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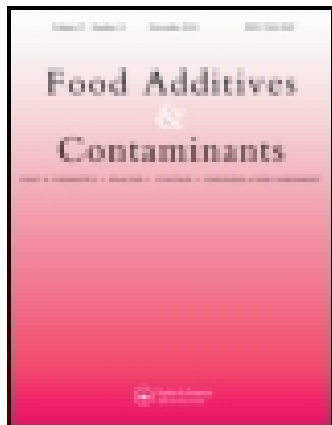
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Determination of the persistence of dimetridazole, metronidazole and ronidazole residues in black tiger shrimp (*Penaeus monodon*) tissue and stability during cooking

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Abstract

The depletion of three banned nitroimidazole drugs (dimetridazole (DMZ), metronidazole (MNZ) and ronidazole (RNZ)) was investigated in black tiger shrimp (*Penaeus monodon*) following in-water medication. The highest concentrations of residues were measured immediately after the 24 h immersion (day 0). At this time, MNZ and MNZ-OH residues were measured in shrimp tissue samples at concentrations ranging from 361 – 4189 and 0.28

– 6.6 $\mu\text{g kg}^{-1}$, respectively. DMZ and its metabolites HMMNI ranged in concentration between 31509 – 37780 and 15.0 – 31.9 $\mu\text{g kg}^{-1}$, respectively. RNZ and HMMNI concentrations ranged 14530 – 24206 and 25.0 – 55 $\mu\text{g kg}^{-1}$, respectively. MNZ, DMZ and RNZ were the more persistent marker residues and can be detected for at least eight days post-treatment. MNZ-OH was only detectable on day 0 following treatment with MNZ. HMMNI residues were only detectable up to day 1 (0.97 – 3.2 $\mu\text{g kg}^{-1}$) or 2 (1.2 – 4.5 $\mu\text{g kg}^{-1}$) following DMZ and RNZ treatment, respectively. The parent drugs, MNZ, DMZ and RNZ were still measurable on day 8 at 0.12 – 1.00, 40.5 – 55 and 8.8 – 18.7 $\mu\text{g kg}^{-1}$, respectively. The study also investigated the stability of nitroimidazole residues under various cooking procedures (frying, grilling, boiling and boiling followed by microwaving). The experiments were carried out in shrimp muscle tissue containing both high and low concentrations of these residues. Different cooking procedures showed the impact on nitroimidazole residue concentration in shrimp tissue – their concentration depleted significantly, but partially, by boiling and/or microwaving but the compounds were largely resistant to conventional grilling or frying. Cooking cannot therefore be considered as a safeguard against harmful nitroimidazole residues in shrimp.

Keywords: nitroimidazole residues, dimetridazole, HMMNI, metronidazole, metronidazole-OH, ronidazole, shrimp, persistence, stability, cooking, UHPLC-MS/MS

Introduction

Shrimp farming has increased significantly over the last couple of decades and by 2007 exceeded the global catch of all marine shrimps (Valderrama and Anderson 2011). In 2010, the value of shrimp production accounted for approximately 15% of the total value of internationally traded fishery products which made shrimp the largest single commodity in

terms of value (Anon. 2012). However, shrimp farming has faced serious disease outbreaks from viral, bacterial and parasitic infections causing significant economic loss (Chou et al. 1995). For instance, diseases outbreaks have resulted in the collapse of the penaeid shrimp aquaculture industry in Taiwan in the late 1980s (Kautsky et al. 2000), caused a \$750 million loss in 1993 in China and \$210 million in 1995–1996 in India (Primavera 1998). Indeed, disease outbreaks have resulted in the dramatic decline in the production during 1994–2004.

The protozoan parasites in shrimp that can cause diseases including several groups such as *Microspora*, *Haplospora* and Gregarina (Johnson 1995; Lightner 1996). For instance, microsporidians can cause a cotton shrimp disease (Hose et al. 1984) whereas the Haplospora group destroy the tissues of the shrimp digestive gland (Johnson 1995). Gregarines are a common intestinal parasite for shrimp. Some parasites in this group, such as *Nematopsis penaeus*, have been reported to cause severe damage in the gut mucosal epithelium of shrimp and associated with mortality and lesions (Fajer-Ávila et al. 2005).

In contrast to terrestrial food producing animals, few veterinary medicines are licensed for treating aquaculture species (Bishop 2005). Consequently, there is a concern about the illegal application of veterinary drugs to prevent or to treat infectious diseases in aquaculture. Several studies were performed to evaluate effectiveness of antibiotics in different farmed aquaculture species (Clopton and Smith 2002; Smith and Clopton 2003; Fajer-Ávila et al. 2005) as well as depletion of these antibiotics (Corliss 1979; Poapolathep et al. 2008). The consumption of shrimps containing banned substances is of continuing concern and there is an ongoing need for knowledge on the persistence of residues in shrimp tissue for risk assessment purposes.

Nitroimidazoles are known to be effective in the treatment of parasitic infections in aquaculture species, but currently metronidazole (MNZ) is licensed only for ornamental fish (Tojo and Santamarina 1998a; Tojo and Santamarina 1998b; Bishop 2005). Nitroimidazoles

and their corresponding hydroxy metabolites are suspected to be carcinogenic and mutagenic to humans (Raether and Hänel 2003). As a consequence the EU banned the use of nitroimidazole drugs (dimetridazole (DMZ), metronidazole (MNZ) and ronidazole (RNZ)) in food-producing animals or in products intended for human consumption under Regulation 2377/90 (Anon. 1990), replaced by 470/2009 (Anon. 1990) and 37/2010 (Anon. 2010). DMZ, MNZ and RNZ are classified as prohibited pharmacologically active substances for which no MRL can be established as they constitute a hazard to the health of the consumer at whatever limit (Anon. 1990). For this reason, confirmation of the presence of dimetridazole, metronidazole and ronidazole residues or their corresponding hydroxy metabolites (namely HMMNI and metronidazole-OH) at any concentration must be considered as a violation of the EU regulation. In order to protect a public health and harmonise trade, DMZ, MNZ, RNZ and their hydroxy metabolites have each been assigned a recommended concentration of $3 \mu\text{g kg}^{-1}$, for the performance of analytical methods, by the EU Reference Laboratory in Berlin (Anon. 2007).

A number of animal studies with different species (laying hens, turkeys and pigs) employing different nitroimidazole drugs (dimetridazole, ipronidazole, metronidazole and ronidazole) have been carried out in the past (Polzer et al. 2004). However, there are no references in the peer-reviewed literature to the depletion studies of nitroimidazoles in farmed aquaculture species. Moreover, most information regarding veterinary drug residues in food is related to their concentrations in raw tissue, but most food of animal origin including aquaculture is consumed after cooking or processing. Therefore, knowledge of the impact of cooking on veterinary drug residues is important for more accurate dietary exposure assessment. A review of the peer reviewed literature has shown that relatively few studies on the effect of cooking on residues have been reported (Rose et al. 1999; Cooper and Kennedy 2007; Mitrowska et al. 2007; Cooper et al. 2011). Furthermore, little is known about the

stability of nitroimidazole residues in food under conventional cooking conditions. To date, the assessment of their stability in food has been limited to the effects of various cooking procedures including boiling, frying and microwaving, on dimetridazole, ronidazole and their metabolite, namely HMMNI, in egg and chicken muscle (Rose et al. 1999).

The objective of this study was to evaluate the elimination of dimetridazole, metronidazole and ronidazole residues in shrimp tissue following an in-water treatment and to determine the stability of these residues under various cooking procedures (frying, grilling, boiling and boiling followed by microwaving).

Materials and methods

Materials and reagents

All chemicals used in this research were analytical grade or better. Dimetridazole (DMZ), metronidazole (MNZ) and ronidazole (RNZ) were purchased from Sigma-Aldrich (Dublin, Ireland). HMMNI, metronidazole-OH (MNZ-OH), dimetridazole-d₃ (DMZ-D₃), HMMNI-d₃, metronidazole-13C₂,15N₂ (MNZ-13C₂,15N₂), metronidazole-OH-d₂ (MNZ-OH-D₂) and ronidazole-D₃ (RNZ-D₃) were purchased from Witega (Berlin, Germany). All standards and internal standards stock solutions were prepared at a concentration of 1 mg mL⁻¹ in MeOH and MeOH-D, respectively.

Two complementary analytical methods covering the range 0.1 – 20 µg kg⁻¹ (low residue method) and 20 – 10000 µg kg⁻¹ (high residue method) were used in this study. A low residue method was reported previously (Gadaj et al. 2014), whereas an additional high residue procedure was developed and validated for the current study since the concentration of nitroimidazole residues detected in treated shrimp tissues at certain time points (day 0 (MNZ), day 0 – 8 (DMZ) and day 0 – 4 (RNZ)) exceeded the range of the initial method used. Consequently, two different working standards mixes were required for calibrations and

controls. A high mixed calibration range was prepared at concentrations of 0.2 (std 1), 0.5 (std 2), 1 (std 3), 2.5 (std 4), 5 (std 5), 10 (std 6), 25 (std 7), 50 (std 8) and 100 (std 9) $\mu\text{g mL}^{-1}$ in MeOH, by serial dilution of the stock solutions. The preparation of standards for a low calibration range is described elsewhere (Gadaj et al. 2014). An intermediate and a working internal standard mix solution were prepared at 100 and 5 $\mu\text{g mL}^{-1}$ in MeOH-D, respectively. All standards and internal standards stock solutions, and intermediate mix solutions were found to be stable for at least one year when stored at $-20\text{ }^{\circ}\text{C}$. Working calibration standard and a working internal standard mix solutions were found to be stable for at least three months when stored at $-20\text{ }^{\circ}\text{C}$.

Analysis of nitroimidazole residues in test samples

A finely chopped portion of each shrimp sample $1\text{ g} \pm 0.01\text{ g}$ was weighed into a 50 mL polypropylene tube. Samples were fortified with 50 μL of a 5 $\mu\text{g mL}^{-1}$ internal standard mix solution and left to stand for 15 min. A 12 mL volume of MeCN was added into each tube. The tube contents were homogenised using an Ultra-Turrax probe blender for 30 s and 9 mL of H_2O was subsequently added. After vortexing for 60 s, 1 g of NaCl and 4 g of MgSO_4 was added to tubes, which were then shaken vigorously by hand for a minimum of 60 s. Samples were centrifuged at 3500 rpm ($2842 \times g$) for 12 min at $4\text{ }^{\circ}\text{C}$. 1 mL of the supernatants were transferred to clean empty 5 mL glass tubes. Following the addition of 1 mL of H_2O , samples were vortexed for 60 s. Extracts were filtered through 0.2 μm PTFE 13 mm Millex-FG syringe filters (Millipore, Cork, Ireland) and 2 μL were injected onto the UHPLC-MS/MS system. The UHPLC-MS/MS conditions are described in detail elsewhere (Gadaj et al. 2014).

Preparation of extracted matrix calibrants and recovery control checks

Extracted matrix calibrants for the high residue method were prepared by fortifying negative shrimp samples ($1\text{ g} \pm 0.01\text{ g}$) prior to extraction with 100 μL of each calibration standard solutions (standards 1 to 9) to give curves in the range 20 – 10000 $\mu\text{g kg}^{-1}$. An

additional four blank samples (recovery controls) were spiked after extraction to monitor for loss of analytes during extraction. Briefly, 6 mL of the supernatants were transferred to clean empty 15 mL polypropylene tubes and two tubes were spiked with 50 μL of std 2 ($0.5 \mu\text{g mL}^{-1}$) and two with 50 μL of std 8 ($50 \mu\text{g mL}^{-1}$). Samples were vortexed for 60 s and 1 mL of the extracts were transferred to clean empty 5 mL glass tubes. The preparation of extracted matrix calibrants and controls checks for the low residue method is described elsewhere (Gadaj et al. 2014).

Method validation

A low residue method validation was reported previously (Gadaj et al. 2014). Briefly, the method was validated according to the EU Commission Decision 2002/657/EC criteria and the following performance studies were carried out: selectivity, specificity, linearity of calibration curve, working range of the method, within laboratory repeatability (WLR), within laboratory reproducibility (WLR), accuracy, precision, decision limit ($CC\alpha$), detection capability ($CC\beta$), matrix effects, absolute recovery and stability. Validation was carried out at 1.5, 3.0 and 4.5 $\mu\text{g kg}^{-1}$.

Accuracy and precision of the method ranged from 96 to 103% and 1.6 to 14.0%, respectively. The $CC\alpha$ values for all nitroimidazoles and their corresponding hydroxy metabolites were below the recommended concentration of 3 $\mu\text{g kg}^{-1}$, ranging 0.07 – 1.0 $\mu\text{g kg}^{-1}$ depending on the analyte.

In the case of a high residue method, the following analytical method performance parameters were investigated: linearity of calibration curve, working range of the method, intra- and inter-assay repeatability, accuracy, precision and absolute recovery. Both intra- and inter-assay repeatability studies were carried out by the same analyst. In total six runs were executed for the validation study. Five portions coming from the same negative control test material were used for the establishment of intra-assay repeatability, whereas two portions

were used for the establishment of inter-assay repeatability, and the method was repeated on five separate days. The validation study was carried out by fortifying shrimp tissue samples at 40, 500 and 5000 $\mu\text{g kg}^{-1}$.

Shrimp experimental treatments and sampling

All shrimp treatment studies were carried out at the Shrimp Biotechnology Business Unit (SBBU), Thailand. Black tiger shrimps (*Penaeus monodon*) were obtained from a farm in the Chachoengsao province. Upon arrival, the experimental shrimps were acclimatised in a 2000 L tank and reared with commercial feed until they reached a weight of 20–25 g. The shrimp were fed four times daily at a 2% rate of total body weight.

A total of four 390 L tanks per drug were supplied with dimetridazole, metronidazole and ronidazole, respectively, at a concentration of 50 mg L^{-1} (volume of 353 L) and a further two tanks with non-medicated static water system. The dose aimed in this bath treatment study was a therapeutic dose as recommended for metronidazole by the Veterinary Formulary (Bishop 2005). Aquaria conditions were maintained as the following: pH 7.5 – 8.5, dissolved oxygen > 5 mg L^{-1} , temperature 27 – 30 $^{\circ}\text{C}$, salinity 15 g L^{-1} , ammonia-nitrogen < 0.03 mg N L^{-1} , nitrite-nitrogen < 1 mg N L^{-1} and alkalinity 80 – 150 $\text{mg CaCO}_3 \text{L}^{-1}$. Thirty five shrimp were immersed for 24 h in each tank containing a nitroimidazole drug.

After the immersion, shrimps were transferred to normal sea water for eight days and collected at specified intervals: immediately (d0), day 1 (d1), day 2 (d2), day 4 (d4) and day 8 (d8). At each sampling time, six shrimp per tank (both medicated and non-medicated) were collected, rinsed with normal sea water, and stored in bags at -20 $^{\circ}\text{C}$ prior to delivery in dry ice. The untreated shrimps were taken from original stock prior to the treatment to be used as a blank tissue. Upon arrival to Teagasc, shrimps were peeled and deveined manually. Shrimps were stored individually in 50 mL polypropylene tubes at -70 $^{\circ}\text{C}$ until assayed, whereas the

shrimps taken from original stock prior to the treatment were homogenised using a mechanical blender and stored in a bag at -70 °C.

Cooking of the incurred shrimp tissue

Methodology

Dimetridazole, metronidazole and ronidazole-treated shrimps from two withdrawal time points (d0 and d8 (DMZ), d0 and d1 (MNZ), and d1 and d8 (RNZ)) were selected ($n = 12$ per time point). To mimic domestic cooking practices, individual intact shrimps (4.60 – 11.82 g) were subjected to one of the following cooking procedures: frying (shallow), grilling (broiling), boiling and boiling followed by microwaving (to mimic preparation of supermarket ready-meals), and were analysed both raw and cooked using an UHPLC-MS/MS method within a single analysis. To minimise variable changes in weight due to water loss during sample freeze-thawing, the 2 g aliquots of raw tissue were weighed out at the same time as the aliquots of cooked shrimp. Samples were then refrozen at -70 °C until assayed. Six replicate shrimps were tested for each of four cooking procedures. Negative controls were included to prove dimetridazole, metronidazole and ronidazole residues did not form during cooking. Negative controls, namely, two raw peeled king prawns purchased from a local supermarket were included for each cooking method, tested before and after cooking.

Boiling

Each of the pre-weighed shrimps was placed in an open 35 mL glass tube containing 15 mL of water sitting in a pot of boiling water on a domestic ceramic hob. Hob heat was reduced and the shrimps were allowed to simmer for 5 min. After 5 min, water in the tubes was decanted to clean polypropylene containers and allowed to cool to room temperature before measuring the total volume of water recovered. The cooked shrimps were placed immediately on tissue paper at 4 °C to halt the cooking process and remove excess water. After 30 min the cooked shrimps were weighed.

Boiling followed by microwaving

Pre-weighed shrimps were boiled as described above including collection of water. After 30 min cooling at 4 °C, the boiled shrimp was placed in a microwavable container covered with cling film and microwaved on full power (800W oven) for 4 min. Cooked shrimps were again cooled for 30 min at 4 °C on tissue paper and reweighed.

Grilling

Pre-weighed shrimps were brushed lightly with a minimal amount of sunflower oil to prevent burning and placed on a rack under a pre-heated domestic grill (high temperature) and grilled for 2 min each side, turning once (this standardised time may be adjusted depending on average size of the shrimps and observed time to cook completely). After cooking, excess oil was removed gently with tissue paper and shrimps were cooled for 30 min at 4 °C on tissue paper to halt the cooking process and reweighed.

Frying

Pre-weighed shrimps were placed in a pre-heated hot frying pan containing a minimal amount of sunflower oil to prevent burning. Shrimps were fried for 1.5 min on each side, turning once. After cooking, shrimps were cooled for 30 min at 4 °C and re-weighed.

Results and discussion

Analytical methodology

Several analytical methods have been reported for nitroimidazole residue analysis in different food matrices based on LC-MS/MS detection and they were found to be capable of the detection of target analytes in the low $\mu\text{g kg}^{-1}$ region, below the recommended concentration (Mottier et al. 2006; Peters et al. 2009; Smith et al. 2009; Mahugo-Santana et al. 2010; Mitrowska et al. 2010; Kaufmann et al. 2011; Gadaj et al. 2014).

Shrimp samples from the immersion treatment experiment were initially analysed using the method that was previously developed by Gadaj et al. (2014) for the analysis of nitroimidazole residues in aquaculture tissue in the range of 0.1 – 20 $\mu\text{g kg}^{-1}$. The analysis of shrimp tissue samples that were collected immediately (d0) after medication with MNZ, and day 0 – 8 and 0 – 4 after medications with DMZ and RNZ, respectively, gave the concentration of the parent drug exceeding the range of the analytical method used. Therefore, a new method for the analysis of high residue samples, covering the range 20 – 10000 $\mu\text{g kg}^{-1}$ was developed and validated.

The linearity of the method was evaluated over the range 20 – 10000 $\mu\text{g kg}^{-1}$ during validation studies. The linearity of the curves (measured as R^2) was satisfactory and greater than 0.99. Shrimp samples were fortified at levels of 40, 500 and 5000 $\mu\text{g kg}^{-1}$. The results of the validation study collected in Table S2, supplementary data, show that the accuracy and precision were in the ranges of 95 – 114% and 1.0 – 12.4%, respectively. Mean recoveries for nitroimidazole drug residues and their hydroxy metabolites were calculated by comparing the concentrations in fortified samples with concentrations in control samples spiked post-extraction to be $91 \pm 5.9\%$ (DMZ), $88 \pm 6.1\%$ (HMMNI) and $90 \pm 4.1\%$ (MNZ), $84 \pm 8.5\%$ (MNZ-OH) and $93 \pm 7.1\%$ (RNZ). Recovery was based on data collected from routine application of the method ($n = 11$ analytical runs).

Reproducibility of analysis

Assay performance was also assessed by reproducibility of the analysis ($n = 3$) of randomly selected shrimp samples subjected to various cooking procedures (boiling, frying and grilling) and containing dimetridazole, metronidazole and ronidazole residues, respectively. The precision was in the range of 1.5 – 16.4%, exceeding 15% only in one case (Table S3, supplementary data). The EU Commission Decision 2002/657/EC states that

precision calculated as CV should be as low as possible and not higher than 23% for mass fractions over $100 \mu\text{g kg}^{-1}$. Therefore, the results obtained in this study were well within the guideline and demonstrate high reproducibility of the analysis.

Evaluation of persistence of metronidazole residues in shrimp tissue

The depletion of DMZ, MNZ and RNZ residues and their hydroxy metabolites (HMMNI and MNZ-OH) were profiled over time as presented in Figure 1. The study served to identify the most suitable nitroimidazole drug marker residues and to investigate the persistence of residues as a function of withdrawal period. In this context residue concentrations at five different time points (d0, d1, d2, d4 and d8) post-treatment were examined in shrimp muscle tissue.

The maximum concentration of nitroimidazole residues measured within the individual shrimp tissue were $361 - 4189 \mu\text{g kg}^{-1}$, $31509 - 37780 \mu\text{g kg}^{-1}$ and $14530 - 24206 \mu\text{g kg}^{-1}$ ($n = 24$) in the case of MNZ, DMZ and RNZ, respectively, and occurred on day 0 post-treatment. Residue concentrations rapidly depleted in tissue samples at later time points (Figure 1). However, for at least eight days post-treatment, nitroimidazole residues were detectable in all medicated shrimp tissues. MNZ-OH residues were only detectable on day 0 post-treatment and the concentrations measured were very low in comparison with MNZ level, and ranged $0.28 - 6.6 \mu\text{g kg}^{-1}$ ($n = 12$). Similarly, HMMNI residues were only detectable up to day 1 ($15.0 - 31.9 \mu\text{g kg}^{-1}$) or 2 ($2.4 \pm 0.80 \mu\text{g kg}^{-1}$) ($n = 24$) following DMZ and RNZ treatment, respectively. The CC α of the low residue method were as follows: $0.11 \mu\text{g kg}^{-1}$ (DMZ), $1.0 \mu\text{g kg}^{-1}$ (HMMNI), $0.07 \mu\text{g kg}^{-1}$ (MNZ), $0.13 \mu\text{g kg}^{-1}$ (MNZ-OH) and $0.33 \mu\text{g kg}^{-1}$ (RNZ) (Gadaj et al. 2014). It can be concluded that even if nitroimidazole drug level in shrimp tissue is decreasing rapidly in the case of the treatment with DMZ, MNZ and RNZ,

the parent drugs themselves are the more persistent marker residues and can still be detected after eight days post-medication.

There have been no reports on the persistence of nitroimidazole drug residues in farmed aquaculture species following treatment. Rodriguez et al. (2011) reported on the uptake of MNZ in *Artemia* (brine shrimp) at different stages of development. *Artemia* were treated using 50 mg MNZ per L as an in-water treatment at room temperature (26 – 28 °C) for 3 h. This study showed that medication levels of 85 – 143 mg kg⁻¹ incurred in *Artemia*. The results from the current study showed a lower incursion level of MNZ in shrimp. This fact may be explained by the different capacities of various crustacean species to accumulate metronidazole. Rodriguez et al. (2011) did not report on the depletion of residues over time because samples were collected immediately post-treatment. No further studies have been reported in fish or crustaceans. Polzer et al. (2004) reported on the depletion of nitroimidazole residues in turkey muscle. These studies showed that DMZ and the HMMNI metabolite rapidly depleted in turkey breast meat from 96 – 2000 µg kg⁻¹ (day 0) to < 0.8 µg kg⁻¹ (day 1) and from approximately 1000 µg kg⁻¹ (day 0) to 0.1 µg kg⁻¹ (day 3), respectively. MNZ and MNZ-OH residues were also monitored in muscle and liver tissue but were < 1 µg kg⁻¹ and not detectable at day 1.

Stability during cooking

Since shrimp is usually cooked before consumption and there is also a wide range of ready-meals containing shrimp available on the market (Radovnikovic et al. 2013), it is reasonable to assess the stability of drug residues during various cooking conditions. In the current study, the stability of dimetridazole, metronidazole and ronidazole residues in shrimp tissue under frying, grilling, boiling and boiling followed by microwaving was examined. Tissue samples coming from shrimp treated with DMZ, MNZ and RNZ, and containing mainly residues of the parent drug in the case of MNZ and both parent drug and its hydroxy metabolite

(HMMNI) in the case of DMZ and RNZ, were used in the study. Changes in nitroimidazole drug and the corresponding hydroxy metabolite residues concentrations for each cooking method are shown in Tables 1 – 6. The concentrations detected in raw (uncooked) tissues were adjusted for sample weight changes resulting from the cooking procedures to give concentrations ‘expected after cooking’. These expected concentrations were then compared with the concentrations detected after cooking. It should be highlighted that due to the limitations in weight of individual shrimps, only one analysis could be carried out for each raw and cooked sample. Therefore, statistical interpretation of the results is difficult and could only be based on the analytical precision of the method, described in Table S2 and elsewhere (Gadaj et al. 2014). However, repeat analysis was carried out on raw shrimp tissue samples tested during the depletion studies and showed good agreement between results on different days. The method reproducibility was additionally assessed on randomly selected cooked samples as described in detail in the paragraph above.

Small reductions in MNZ concentrations of up to 25% were evident in the frying and grilling experiments in shrimp tissue samples from the high level treatment group (Table 1). Further frying and grilling studies were carried out using shrimp samples containing MNZ residues in the low range from 0.32 to 12.5 $\mu\text{g kg}^{-1}$. Apparent small increases in MNZ were observed, in two out of three samples following frying. In contrast, grilling the low concentration shrimps showed that MNZ concentrations consistently increased to a greater degree (mean 155% increase). It should be noted that decreases in water content of samples were monitored during the cooking processes and were factored into residue concentration calculations. It could be postulated that grilling may result in the release of additional tissue-bound MNZ residues; however, as increases were not seen in the high concentration samples following cooking, this cannot be claimed with any certainty.

Additional cooking studies were conducted to investigate the impact of boiling and boiling combined with microwaving on MNZ concentration in shrimp (Table 2). Mean MNZ residues were found to decrease by 42% and 56% in the high and low concentration groups after boiling, respectively. Subsequent analysis showed that 36% and 31% of MNZ residues in shrimps from the high and low concentration groups were extracted into the water. Following the combined boiling and microwaving treatment, mean MNZ concentrations decreased by 84% and 89% in the high and low concentration groups, respectively. Similarly, analysis showed that 34% and 36% of MNZ residues from the high and low concentration groups were extracted into the water.

MNZ-OH concentrations were also monitored during the cooking studies but only two samples contained residues and these were reduced marginally following cooking. MNZ-OH does not occur as a major residue in shrimp and does not appear to form during cooking.

In the case of DMZ, the frying experiment showed a small mean reduction of 12% in shrimp tissue samples from the high level treatment group (Table 3). A similar reduction of 13% was observed in the high concentration grilling experiment. Further frying and grilling studies were carried out using shrimp samples containing DMZ residues in the low range from 54 to 160 $\mu\text{g kg}^{-1}$. Similar small reductions in DMZ residues were evident in half of the samples, although small apparent increases of up to 11% were evident in the other half.

Additional cooking studies were conducted to investigate the impact of boiling and boiling combined with microwaving on DMZ concentration in shrimp (Table 4). Mean DMZ residues were found to decrease by 35% and 54% in the high and low concentration groups after boiling, respectively. Subsequent analysis showed that 42% and 40% of DMZ residues in shrimps from the high and low concentration groups were extracted into the water. Following the combined boiling and microwaving treatment, mean DMZ concentrations decreased by 52% and 65% in the high and low concentration groups, respectively. Similarly,

analysis showed that 45% and 55% of DMZ residues from the high and low concentration groups were extracted into the water.

HMMNI concentrations, following DMZ treatment, were also monitored during the cooking studies but only samples from the high level treatment group contained detectable residues. This showed that frying, grilling, boiling and boiling followed by microwaving reduced mean HMMNI residues in shrimp muscle tissue by 17%, 14%, 45% and 44%, respectively. Moreover analysis showed that 40% of HMMNI residues were extracted into the water in both boiling and boiling followed by microwaving experiment.

In the case of RNZ (Table 5), mean reductions of 7% and 14% were observed following frying and grilling of shrimp samples containing high levels of RNZ, respectively. Further frying and grilling studies were carried out using shrimp samples containing RNZ residues in the low range from 2.9 to 15.7 $\mu\text{g kg}^{-1}$. One out of three low range samples showed a large increase in RNZ following frying, whilst grilling resulted in consistent increases (mean 108%). Additional cooking studies were conducted to investigate the impact of boiling and boiling combined with microwaving on RNZ concentration in shrimp muscle tissue (Table 6). Mean RNZ residues were found to decrease by 43% and 15% in the high and low concentration groups after boiling, respectively. Subsequent analysis showed that 49% and 70% of RNZ residues from the high and low concentration groups were extracted into the water. Following the combined boiling and microwaving treatment, mean RNZ concentrations decreased by 73% and 91% in the high and low concentration groups respectively. Similarly, analysis showed that 43% and 75% of RNZ residues from the high and low concentration groups were extracted into the water.

HMMNI concentrations, following RNZ treatment, were also monitored during the cooking studies but only samples from the high level treatment group contained residues. This showed that frying, grilling and boiling followed by microwaving increased mean HMMNI

residues in the shrimp tissue by 140%, 112% and 920%, respectively, whereas boiling reduced them by 7%. Moreover, analysis showed that HMMNI residues were extracted into the water, to a small but approximately equal degree, in both boiling and boiling followed by microwaving experiment (Table 6). This striking effect of microwaving causing a large increase in HMMNI residues following RNZ treatment (but not DMZ treatment) cannot be easily explained. As suggested previously by Rose et al. (1999), the effect of some conversion from parent drug to its metabolite might occur during various heat processing conditions and superimpose over the other effects. Given that microwaving was applied to single, boiled shrimps with no accompanying liquid or sauce, causing a major loss of water and potentially higher internal temperatures than would be experienced in normal domestic cooking of shrimps in a meal of mixed ingredients, it is possible this increase in HMMNI following microwaving may not be replicated in the kitchen. However, there is a clear pattern across all cooking methods of HMMNI production in shrimps when high levels of RNZ are present. This is unlikely to represent a significant safety issue since HMMNI concentrations derived from RNZ following cooking represent less than 3% of the accompanying RNZ concentrations across all cooking methods.

A review of the peer reviewed literature showed that no cooking study has been carried out on nitroimidazole drug residues in shrimp or any other aquaculture tissue or food material. Rose et al. (1999) reported on the effect of frying, boiling and microwaving on dimetridazole, ronidazole and HMMNI (metabolite of each drug) residues in egg and chicken muscle. As was also seen in the current study, the analysis of water used for boiling showed that residues of DMZ and its metabolite were extracted from meat to water. Similarly, the results of frying and microwaving experiments showed reductions in residues concentrations which were accounted for by losses in the juices expressed during cooking. In the case of RNZ, a reduction of residue concentrations was observed following microwaving.

Conclusions

The results of the current study demonstrate that dimetridazole, metronidazole and ronidazole are the major residues found in shrimp muscle tissue following an in-water medication. Nitroimidazole hydroxy metabolites were also detected, but only at earlier withdrawal periods. The depletion profiles of the studied drugs show that DMZ and RNZ occur at higher concentrations and are more persistent than MNZ in shrimp following an in-water medication treatment.

The effect of different cooking procedures was investigated and showed that a boiling-microwaving combination treatment resulted in the greatest reduction of residues in tissue, followed by boiling, then grilling and frying which had similar and less pronounced effects on residue concentrations. There was no clear difference in the stability of the three nitroimidazoles – DMZ was more resilient to the most severe cooking method (boiling and microwaving) than MNZ and RNZ, although DMZ residues in shrimp meat were still degraded by 52 – 65%. The results indicate that boiling of shrimps prior to further cooking can reduce nitroimidazole exposure, primarily via partial extraction of residues into the water, but will not eliminate residues. Indeed there is the potential that nitroimidazole residues may be converted to more toxic substances following frying, grilling or microwaving. Therefore, cooking cannot be considered as a safeguard against ingestion of harmful nitroimidazole residues.

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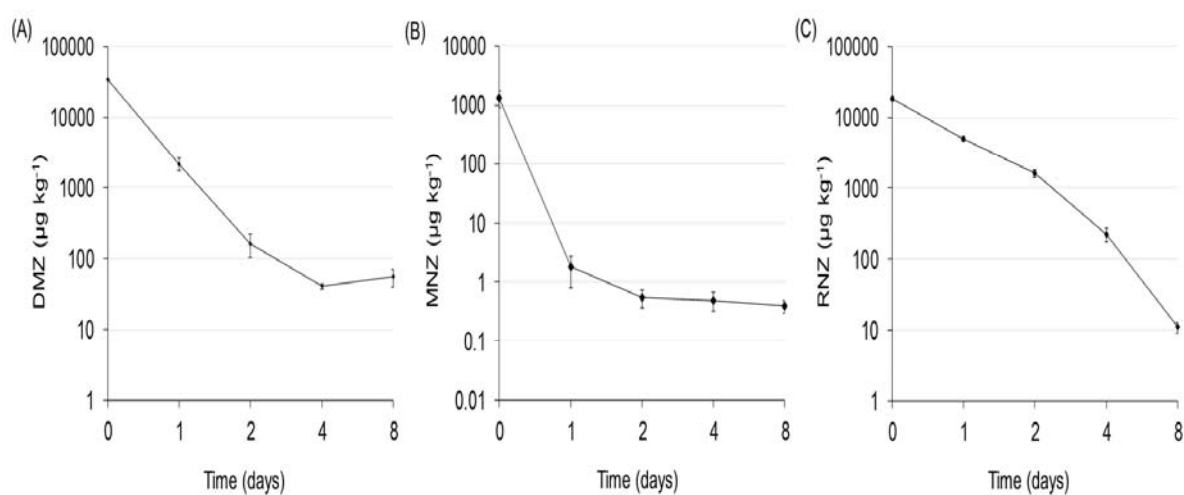
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Figure 1: Depletion profile of (A) dimetridazole, (B) metronidazole and (C) ronidazole in shrimp tissue (95% CL, $n = 24$ results per time point).



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Table 1

Effect of frying and grilling on the concentration of MNZ and MNZ-OH residues in shrimp tissue.

Sample	Weight (g)		Residue							
	Before	After	Metronidazole				Metronidazole-OH			
			Raw ($\mu\text{g kg}^{-1}$)	Cooked ($\mu\text{g kg}^{-1}$)		Residue change (%)	Raw ($\mu\text{g kg}^{-1}$)	Cooked ($\mu\text{g kg}^{-1}$)		Residue change (%)
Expected	Observed	Expected	Observed	Expected	Observed					
<i>Frying (high residue)</i>										
1	7.08	5.75	2239	2757	2327	-16	ND	N/A	ND	N/A
2	7.44	6.10	1238	1510	1509	0	ND	N/A	ND	N/A
3	9.34	7.67	734	894	861	-4	ND	N/A	ND	N/A
<i>Frying (low residue)</i>										
4	8.65	6.46	1.9	2.6	1.1	-59	ND	N/A	ND	N/A
5	7.81	6.23	1.6	2.0	2.4	+17	ND	N/A	ND	N/A
6	5.23	4.09	1.8	2.4	2.6	+9	ND	N/A	ND	N/A
<i>Grilling (high residue)</i>										
7	7.50	5.15	794	1156	960	-17	ND	N/A	ND	N/A
8	7.19	4.65	2892	4472	3334	-25	ND	N/A	ND	N/A
9	5.30	3.36	4189	6608	5883	-11	8.1	12.7	10.7	-16
<i>Grilling (low residue)</i>										
10	6.88	4.18	0.69	1.1	3.1	+176	ND	N/A	ND	N/A
11	7.41	4.87	0.39	0.59	2.1	+251	ND	N/A	ND	N/A
12	7.44	4.95	0.32	0.48	0.66	+37	ND	N/A	ND	N/A

Notes: Expected concentrations are corrected for weight loss during cooking. ND, None detected. N/A, Not applicable.

Table 2

Effect of boiling and boiling followed by microwaving on the concentration of MNZ and MNZ-OH residues in shrimp tissue.

Sample	Weight (g)		Residue								
	Before	After	Metronidazole				Metronidazole-OH				
			Raw ($\mu\text{g kg}^{-1}$)	Cooked ($\mu\text{g kg}^{-1}$)		Water/juices ($\mu\text{g kg}^{-1}$)	Residue change including water/juices (%)	Raw ($\mu\text{g kg}^{-1}$)	Cooked ($\mu\text{g kg}^{-1}$)		Residue change (%)
Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed
<i>Boiling (high residue)</i>											
13	10.95	7.46	870	1277	764	187	-9	ND	N/A	ND	N/A
14	6.31	4.14	1657	2526	1364	256	-9	ND	N/A	ND	N/A
15	9.11	6.12	830	1235	750	185	-1	ND	N/A	ND	N/A
<i>Boiling (low residue)</i>											
16	5.70	3.46	1.4	2.3	0.93	0.19	-25	ND	N/A	ND	N/A
17	8.82	6.21	12.5	17.7	5.5	1.2	-53	0.12	0.17	0.13	-24
18	7.83	5.37	4.5	6.5	4.0	0.94	+3	ND	N/A	ND	N/A
<i>Boiling followed by microwaving (high residue)</i>											
19	9.15	1.75	717	3748	353	104	-66	ND	N/A	ND	N/A
20	8.01	2.34	1245	4262	1005	213	-44	ND	N/A	ND	N/A
21	8.95	1.55	931	5376	852	241	-39	ND	N/A	ND	N/A
<i>Boiling followed by microwaving (low residue)</i>											
22	8.47	1.42	1.8	10.9	0.96	0.31	-58	ND	N/A	ND	N/A
23	8.48	1.84	0.66	3.0	0.39	0.13	-52	ND	N/A	ND	N/A
24	11.29	1.93	1.4	8.4	0.97	0.40	-49	ND	N/A	ND	N/A

Notes: Expected concentrations are corrected for weight loss during cooking. ND, None detected. N/A, Not applicable.

Table 3

Effect of frying and grilling on the concentration of DMZ and HMMNI residues in shrimp tissue.

Sample	Weight (g)		Residue							
	Before	After	Dimetridazole				HMMNI			
			Raw ($\mu\text{g kg}^{-1}$)	Cooked ($\mu\text{g kg}^{-1}$)		Residue change (%)	Raw ($\mu\text{g kg}^{-1}$)	Cooked ($\mu\text{g kg}^{-1}$)		Residue change (%)
		Expected	Observed		Expected		Observed			
<i>Frying (high residue)</i>										
25	7.73	6.22	34668	43085	38907	-10	21.9	27.2	23.8	-13
26	5.44	4.42	36054	44374	40451	-9	23.0	28.3	21.6	-24
27	7.50	5.81	37780	48769	40349	-17	21.0	27.1	23.1	-15
<i>Frying (low residue)</i>										
28	5.36	3.90	83	114	101	-12	ND	N/A	ND	N/A
29	7.62	5.83	54	70	78	+11	ND	N/A	ND	N/A
30	5.47	3.92	54	75	78	+3	ND	N/A	ND	N/A
<i>Grilling (high residue)</i>										
31	8.16	4.90	32323	53827	44958	-16	32.2	54	40.4	-25
32	11.82	8.23	33509	48126	47766	-1	39.2	56	61	8
33	6.50	3.60	33239	60015	46875	-22	33.6	61	45.7	-25
<i>Grilling (low residue)</i>										
34	8.70	5.96	160	233	249	+7	ND	N/A	ND	N/A
35	7.39	5.08	95	139	111	-20	ND	N/A	ND	N/A
36	11.59	8.00	110	159	136	-15	ND	N/A	ND	N/A

Notes: Expected concentrations are corrected for weight loss during cooking. ND, None detected. N/A, Not applicable.

Table 4

Effect of boiling and boiling followed by microwaving on the concentration of DMZ and HMMNI residues in shrimp tissue.

Sample	Weight (g)		Residue									
	Before	After	Dimetridazole					HMMNI				
			Raw ($\mu\text{g kg}^{-1}$)	Cooked ($\mu\text{g kg}^{-1}$)		Water/juices ($\mu\text{g kg}^{-1}$)	Residue change including water/juices (%)	Raw ($\mu\text{g kg}^{-1}$)	Cooked ($\mu\text{g kg}^{-1}$)		Water/juices ($\mu\text{g kg}^{-1}$)	Residue change including water/juices (%)
Expected	Observed	Expected	Observed	Expected	Observed							
<i>Boiling (high residue)</i>												
37	6.24	3.58	34484	60107	32331	7780	+5	11.3	19.7	8.6	2.3	-10
38	8.03	5.41	32140	47704	34177	7422	+13	19.5	28.9	16.8	4.3	-3
39	10.11	7.19	33328	46862	31978	6998	+4	25.3	35.6	22.4	5.1	-3
<i>Boiling (low residue)</i>												
40	7.76	5.15	118	178	92	18.6	-19	ND	N/A	ND	ND	N/A
41	7.02	4.40	57	91	48.2	11.8	-2	ND	N/A	ND	ND	N/A
42	5.60	3.10	77	139	44.6	15.3	-20	ND	N/A	ND	ND	N/A
<i>Boiling followed by microwaving (high residue)</i>												
43	7.33	2.28	33659	108210	49198	6694	-10	34.8	112	62	6.9	-1
44	7.01	1.94	34434	124424	58567	7023	-8	32.7	118	67	6.2	-2
45	7.12	2.14	31509	104834	54102	6395	-4	88	293	167	14.0	-8
<i>Boiling followed by microwaving (low residue)</i>												
46	6.74	2.28	85	252	96	25.3	+6	ND	N/A	ND	ND	N/A
47	10.76	2.61	91	375	144	25.2	-16	ND	N/A	ND	ND	N/A
48	7.55	1.58	69	330	99	16.2	-19	ND	N/A	ND	ND	N/A

Notes: Expected concentrations are corrected for weight loss during cooking. ND, None detected. N/A, Not applicable.

Table 5

Effect of frying and grilling on the concentration of RNZ and HMMNI residues in shrimp tissue.

Sample	Weight (g)		Residue							
	Before	After	Ronidazole				HMMNI			
			Raw ($\mu\text{g kg}^{-1}$)	Cooked ($\mu\text{g kg}^{-1}$)		Residue change (%)	Raw ($\mu\text{g kg}^{-1}$)	Cooked ($\mu\text{g kg}^{-1}$)		Residue change (%)
Expected	Observed	Expected	Observed	Expected	Observed					
<i>Frying (high residue)</i>										
49	7.30	5.64	5044	6528	5997	-8	10.7	13.8	34.8	151
50	7.76	6.81	5065	5771	6113	+6	11.9	13.6	19.1	41
51	9.11	6.43	5438	7704	6260	-19	8.4	11.9	38.9	227
<i>Frying (low residue)</i>										
52	5.14	3.93	9.5	12.4	11.6	-6	ND	N/A	ND	N/A
53	7.11	5.76	14.1	17.4	17.1	-2	ND	N/A	ND	N/A
54	4.71	3.27	3.8	5.5	10.5	+89	ND	N/A	ND	N/A
<i>Grilling (high residue)</i>										
55	6.60	4.29	4778	7351	5708	-22	13.1	20.2	30.7	52
56	5.68	3.57	4415	7025	5865	-17	7.8	12.4	33.5	170
57	8.96	6.16	4465	6495	6317	-3	8.2	11.9	25.7	115
<i>Grilling (low residue)</i>										
58	5.97	3.69	6.4	10.4	12.6	+21	ND	N/A	ND	N/A
59	6.70	4.37	2.9	4.4	11.9	+168	ND	N/A	ND	N/A
60	6.80	4.50	4.0	6.0	14.1	+136	ND	N/A	ND	N/A

Notes: Expected concentrations are corrected for weight loss during cooking. ND, None detected. N/A, Not applicable.

Table 6

Effect of boiling and boiling followed by microwaving on the concentration of RNZ and HMMNI residues in shrimp tissue.

Sample	Weight (g)		Residue									
	Before	After	Ronidazole					HMMNI				
			Raw ($\mu\text{g kg}^{-1}$)	Cooked ($\mu\text{g kg}^{-1}$)		Water/juices ($\mu\text{g kg}^{-1}$)	Residue change including water/juices (%)	Raw ($\mu\text{g kg}^{-1}$)	Cooked ($\mu\text{g kg}^{-1}$)		Water/juices ($\mu\text{g kg}^{-1}$)	Residue change including water/juices (%)
		Expected	Observed					Expected	Observed			
<i>Boiling (high residue)</i>												
61	8.85	5.74	4373	6743	4030	998	+5	7.1	10.9	14.8	4.9	170
62	7.95	4.58	4645	8063	4321	1038	+4	14.1	24.5	18.0	5.9	68
63	6.40	3.65	4509	7905	4392	872	+8	12.4	21.7	15.1	4.0	57
<i>Boiling (low residue)</i>												
64	6.85	4.30	13.2	21.0	13.5	3.7	+42	ND	N/A	ND	ND	N/A
65	8.63	6.07	9.2	13.1	12.5	3.5	+66	ND	N/A	ND	ND	N/A
66	9.28	6.23	14.2	21.2	20.5	4.1	+56	ND	N/A	ND	ND	N/A
<i>Boiling followed by microwaving (high residue)</i>												
67	9.64	2.13	5758	26059	6102	1301	-39	11.4	52	738	7.7	1442
68	6.89	1.71	4895	19725	8868	856	-12	12.9	52	393	4.6	743
69	9.31	1.79	3646	18962	2508	968	-38	9.9	51	450	5.0	865
<i>Boiling followed by microwaving (low residue)</i>												
70	6.60	1.22	15.7	85	11.0	4.5	-13	ND	N/A	ND	ND	N/A
71	6.26	1.19	8.6	45.0	1.3	4.0	+26	ND	N/A	ND	ND	N/A
72	6.20	1.06	5.8	34.2	3.8	2.8	+36	ND	N/A	ND	ND	N/A

Notes: Expected concentrations are corrected for weight loss during cooking. ND, None detected. N/A, Not applicable.

Table S1

UHPLC-MS/MS conditions.

Analyte	T _R ^a (min)	Transition (<i>m/z</i>)	Dwell time (s)	Cone (V)	CE ^b (eV)	IS ^c
DMZ	2.67	141.9 → 96.0 ^d 141.9 → 81.0	0.075	23	15 22	DMZ-D ₃
DMZ-D ₃	2.64	144.9 → 99.0	0.075	25	15	IS
HMMNI	1.81	157.9 → 139.9 ^d 157.9 → 55.1	0.020	20	12 17	HMMNI-D ₃
HMMNI-D ₃	1.79	160.9 → 142.9	0.020	20	12	IS
MNZ-OH	1.46	187.9 → 123.0 ^d 187.9 → 126.0	0.100	21	12 16	MNZ-OH-D ₂
MNZ-OH-D ₂	1.46	189.9 → 128.0	0.100	21	17	IS
MNZ	2.17	172.0 → 128.0 ^d 172.0 → 82.0	0.040	20	14 23	MNZ-13C ₂ ,15N ₂
MNZ-13C ₂ ,15N ₂	2.17	176.0 → 132.0	0.040	21	14	IS
RNZ	2.14	200.9 → 139.9 ^d 200.9 → 55.0	0.040	16	11 20	RNZ-D ₃
RNZ-D ₃	2.12	203.9 → 142.9	0.040	16	11	IS

^aT_R, retention time. ^bCE, collision energy. ^cIS, internal standard. ^dQuantitation ion.

Table S2

Intra- and inter-assay validation data.

	Dimetridazole ($\mu\text{g kg}^{-1}$)			HMMNI ($\mu\text{g kg}^{-1}$)			Metronidazole ($\mu\text{g kg}^{-1}$)			Metronidazole- OH ($\mu\text{g kg}^{-1}$)			Ronidazole ($\mu\text{g kg}^{-1}$)		
	40	50	500	40	50	500	40	50	500	40	50	500	40	50	500
		0	0		0	0		0	0		0	0		0	0
<i>Intra-assay</i>															
Mean	44.	53	515	43.	53	516	43.	51	502	43.	52	473	44.	52	479
($\mu\text{g kg}^{-1}$)	8	3	2	2	1	5	4	6	5	8	8	5	6	8	9
SD ($\mu\text{g kg}^{-1}$)	1.6	7	49	1.7	11	132	0.9	11	71	3.0	14	113	2.5	7	203
CV (%)	3.7	1.4	1.0	4.0	2.0	2.6	2.2	2.2	1.4	6.9	2.7	2.4	5.5	1.3	4.2
Accuracy (%)	112	10	103	108	10	103	109	10	101	109	10	95	111	10	96
		7			6			3			6			6	
<i>Inter-assay</i>															
Mean	44.	53	503	43.	52	510	43.	51	501	45.	52	475	45.	52	482
($\mu\text{g kg}^{-1}$)	5	0	9	5	5	2	1	7	9	7	9	8	0	9	6
SD ($\mu\text{g kg}^{-1}$)	2.1	17	113	5.4	17	189	3.0	12	103	4.3	11	102	3.0	9	153
CV (%)	4.7	3.3	2.2	12.	3.2	3.7	6.9	2.3	2.1	9.3	2.0	2.2	6.7	1.8	3.2
				4											
Accuracy (%)	111	10	101	109	10	102	108	10	100	114	10	95	113	10	97
		6			5			3			6			6	

Table S3Method reproducibility results for cooked shrimp samples ($n = 3$).

Sample	Analyte	Sample treatment	Mean \pm SD ($\mu\text{g kg}^{-1}$)	CV (%)
1	Dimetridazole	Boiling	47.8 \pm 0.7	1.5
2		Frying	130 \pm 5.0	3.9
3		Grilling	123 \pm 8.0	6.7
4	Metronidazole	Boiling	4.8 \pm 0.2	4.2
5		Frying	1.3 \pm 0.1	8.9
6		Frying	2.1 \pm 0.1	5.9
7	Ronidazole	Boiling	16.9 \pm 0.6	3.4
8		Frying	22.9 \pm 0.7	3.1
9		Grilling	23.3 \pm 3.8	16.4