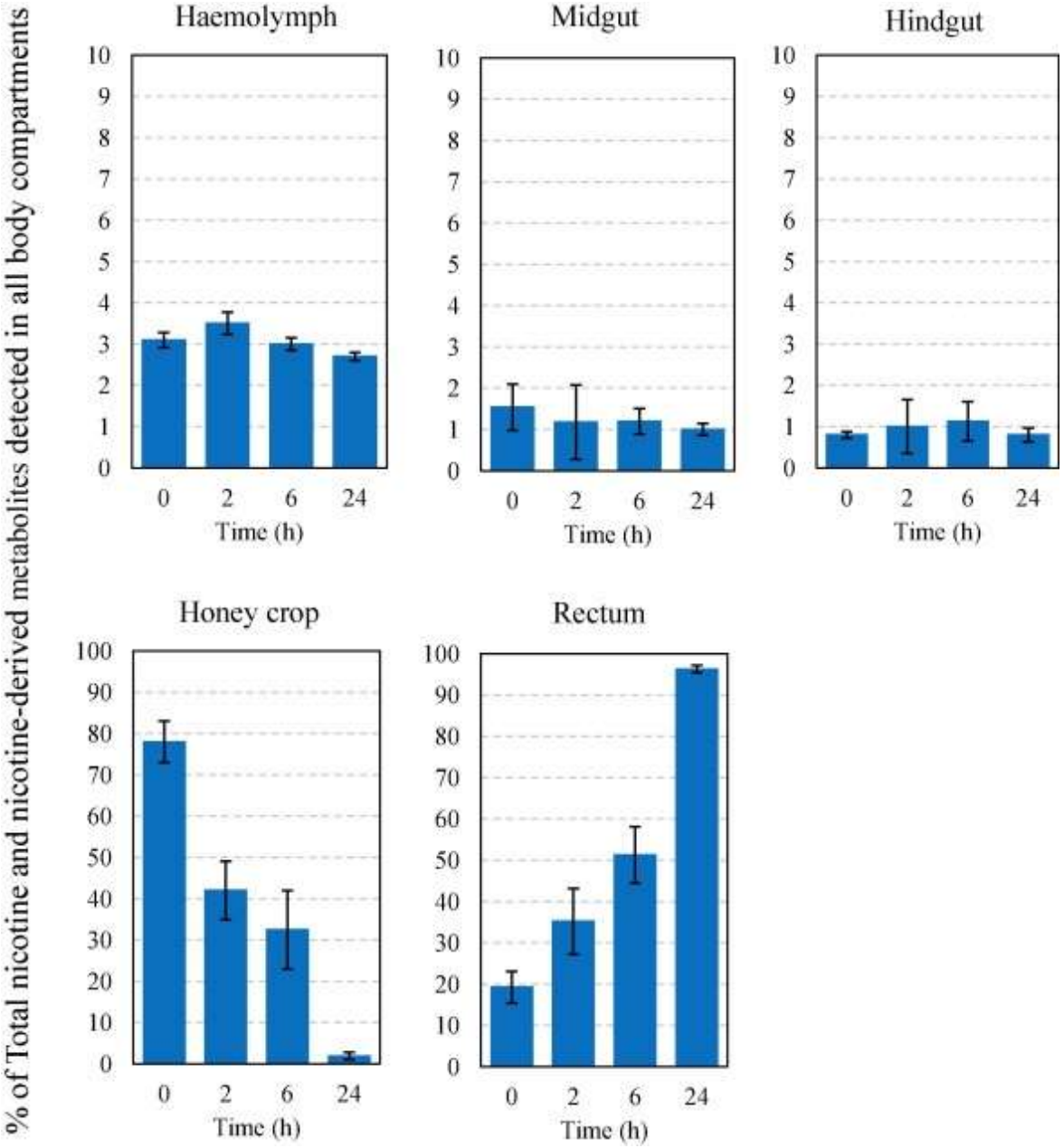


Figure 3. The distribution kinetics of nicotine and its metabolites in the haemolymph, midgut, hindgut, honey crop and rectum during the 24 h after nicotine treatment.

Nicotine and nicotine metabolites were present in all tissue samples from the digestive tract. The honey crop and rectum contained the highest levels of nicotine and nicotine metabolites at all time points. Only nicotine, at low levels, was detected in the haemolymph. Data represent the mean of six experiments ± SE and are expressed as percentage of the total nicotine and nicotine metabolites detected (n=6; averaged values of 10 bees were used for each colony). Note that the bars in the graph labelled 'Haemolymph' represent nicotine only.

Nicotine and nicotine-derived metabolites were also detected in the midgut and hindgut (ileum and Malpighian tubules) at levels below 2% of the total detectable metabolites in the digestive tract (Fig. 3). In the honey crop only nicotine was detected. Only nicotine was detectable in haemolymph as well; the level was never more than 3.5% of the level of nicotine and nicotine-derived metabolites detected in the digestive tract. No glutathione or mercapturic acid derivatives or any other likely conjugation derivatives were detected in any of the samples.

During the 24 h sampling period, the honey crop and rectum always contained the highest levels of nicotine and nicotine-derived metabolites – as the level of nicotine decreased in the honey crop over the 24 h sampling period from 78% to under 2%, the level of nicotine metabolites increased from 19% to 97% in the rectum (Fig. 3). In the midgut and hindgut, the level of nicotine and nicotine-derived metabolites remained relatively constant over the 24 h period as nicotine steadily moved through the digestive tract from the honey crop to be processed before finally being excreted into the rectum for elimination. The level of nicotine in the haemolymph was low, fluctuating only between 2.7 to 3.5% during the 24 hr period, reaching T_{max} after 2 h and decreasing after 24 h to 2.7%. Immediately after the bees had been switched to a nicotine-free diet (0 h), nicotine accounted for 80% of the total nicotine and nicotine-derived compounds detected in the digestive tract and haemolymph. After 24 h, less than 10% could be attributed to unmetabolised nicotine (Fig. 4). Over the 24 h period nicotine steadily decreased while the major metabolite, 4-hydroxy-4-(3-pyridyl) butanoic acid, increased at the same rate as illustrated in Fig. 4.

The rectum contained 18.9% of the total radioactivity detected in the body of the bee; this increases to 23% if the haemolymph and digestive tract tissues are included, as in the LC-MS metabolite study. The structure of the predominant nicotine-derived metabolite identified in the rectum, 4-hydroxy-4-(3-pyridyl)-butanoic acid, does not contain the carbon from the methyl-group which carries the radiolabel in the nicotine radiochemical used and so did not contribute to the radioactivity observed in the rectum. However, the presence of nicotine, cotinine, cotinine N-oxide, 3'-hydroxy cotinine and nicotine oxide can account for the radioactivity observed in the rectum.

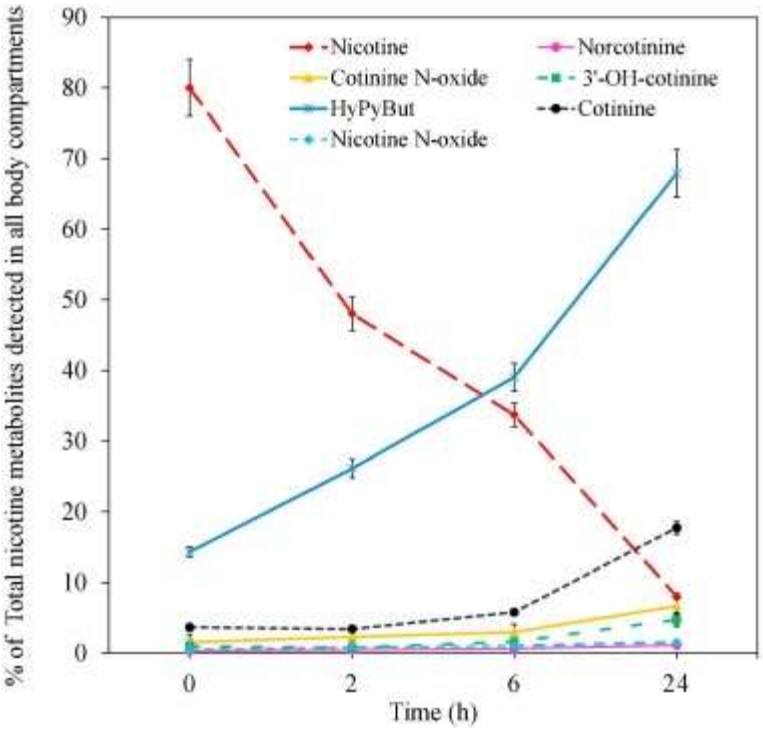


Figure 4. Distribution kinetics of nicotine and its metabolites in the honey bee.

Nicotine and its metabolites were detected and quantified during the 24 h after nicotine treatment (i.e. after being switched to a nicotine free-diet). Nicotine metabolism was comprehensive: less than 10% of the total nicotine and nicotine metabolites detected could be attributed to unmetabolised nicotine after 24 h. Data represent the mean of six experiments ± SE and are expressed as percentage of the total nicotine and nicotine metabolites detected (n=6; averaged values of 10 bees were used for each colony).

4. Discussion

Nicotine is extensively metabolised by honey bees. Less than 10% of the total nicotine and nicotine-derived metabolites detected 24 h after switching to a nicotine-free diet could be attributed to unmetabolised nicotine (Fig. 4). Virtually no unmetabolised nicotine was recovered from the rectum (Fig. 2 and Supplementary Table A.1). Nicotine metabolites were present in all tissues sampled with the exception of the honey crop, which contained only nicotine. Accumulation of nicotine and nicotine-derived metabolites was greatest in the honey crop and rectum (Figs. 1 and 3). This was expected, since hive bees temporarily store food in their honey crops, which is then available for trophallaxis, and caged bees do not defecate, which leads to the accumulation of waste products in the rectum over time. In order to determine the extent of nicotine absorption from the midgut and subsequent level of circulation in the haemocoel, haemolymph samples were analysed as well. The data showed a very small amount of nicotine only in the haemolymph.

Nicotine may follow several potential routes in the body of the bee. Once ingested, nicotine may temporarily be stored in the crop along with the consumed sugar solution. We found no evidence that any form of nicotine metabolism is taking place in the crop. *In vitro* studies have shown that the honey bee crop is permeable to un-ionised lipophilic pesticides to varying degrees (Conner et al., 1978). However, it is unlikely that any significant amount of nicotine is absorbed from the honey bee crop due to the presence of a cuticular lining (Crailsheim, 1988) and the predominant ionised state of nicotine in acid or physiological pH environments below pH 8. Nicotine is likely to pass from the crop to the midgut, which is thought to be the main site of detoxification in bees (Corona and Robinson, 2006; Mao et al., 2009). The insect midgut is rich in ABC or multidrug transporters (Bretschneider et al., 2016; Hawthorne and Dively, 2011; Labbé et al., 2011; Lanning et al., 1996; Liu et al., 2011) and it is likely that nicotine is taken up by the midgut epithelial cells, where most of it is metabolised before it is transported back into the midgut lumen across the apical membrane or into the haemolymph across the basal membrane. From the midgut, nicotine and its metabolites are passed to the hindgut and then to the rectum. Like the crop, the rectum is cuticle lined and is impermeable to nicotine and any nicotine-derived metabolites. Although nicotine and nicotine-derived metabolites were detected in the midgut and hindgut, no accumulation of nicotine or any nicotine metabolite was observed, which suggests rapid transit through the digestive tract.

The midgut epithelium of insects has no cuticular layer and it is known to be permeable to small organic molecules (Huang et al., 2015; Klein et al., 1996; Sacchi and Wolfersberger, 1996; Turunen and Crailsheim, 1996). Nicotine, in its lipophilic neutral form, may passively diffuse across the epithelium of the midgut into the haemolymph (transcellular route) as is evident from the presence of radioactivity (Fig. 1) and the detection of nicotine in the haemolymph. It can also cross the epithelium through the paracelluar route (Huang et al., 2015). Passive movement of nicotine-derived metabolites across membrane barriers will be slower due to their higher polarity, and only nicotine was detected in the haemolymph.

Insect Malpighian tubules are rich in ABC transporters (Dermauw and Van Leeuwen, 2014) and actively excrete a wide range of xenobiotics, including nicotine (Gaertner et al., 1998; Maddrell and Gardiner, 1976). Nicotine could be rapidly transported into the

tubules, then entering the ileum and rectum. However, very low levels of nicotine in the rectum suggest this is not the case. Insect Malpighian tubules can transport toxins such as nicotine unchanged, or metabolise them before transport into the tubule lumen (Gaertner et al., 1998; Maddrell and Gardiner, 1976). In *Drosophila melanogaster* the Malpighian tubules may even be the dominant tissue for xenobiotic metabolism (Yang et al., 2007). Almost no unmetabolised nicotine is present in the honey bee rectum, suggesting that the Malpighian tubules are also able to metabolise nicotine. The low levels of nicotine in the haemolymph may mean that the Malpighian tubules are very efficient in removing nicotine from the haemolymph and metabolising it; or alternatively that nicotine is mainly metabolised in the midgut and passive diffusion of its metabolites into the haemolymph is restricted.

Based on our previous study that utilised untargeted metabolomic profiling to investigate the effects of dietary nicotine on honey bees (Du Rand et al., 2015), we predicted that the biotransformation of nicotine would resemble that observed in caterpillars (Snyder et al., 1994; Wink and Theile, 2002) with cotinine N-oxide as the major metabolite. In contrast, the targeted nicotine metabolite profiling reported in this study clearly demonstrated that the major metabolic pathway of ingested nicotine in honey bees is the formation of 4-hydroxy-4-(3-pyridyl) butanoic acid. This compound, previously unreported in insects, was present at levels fourfold higher than any other metabolite identified (Fig. 2). 4-Hydroxy-4-(3-pyridyl) butanoic acid is the end result of the 2'C-oxidation of nicotine (illustrated in Fig. 5). In mammals, the major metabolic pathway identified in many species is the 5'C-oxidation of nicotine to form cotinine (Hukkanen et al., 2005). In humans 75% of nicotine is oxidized to cotinine which is subsequently hydroxylated, demethylated, oxidized or glucuronidated, to form several cotinine-derived metabolites, with about 15% of the nicotine dose excreted as unmetabolised nicotine and various minor nicotine-derived metabolites as highlighted in Fig. 5 (Hukkanen et al., 2005). 2'C-Oxidation is a minor catabolic pathway with only about 10% of the nicotine dose oxidised to form 4-hydroxy-4-(3-pyridyl)-butanoic acid in humans (Hecht et al., 1999).

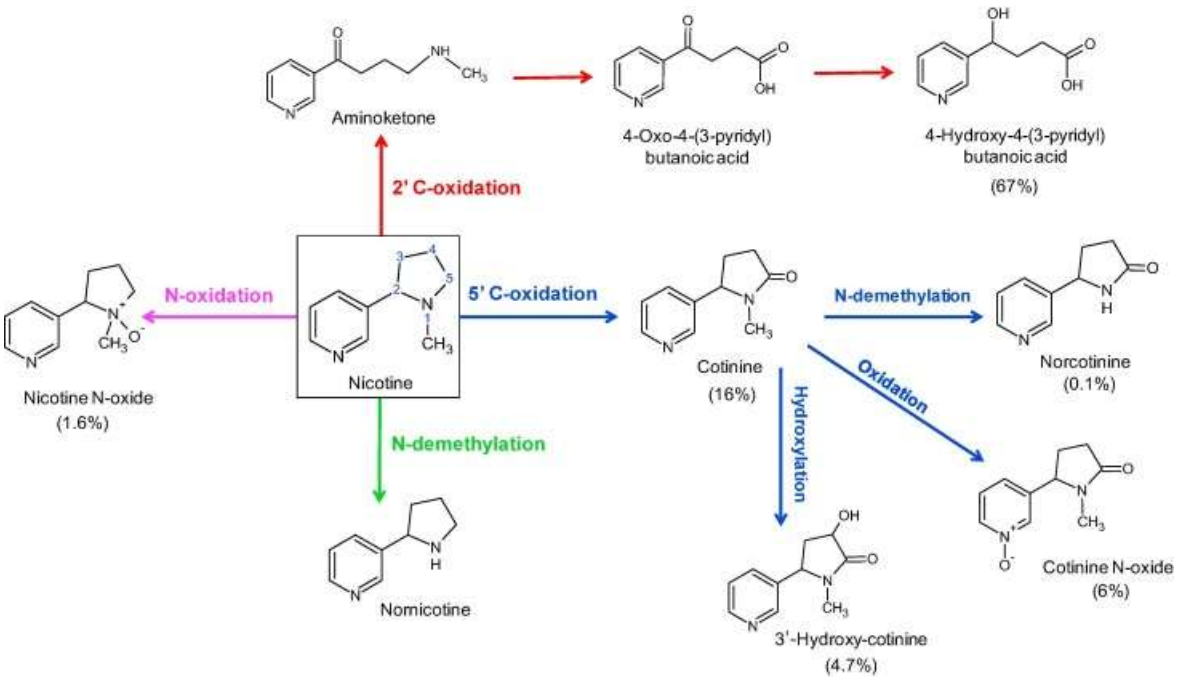


Figure 5. Conversion of nicotine to its major metabolites.

Relative amounts (%) of nicotine and its metabolites (found in the honey bee rectum 24 h after being switched to a nicotine-free diet) are shown in parentheses. In humans, the metabolic fate of nicotine involves the formation of a number of phase I metabolites through oxidation (blue, red, purple) and N-demethylation (green) and also phase II metabolites through conjugation with glucuoronic acid (not shown). Up to 75% of nicotine is oxidized to cotinine through the 5'C-oxidation pathway in humans. In honey bees and nicotine-resistant aphids, the 2'C-oxidation of nicotine is the predominate catabolic pathway (red). The tobacco hornworm and other nicotine-resistant Lepidoptera mainly rely on the rapid extraction of unmetabolised nicotine to escape the toxic effects of nicotine, although nicotine is catabolised via the 5'C- and N-oxidation pathways to some extent as well (blue and purple). Diagram adapted from (Hukkanen et al., 2005).

In the aphid *M. persicae*, in which nicotine resistance is conferred enzymatically by CYP6CY3 and not through rapid excretion of unmetabolised nicotine as in the Lepidoptera, nicotine is primarily metabolised to aminoketone (Bass et al., 2013; Wink and Theile, 2002). Aminoketone is formed through the 2'C-oxidation of nicotine and is the first intermediate in the formation of 4-hydroxy-4-(3-pyridyl)-butanoic acid (Fig. 5). Based on substrate depletion, CYP6CY3 metabolises nicotine to aminoketone through 2'C-oxidation more efficiently or at higher rates than CYP2A6, the main high affinity metaboliser of nicotine in humans (Hukkanen et al., 2005), can convert nicotine to cotinine via 5'C-oxidation. In the Lepidoptera, the degree of 5'C-oxidation of nicotine to cotinine N-oxide is low in nicotine tolerant species such as *M. sexta*, yet nicotine is almost completely eliminated within 24 h due to the efficient excretion of unchanged nicotine, comparable to the rate at which nicotine is eliminated in honey bees in the form of nicotine

oxidation products (Fig. 4). In contrast, the degree of nicotine oxidation to cotinine N-oxide is high in nicotine sensitive species such as *Smerinthus ocellatus* (Wink and Theile, 2002), yet nicotine is only eliminated from these insects after 48 h.

Our data suggest that in nicotine-tolerant insects that do not rely on the rapid excretion of unchanged nicotine as a strategy to cope with high levels of nicotine, the conversion of nicotine to aminoketone and ultimately 4-hydroxy-4-(3-pyridyl)-butanoic acid via 2'C-oxidation is a more efficient process than converting nicotine to cotinine through 5'C-oxidation. This is not only because it is likely a more rapid process but also 4-hydroxy-4-(3-pyridyl)-butanoic acid is more polar and has a lower LogP-value (Pubchem; Chemspider) compared to cotinine and the other N-oxide metabolites, which translates into slower passive transport across membranes. The fast conversion of nicotine to a metabolite with low membrane clearance ensures that the xenobiotic is trapped in the gut lumen preventing circulation at high levels in the haemocoel.

The other metabolites identified, besides 4-hydroxy-4-(3-pyridyl)-butanoic acid, included cotinine, cotinine N-oxide, 3'-hydroxy-cotinine, nicotine N-oxide, and very small amounts of norcotinine (Fig. 2). These metabolites, of which representative MRM chromatogram profiles are shown in Fig. 6, have all been observed in mammals and are the products of phase I oxidation, hydroxylation and N-demethylation reactions (Hukkanen et al., 2005; Rangiah et al., 2011). Given that 2'C-oxidation is quantitatively the dominant catabolic pathway observed in this study, it is likely that the 2'C-oxidation intermediates, 4-(methylamino)-1-(3-pyridyl)-1-butanone or aminoketone and 4-oxo-4-(3-pyridyl)-butanoic acid, are also present, but their presence has to be confirmed experimentally.

The particular enzymes responsible for nicotine metabolism in honey bees have not been identified. As yet there is no experimental evidence that nicotine induces any of the phase I detoxification enzymes in adult bees that are responsible for nicotine metabolism in other insects and mammals (Bass et al., 2013; Hukkanen et al., 2005; Kliot et al., 2014). Nonetheless, the presence of phase I metabolites together with the results from other studies investigating the metabolism of xenobiotics in bees (Johnson et al., 2012; Mao et al., 2013, 2011, 2009), points to constitutively expressed cytochrome P450-dependent metabolism of nicotine in honey bees. Members of the CYP6 and CYP9 families are generally associated with detoxification functions in insects (Li et al., 2007). In honey bees

CYP6AS, CYP6BD1, CYP9Q1 and CYP9Q3 have been linked to plant secondary metabolite metabolism (Mao et al., 2013, 2011, 2009). It is likely that that one or more members of the CYP6 and CYP9 families that are constitutively expressed in honey bees are responsible for the observed phase I detoxification products of nicotine.

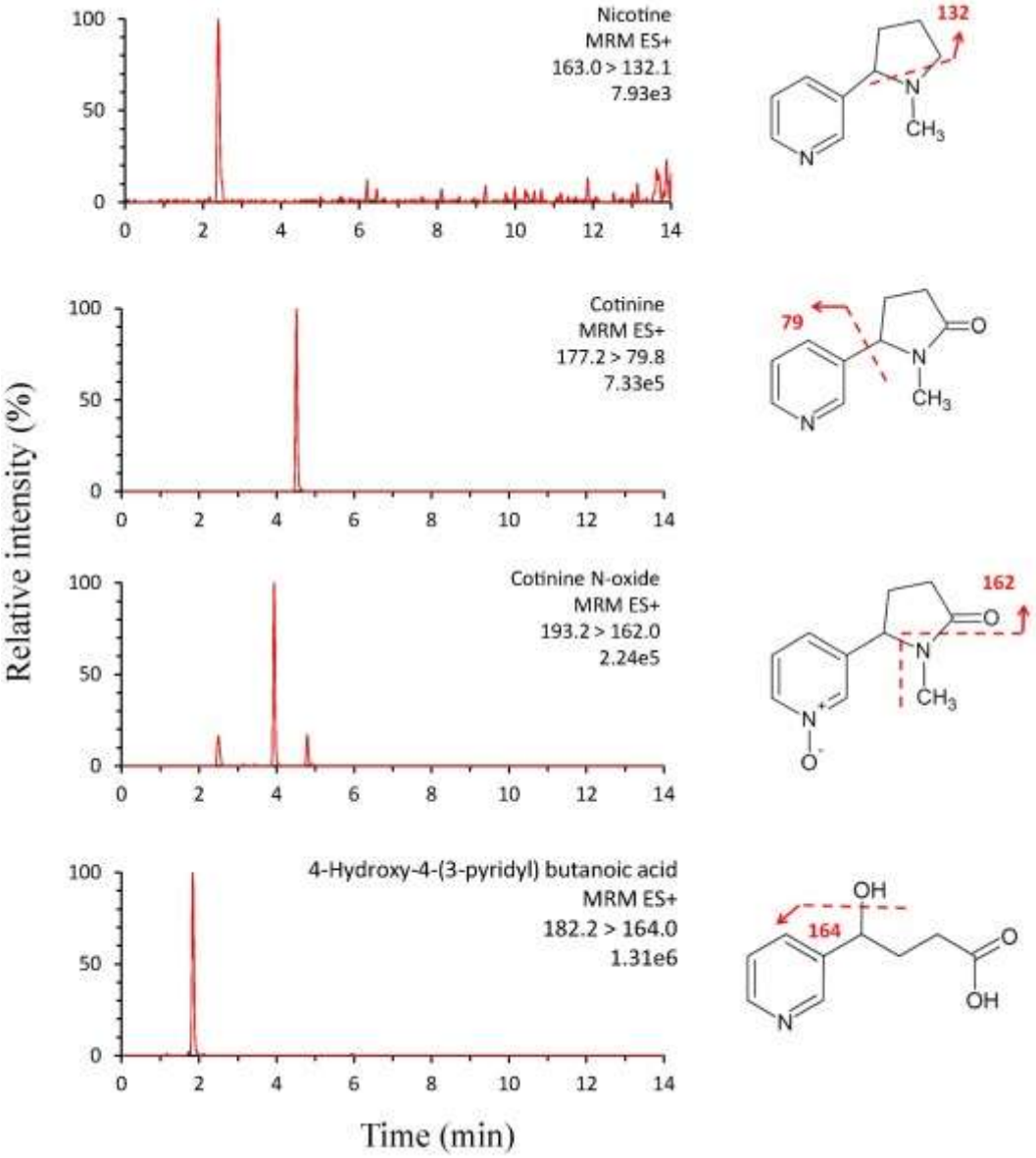


Figure 6. Representative LC–MS (MRM) chromatograms and proposed fragmentation of nicotine and the three nicotine-derived metabolites that were present at the highest levels in the rectum.

4-Hydroxy-4-(3-pyridyl) butanoic was the major metabolite detected (67%), with lower levels of cotinine (16%) and cotinine N-oxide (6%). Only a very small amount of nicotine was detected (1.2%).

In humans and other mammals, nicotine biotransformation involves conjugation pathways. Du Rand et al. (2015) reported that nicotine induces the expression of glutathione S-transferase D1 (GSTD1) in bees, raising the possibility that phase II conjugation products

could be present in honey bees as well. The putative function of GSTD1 in honey bees is the direct detoxification of xenobiotics through conjugation to glutathione (Kanehisa et al., 2014) and in the Drosophilidae it has been shown to catalyse the first step in the mercapturic acid detoxification pathway (Gloss et al., 2014). However, no metabolites produced by phase II enzymes were detected: either they occur at very low levels or are not produced at all by honey bees.

It has been proposed that gut microbes in herbivorous insects could play a role in detoxifying plant secondary metabolites (Hansen and Moran, 2014). Gut microbiota mediate caffeine detoxification in the coffee berry borer (*Hypothenemus hampei*), the primary insect pest of coffee (Ceja-Navarro et al., 2015). As yet, there is no evidence for the involvement of gut microbiota in detoxification of secondary metabolites in honey bees (Engel et al., 2012; Saraiva et al., 2015). In insects in general there is little experimental evidence for the involvement of gut microbes in detoxification, compared to the large body of literature describing mechanisms for overcoming plant chemical defences (Hansen and Moran, 2014).

In summary, our results demonstrate that formation of 4-hydroxy-4-(3-pyridyl) butanoic acid is quantitatively the most significant pathway of nicotine metabolism in honey bees and that the rapid excretion of unmetabolised nicotine does not contribute significantly to their nicotine tolerance. In nicotine-tolerant insects that do not rely on the rapid excretion of nicotine as in the Lepidoptera (Snyder et al., 1994; Wink and Theile, 2002), it is possible that the 2'C-oxidation of nicotine is the conserved metabolic pathway instead of the generally accepted 5'C-oxidation pathway. Knowledge of *in vivo* metabolic pathways of dietary toxins in honey bees adds to better understanding of how this pollinator copes with the broad diversity of xenobiotics encountered in nectar and pollen. Knowledge of the mechanistic basis of xenobiotic tolerance in bees will serve as a basis to promote bee health and to investigate interactions of pesticides with other stressors, especially pathogens and poor nutrition.

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