JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Investigation into the Possible Natural Occurence of Semicarbazide in *Macrobrachium rosenbergii* Prawns

Christof Van Poucke,*[,†] Christ'l Detavernier,[†] Mathieu Wille,[‡] Jan Kwakman,[§] Patrick Sorgeloos,[‡] and Carlos Van Peteghem[†]

[†]Ghent University, Department of Bioanalysis, Laboratory of Food Analysis, Harelbekestraat 72, 9000 Ghent, Belgium

[‡]Ghent University, Laboratory of Aquaculture & Artemia Reference Center, Rozier 44, 9000 Ghent, Belgium

[§]Seafood Importers and Producers Alliance (SIPA), Heiveldekens 4, 2500 Kontich, Belgium

ABSTRACT: In the past year there has been an increased incidence in Belgium of cases of positive semicarbazide (SEM) tests in imported freshwater *Macrobrachium rosenbergii* prawns, seemingly indicating the possible abuse of nitrofurazone, a banned antimicrobial agent. This was in contrast to all other European countries where no significant increase in SEM-positive samples was detected. A possible explanation for this discrepancy between Belgium and the other European Union member states could be the fact that only in Belgium were whole prawns (meat + shell) analyzed for the presence of tissue-bound metabolites of nitrofurans, whereas in the other countries only the edible part (meat) of these prawns was analyzed. To investigate the possible natural occurrence of SEM in freshwater prawns, an animal trial was set up. In this experiment two groups of 10 juvenile *M. rosenbergii*, previously raised under standardized laboratory conditions, were stocked into two separate aquaria, a control group under reference conditions (no addition of nitrofurazone) and a group exposed to a daily dose of 50 mg of nitrofurazone L^{-1} of culture water. Results of this animal trial proved that SEM naturally occurs in *M. rosenbergii* prawns but that at the current minimum required performance limit (MRPL) no tissue-bound SEM can be found in the meat of nontreated animals. In addition to this animal trial, commercial samples of other crustacean species, the shell and meat of which were analyzed separately, were also analyzed for the presence of SEM.

KEYWORDS: nitrofurazone, semicarbazide, *Macrobrachium rosenbergii*, animal trial, shell

1. INTRODUCTION

Nitrofurazone (5-nitro-2-furaldehyde semicarbazone) is an antibacterial drug that is often used in shrimp farming and poultry production because of its growth-promoting properties and to prevent and control bacterial infections.[1] After administration of nitrofurazone to chickens or pigs, the compound is rapidly metabolized to semicarbazide (SEM) (Figure 1), which can easily bind to tissue proteins.[2,3] Because of its carcinogenic properties, within the European Union the use of nitrofurazone in animal breeding is prohibited,[4] and no foodstuffs containing its residues may be imported.[5] To detect the possible abuse of nitrofurazone, it is generally accepted to determine tissue-bound SEM. In the search for harmonization, a minimum required performance limit (MRPL) of 1 μg kg^{-1} is fixed within the European Union for this banned compound.[6]

In the period 2008−2009 there was an increased incidence in Belgium of cases of positive SEM tests in imported freshwater prawns, *Macrobrachium rosenbergii*, seemingly indicating the possible abuse of nitrofurazone, a banned antimicrobial agent. This was in contrast to all other European Union (EU) countries, where no significant increase in SEM positive samples was detected. A possible explanation for this discrepancy between Belgium and the other EU member states could be the fact that only in Belgium were whole prawns analyzed for the presence of tissue-bound metabolites of nitrofurans, whereas in the other countries only the edible part (meat) of these prawns was analyzed.[7]

This paper describes the research undertaken to investigate the possible natural occurrence of SEM in *M. rosenbergii* prawns.

2. MATERIALS AND METHODS

2.1. Reagents and Chemicals. SEM, nitrofurazone, and 2-nitrobenzaldehyde (NBA) were purchased from Sigma-Aldrich (Bornem, Belgium). 3-Amino-2-oxazolidinone-D4 (AOZ-D4) was purchased from Witega (Berlin, Germany).

Formic acid, glacial acetic acid, fuming hydrochloric acid 37%, acetonitrile, and methanol for HPLC were purchased from VWR (Leuven, Belgium); ethyl acetate was supplied by Acros (Geel, Belgium). Water was purified using a Milli-Q system (Millipore, Bedford, MA).

2.2. Total SEM Procedure . For the determination of total SEM (free + tissue-bound), internal standard AOZ-D4 is added at a concentration of 2 μg kg^{-1} to 1 g (±0.05g) of sample. The sample is then incubated overnight at 37 °C with 5 mL of a 0.2 M HCl solution and 50 μL of a 100 mM 2-nitrobenzaldehyde (NBA) solution. After overnight hydrolysis and derivatization, the pH of the sample is adjusted between 6.5 and 7.5. Next the sample is extracted twice with 4 mL of ethyl acetate. The extracts are combined and evaporated under nitrogen gas to dryness at 37 °C (maximum). The dried residue is dissolved in 150 μL of water/acetonitrile (90:10; v/v) containing 0.1% acetic acid and analyzed with LC-MS/MS.

Received: August 24, 2010
Accepted: January 18, 2011
Revised: January 12, 2011
Published: February 07, 2011

 © 2011 American Chemical Society

2.3. Tissue-Bound SEM Procedure. To remove any free SEM and determine only the tissue-bound portion of the total SEM, the sample is subjected to a washing procedure. The sample is subsequently washed with 6 mL of methanol/water (50:50; v/v), 6 mL of methanol/water (75:25; v/v), 6 mL of methanol, and 2 mL of water. Between each washing step the supernatant is discarded. Next the washed sample is treated as described for total SEM (section 2.1).

2.4. LC-MS/MS Analysis. Analyses were performed on an Acquity UPLC coupled to a Quattro Premier XE mass spectrometer (Waters, Milford, MA). A Symmetry C_{18} column (2.1 × 150 mm; 5 μm) (Waters) was used for chromatographic analysis. A Symmetry guard column (3.5 μm; 2.1 × 10 mm) was used prior to the analytical column. The injection volume was 10 μL, a flow of 0.3 mL min^{-1} was used, and the column was operated at room temperature. The mobile phase consisted of water with 0.1% acetic acid (mobile phase A) and acetonitrile/water (90:10; v/v) with 0.1% acetic acid (mobile phase B), and a gradient program (Table 1) with a run time of 25 min was used.

The mass spectrometer was operated in the electrospray positive (ESP+) mode. Capillary voltage was set at 4 kV, extractor at 3 V, source block temperature at 120 °C, and desolvation temperature at 300 °C. High-purity nitrogen was used as the drying gas and ESI nebulizing gas. For collision-induced dissociation, argon was used as the collision gas. Data were collected in the multiple reaction monitoring (MRM) mode (Table 2).

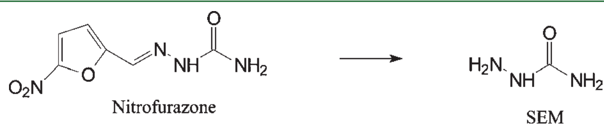

Figure 1. Metabolization of nitrofurazone to semicarbazide (SEM).

Table 1. Gradient Program

time (min)	A	B	curve
0	90	10	1
1	90	10	1
14	45	55	6
16	10	90	6
18	10	90	1
19	90	10	6
25	90	10	1

Table 2. MRM Conditions Used

compound	precursor ion (m/z)	fragment ion (m/z)	collision energy (eV)	cone voltage (V)
NPSEM	209.2	166.1 192.1	10	28
NPAOZ-D_4 (internal standard)	240.0	134.0	10	28

2.5. Experimental Animals. The juvenile *M. rosenbergii* used in this experiment were offspring from breeders with a minimum of three generations (F3) in captivity at the Laboratory of Aquaculture and Artemia Reference Center of Ghent University. The original stock was imported from Thailand as adult breeders. Broodstock rearing, egg incubation, and hatching followed the techniques described by Cavalli et al.[8,9] Twenty four hours after hatching, the larvae were transferred into 10 L cylindroconical jars connected to a single recirculation system as was described by Cavalli et al.[10] Newly hatched *Artemia franciscana* nauplii (EG type, INVE Aquaculture, Baasrode, Belgium) were used as live feed and dosed to the culture water ad libitum (density always >6 nauplii mL^{-1}). The *Artemia* ration was split over two feedings at 9:00 a.m. and 5:00 p.m. Salinity during larval rearing was 12 g L^{-1}; temperature was controlled around 28 °C, and photoperiod was 12 h light/12 h dark. After metamorphosis into postlarvae, the animals were moved to rectangular 30 L PVC tanks connected to a biofilter system to be further grown to the juvenile stage. Salinity of the rearing water was gradually lowered until completely fresh. The animals were at the same time weaned from *Artemia* nauplii to a formulated feed (A2 monodon grower, Crevetec, Belgium).

2.6. Exposure of *M. rosenbergii* to Nitrofurazone. Two groups of 10 juvenile *M. rosenbergii*, raised as described above, were then stocked into two separate rectangular PVC tanks of 80 L. The control group was kept under normal conditions (no addition of nitrofurazone), and the other group was exposed to a daily dose of 50 mg of nitrofurazone L^{-1} of culture water. No water recirculation or filter system was applied. Every day most (>95%) of the water in the tanks was removed, and the tanks were refilled with clean water, after which the treatment group was again dosed with 50 mg of nitrofurazone L^{-1}. The animals were fed the standard formulated feed during the exposure. Environmental conditions were similar to those described above.

Table 3. Results of the Analysis of Meat and Shell of Different Crustacean Species for Tissue-Bound SEM (n = 1)

species	origin	bound SEM in meat	bound SEM in shell
Penaeus vannamei	Thailand[a]	absent (<0.5 μg kg^{-1})	2.2 μg kg^{-1}
Penaeus vannamei	Vietnam[a]	absent (<0.5 μg kg^{-1})	2.6 μg kg^{-1}
Penaeus vannamei	Ecuador[a]	absent (<0.5 μg kg^{-1})	2.1 μg kg^{-1}
Penaeus monodon	Gujarat, India[a]	absent (<0.5 μg kg^{-1})	1.5 μg kg^{-1}
Penaeus monodon	Indonesia[a]	absent (<0.5 μg kg^{-1})	2.3 μg kg^{-1}
Nephrops norvegicus	Atlantic Ocean; Scotland[a]	absent (<0.5 μg kg^{-1})	absent (<0.5 μg kg^{-1})
Scylla serrata	Madagascar[b]	absent (<0.5 μg kg^{-1})	12.6 μg kg^{-1}
Scylla serrata	Vietnam[b]	absent (<0.5 μg kg^{-1})	5.0 μg kg^{-1}
Scylla serrata	Vietnam[b]	absent (<0.5 μg kg^{-1})	4.1 μg kg^{-1}
Somanniathelpusa sinensis	Vietnam[b]	0.6 μg kg^{-1}	8.4 μg kg^{-1}
Portunus pelagicus	China[b]	absent (<0.5 μg kg^{-1})	4.1 μg kg^{-1}
Portunus pelagicus	Vietnam[b]	absent (<0.5 μg kg^{-1})	3.3 μg kg^{-1}

[a] From commercial aquaculture farms. [b] Wild caught.

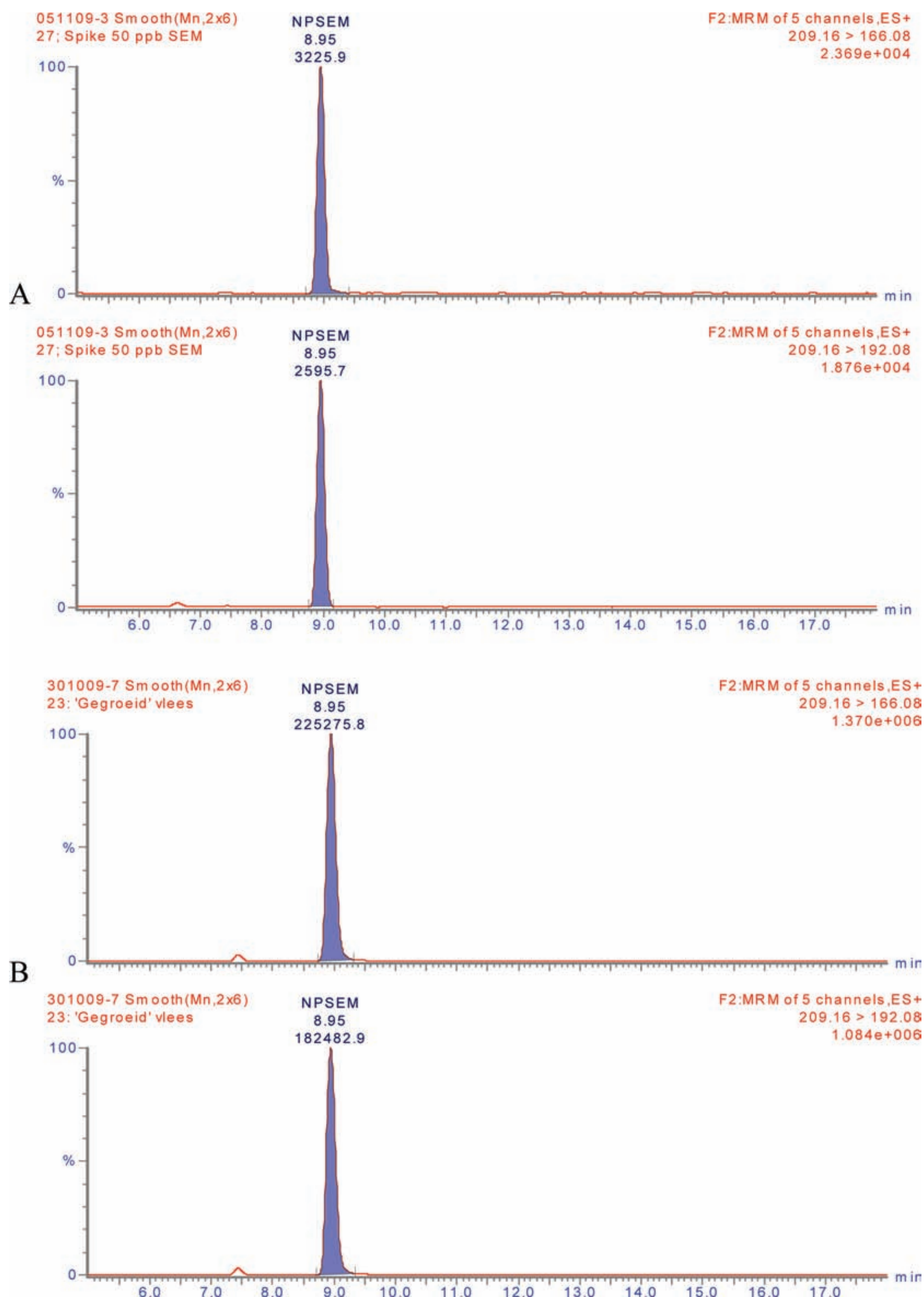

Figure 2. Chromatograms showing the two diagnostic ions of SEM in (A) blank prawn meat spiked at 50 ppb SEM and (B) meat sample of *M. rosenbergii* prawns exposed to a daily dose of 50 mg of nitrofurazone/L of culture water and analyzed as described under section 2.3 (tissue-bound SEM).

After 1 week of exposure, the animals were netted out of the tanks, rinsed with tapwater, and immediately transported to the Laboratory of Food Analysis of Ghent University for further processing.

3. RESULTS AND DISCUSSION

To explain the discrepancy between the results obtained in Belgium and those in the rest of the EU, a batch of prawn samples that tested positive for SEM during official control was reanalyzed. From this batch, samples were analyzed as whole prawns, and both the meat and the shell of the *M. rosenbergii* prawns were also analyzed separately for the presence of tissue-bound SEM. Whereas in the whole prawns the tissue-bound SEM concentration was around 7 μg kg^{-1}, no residues could be detected (below

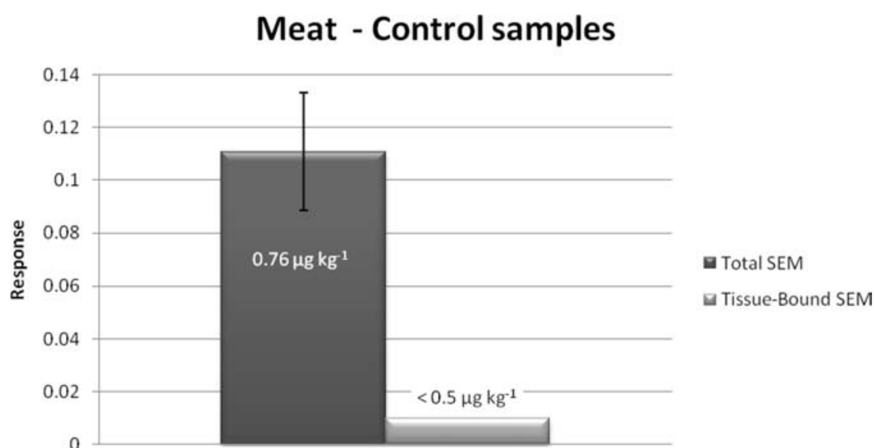

Figure 3. Overview of total and tissue-bound SEM concentrations in the meat of nontreated *M. rosenbergii* prawns (control).

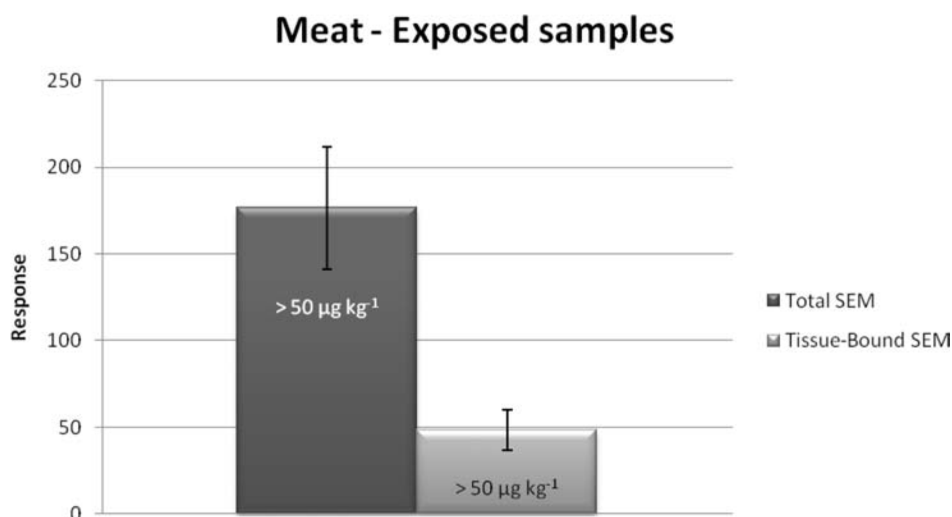

Figure 4. Overview of total and tissue-bound SEM concentrations in the meat of *M. rosenbergii* prawns exposed to a daily dose of 50 mg of nitrofurazone/L ofculture water (exposed).

LOD, <0.5 μg kg^{-1}) in the edible part (meat) of the prawns. However, in the shell of this batch of *M. rosenbergii* prawns, around 25 μg kg^{-1} was detected. These results confirmed the hypothesis that SEM could only be detected in the shell of these prawns. The discrepancy in the incidence of positive samples between Belgium and other EU member states was clearly caused by the fact that only in Belgium were whole prawns analyzed for the presence of tissue-bound metabolites of nitrofurans, whereas in the other countries only the edible part (meat) of these prawns was analyzed.

To determine if the observed phenomenon (SEM mainly detectable in the shell) was species dependent, commercial samples of other crustacean species were analyzed for the presence of tissue-bound SEM. The results of these analyses are presented in Table 3. Although the detected concentrations of tissue-bound SEM in the shell of these other crustacean species were significantly lower than the concentrations in the *M. rosenbergii* prawns, SEM was also here detected at higher concentrations in the shell than in the meat. Moreover, in most samples tissue-bound SEM was found only in the shell, whereas in the meat no residues could be detected. The fact that in comparison with the shell of other tested crustacean products *M. rosenbergii* shells contain the highest tissue-bound SEM concentrations may explain why the increased incidence in SEM -positive samples was reported for only this species.

Because the experiments described above were performed on imported crustaceans, most of them cultivated in commercial aquaculture farms, this still did not exclude the possibility that the detected SEM could not be caused by the illegal use of nitrofurazone. Consequently, it was decided to set up an animal trial with *M. rosenbergii* prawns that were raised under standardized laboratory conditions.

Two groups of 10 juveniles of *M. rosenbergii* prawns were stocked for 1 week into two separate aquaria, the control group under normal conditions (no addition of nitrofurazone), the other group being exposed to a daily dose of 50 mg of nitrofurazone L^{-1} of culture water. Meat and shell of replicate samples taken from the two initial tanks of these freshwaters prawns were analyzed for both total (sum of free and tissue-bound) and tissue-bound SEM concentrations. For the calculation of the concentration present in the samples blank prawn samples were spiked with different concentrations of SEM before extraction. The samples of this matrix-matched calibration curve were then analyzed as described under section 2.2 or 2.3. In many

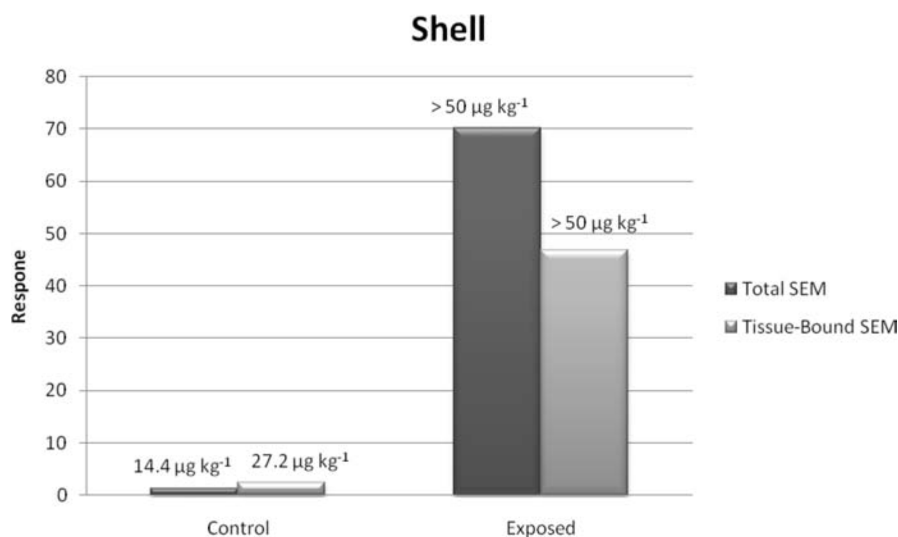

Figure 5. Overview of total and tissue-bound SEM concentrations in the shell of both nontreated *M. rosenbergii* prawns (control) and *M. rosenbergii* prawns exposed to a daily dose of 50 mg of nitrofurazone/L of culture water (exposed).

cases however, the detected concentrations were much higher than the highest spiked sample (50 μg kg^{-1}) (Figure 2). Consequently it was decided to express the obtained results as responses. This response was calculated by dividing the area of SEM by the area of the internal standard AOZ-D4, consequently correcting for any losses during cleanup and instrumental variability. When possible (e.g., below 50 μg kg^{-1}), average concentrations are given. An overview of all results is shown in Figures 3−5.

In the meat of the nontreated prawns total SEM concentrations were below the MRPL of 1 μg kg^{-1}, and after the samples had been washed to determine the tissue-bound fraction, SEM could no longer be detected (<0.5 μg kg^{-1}) (Figure 3) . After exposure of these prawns during 1 week to nitrofurazone, both free and tissue-bound SEM concentrations increased drastically. Tissue-bound SEM concentrations in the meat of exposed prawns were about 4 times lower than the total SEM concentration (Figure 4). In the shell of the control samples comparable results were obtained for both total and tissue-bound SEM concentrations, indicating that SEM is mainly tissue-bound in the shell of *M. rosenbergii* prawns. Moreover, as these prawns were cultivated under standardized laboratory conditions and consequently the treatment with nitrofurazone can be excluded, these results prove that tissue-bound SEM naturally occurs in the shell of *M. rosenbergii* prawns. After treatment of these prawns with a daily dose of 50 mg of nitrofurazone L^{-1} of culture water, the response increases by a factor 20−50 for tissue-bound and total SEM, respectively. Whereas in the meat of the exposed samples only about a fourth of the total SEM concentration was tissue-bound, in the shell >65% is tissue-bound (Figure 5), and whereas total SEM concentrations are much higher in the meat than in the shell of exposed prawns, tissue-bound concentrations are comparable. Although no tissue-bound SEM could be detected in the meat of the nontreated prawns, these results do not exclude the possibility that concentrations below the LOD of the used method (0.5 μg kg^{-1}) may be present. Moreover, from the presented results it is clear that even the slightest amount of shell that would not be removed from the portion used for analysis could result in the detection of tissue-bound SEM.

Results from this paper and those obtained in the past by other research groups[11−15] clearly demonstrate that SEM does not result only from the abuse of nitrofurazone. Moreover, the results of this study even show that tissue-bound SEM can have a source other than the abuse of nitrofurazone. Consequently, SEM can no longer be used as an unambiguous marker for nitrofurazone abuse, and there is a clear need for an alternative marker to prove such abuse.

AUTHOR INFORMATION

Corresponding Author

* Phone: +32 264 81 34. Fax: +32 264 81 99. E-mail: christof.vanpoucke@UGent.be.

REFERENCES

(1) Pereira, A. S.; Pampana, L. C.; Donato, J. L.; De Nucci, G. Analysis of nitrofuran metabolic residues in salt by liquid chromatography−tandem mass spectrometry. *Anal. Chim. Acta* **2004**, *514* (1), 9–13.

(2) Cooper, K. M.; Mulder, P. P. J.; van Rhijn, J. A.; Kovacsics, L.; McCracken, R. J.; Young, P. B.; Kennedy, D. G. Depletion of four nitrofuran antibiotics and their tissue-bound metabolites in porcine tissues and determination using LC-MS/MS and HPLC-UV. *Food Addit. Contam.* **2005**, *22* (5), 406–414.

(3) McCracken, R. J.; Van Rhijn, J. A.; Kennedy, D. G. The occurrence of nitrofuran metabolites in the tissues of chickens exposed to very low dietary concentrations of the nitrofurans. *Food Addit. Contam.* **2005**, *22* (6), 567–572.

(4) European Commission, Commission Regulation 37/2010. *Off. J. Eur. Communities* **2010**, *L15*, 1.

(5) European Commission, Council Regulation 470/2009/EC. *Off. J. Eur. Communities* **2009**, *L152*, 11.

(6) European Commission, Commission Decision 2003/181/EC. *Off. J. Eur. Communities* **2003**, *L71*, 17.

(7) De Poorter, G. Note RV/LABO/318833. http://www.favv.be/laboratoires/laboratoiresagrees/notesdeservice/_documents/2009-06-08_nota_nitrofuran_garnaalachtigen.rect._fr-nl.pdf (11/01/2011).

(8) Cavalli, R. O.; Lavens, P.; Sorgeloos, P. Performance of *Macrobrachium rosenbergii* broodstock fed diets with different fatty acid composition. *Aquaculture* **1999**, *179* (1−4), 387–402.

(9) Cavalli, R. O.; Berghe, E. V.; Lavens, P.; Thuy, N. T. T.; Wille, M.; Sorgeloos, P. Ammonia toxicity as a criterion for the evaluation of larval quality in the prawn *Macrobrachium rosenbergii*. *Comp. Biochem. Physiol. C−Toxicol. Pharmacol.* **2000**, *125* (3), 333–343.

(10) Cavalli, R. O.; Lavens, P.; Sorgeloos, P. Reproductive performance of *Macrobrachium rosenbergii* females in captivity. *J. World Aquacult. Soc.* **2001**, *32* (1), 60–67.

(11) Becalski, A.; Lau, B. P. Y.; Lewis, D.; Seaman, S. W. Semicarbazide formation in azodicarbonamide-treated flour: a model study. *J. Agric. Food Chem.* **2004**, *52* (18), 5730–5734.

(12) Hoenicke, K.; Gatermann, R.; Hartig, L.; Mandix, M.; Otte, S. Formation of semicarbazide (SEM) in food by hypochlorite treatment: is SEM a specific marker for nitrofurazone abuse?. *Food Addit. Contam.* **2004**, *21* (6), 526–537.

(13) Stadler, R. H.; Mottier, P.; Guy, P.; Gremaud, E.; Varga, N.; Lalljie, S.; Whitaker, R.; Kintscher, J.; Dudler, V.; Read, W. A.; Castle, L. Semicarbazide is a minor thermal decomposition product of azodicarbonamide used in the gaskets of certain food jars. *Analyst* **2004**, *129* (3), 276–281.

(14) Barnabas, S.; Miklos, S. Semicarbazide-residue formed in foods, as non-specific marker of the prohibited nitrofurazone. *Magyar Allatorvosok Lapja* **2005**, *127* (3), 178–183.

(15) Sarnsonova, J. V.; Douglas, A. J.; Cooper, K. M.; Kennedy, D. G.; Elliott, C. T. The identification of potential alternative biomarkers of nitrofurazone abuse in animal derived food products. *Food Chem. Toxicol.* **2008**, *46* (5), 1548–1554.