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Determination and Investigation the Nitrofurazone's Metabolite Semicarbazide in *Macrobrachium Nipponese* Prawns

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Abstract: Semicarbazide (SEM) is the metabolite of the illegal veterinary nitrofurazone (NFZ) in aquatic animal. However there are increasing occurrences that SEM has been found in *Macrobrachium Nipponese* (*M. Nipponese*) prawns during the routine quality controls testing for aquatic food in China since 2008. To ascertain the presence and source of SEM, prawn samples were collected from different resources (i.e., aquatic product markets, natural waters, aquaculture farms) for qualitation and quantitation, and we incubated *M. Nipponese* larvae in the laboratory under control for observing the SEM. SEM was detected out in all the samples analyzed, including the wild-caught samples, and it presents a regular content relationship among three parts of prawns (i.e., the shell > head > muscle). This relationship was unrelated to sample resources. In addition, a natural increase of SEM was observed after the larvae metamorphosing into postlarvae. These findings in *M. Nipponense* prawns can provid new evidences for further confirmation and research of the natural occurrence of SEM.

Keywords: Semicarbazide, Nitrofurazone, *Macrobrachium Nipponese* Prawn, Natural Occurrence, Shell

1. Introduction

Semicarbazide (SEM) is the metabolite of the illegal veterinary nitrofurazone (NFZ) in aquatic animal [1, 2], and SEM is as the marker residue for detecting nitrofurazone (NFZ) in animal-derived food since 2003 in China. NFZ is one of the nitrofuran antibiotics prohibited for use in food animals according to the Banned Drugs and Substances in Feed and Animal Production [3] issued by China. The determination of the lowest detection limitation for the nitrofurans metabolite residues in animal-derived food is 1μg/kg of total SEM in Chinese current regulation [4], in accordance with the minimum required performance limit (MRPL) 1μg/kg of tissue-bound SEM by Commission Decision 2003/181/EC [5] within European Union.

With the increase of cultivation and consumption of *M. Nipponese* in China, more and more attention has been focused on the safety of this species. *M. Nipponese* has been listed on the monitoring object of illegal veterinary drugs since 2007[6]. However, positive-SEM in many batches of *M. Nipponese* samples has been frequently detected during the routine quality controls testing for aquatic food during 2008-2014, which generally indicated the illegal use of NFZ. The occurrence of SEM in *M. Nipponese* samples has exerted a serious influence on aquatic marketing in China. This urges us to investigate the occurrence of SEM in *M. Nipponese*. Similar occurrence was also happened in another species. The EU issued 54 nitrofuran-related warnings and rejected consignments of *M. Rosenbergii* from Bangladesh through the Rapid Alert System for Food and Feed [7], since SEM was found in the export-oriented *M. Rosenbergii* prawns. Afterwards, McCracken et al. and Poucke et al. determined SEM in wild-caught *M. Rosenbergii*, and suggested that SEM naturally occurred in *M. Rosenbergii*, unrelated to the use of illegal drug [8~9]. However, the natural source of SEM occurred in prawns remains unclear. Lacking of sufficient evidence, SEM is still used as the marker for the illegal veterinary drug NFZ by many countries.

In this study, we used LC- MS/MS to determine SEM in three parts (muscle, shell and head) of *M. Nipponese* prawns,

which were caught from aquatic product markets, natural waters, aquaculture farms. Secondly, we established another qualitative method by higher resolution LC-MS/MS for reanalyzing the SEM in the positive samples. Finally, we studied the variation of the SEM concentration in artificially hatched *M. Nipponese* in the larva stage, inquiring whether there would be an increase of SEM in prawns with their process of growing up under non-NFZ condition. We hope present work will provide valuable information for the research of SEM in *M. Nipponese* prawns, as well as a new reference for strategic decision on whether SEM can continue to serve as the marker of the illegal veterinary drug NFZ for domestic and overseas supervision.

2. Experimental

2.1. Reagents and Chemicals

Methanol, ethyl acetate and hexane (n-hexane, 95%) of were HPLC grade (J. T. Baker). 2-Nitrobenzaldehyde (2-NBA, 98%) was bought from Alfa Aesar (Tianjin, China). Ammonium acetate (HPLC, 99.0%) was from Sigma-Aldrich (St.Louis.MO.USA). Water (18MΩ) was obtained from a Milli-Q gradient A10 purification system (Millipore, France). All other chemicals were of pro analysis grade or better.

Semicarbazide (SEM, 99.5%) and internal standard semicarbazide (^{13}C, $^{15}N_2$ –SEM, 99%) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Both of stock standard solutions of SEM and ^{13}C, $^{15}N_2$ –SEM at 1 μg/mL were prepared by dissolving in methanol and stored at 4℃ in the dark. Work standard solutions were prepared by diluting the stock standard solutions in methanol to get the concentration of 200 ng/mL.

2.2. Sampling Work and Sample Treatment

The *M. Nipponense* prawns were successively collected from Chinese natural waters (EastDongting Lake, SouthDongting Lake, Qiandao Lake, Weishan Lake, Shangdong reservoirs, Tianjing reservoirs), four aquaculture farms in Nanjiang City, Huzhou City and Tianjing City, and various aquatic product markets in Shanghai City and Nanjing City. Shell, muscle and head parts of prawns were analyzed for the SEM, respectively. The weight of every batch of samples was up to 500g. Blank samples were prepared by the *Penaeus Vanmamei* prawns from aquatic product market. All living prawns were transported to the laboratory and frozen at -40℃. The shell and head of prawns was removed and shredded into pieces. The muscle was minced in a blender.

Along with the sampling work, we also collected feedstuffs samples and cultural water samples from every aquaculture farm and aquatic product market for detecting the illegal drug nitrofurazone. We caught sediment samples and water from culturing pond in aquaculture farm and natural waters for detecting the SEM residue. The pellet samples were shattered by shredder, and then were filtered through nylon filter. The water samples were kept under the acid condition.

2.3. Total and Tissue-Bound SEM Extraction Procedure

Internal standard $^{13}C^{15}N_2$-SEM was spiked at a concentration of 5 ng/g to sample. The sample was hydrolyzed with 0.3mol/L HCl solution (5mL for muscle, 7 mL for shell) and was derivated by 2-NBA (150μL, 0.1mol/L in dimethyl sulfoxide). The reaction mixture was kept at 37 ℃ for 16h. After derivation reaction, the pH of the sample was adjusted between pH 7 and pH 7.5 with potassium phosphate, and then the sample was extracted with 8mL of ethyl acetate. The supernatant was evaporated under nitrogen gas to dryness at 40℃. The dried residue was dissolved in 2mL hexane solution and shaking for 10s, then added 1 mL methanol–water (5:95; v/v) and shaking for 1 min. Afterwards, the mixture was centrifuged at 2500rpm/ min for 5min and the subnatant (methanol–water) was filtered with 0.22μm filters, prepared for analysis by LC-MS/MS.

Tissue-bound SEM was determined by using the method reported previously for the determination of nitrofuran metabolites [10~11]. Shortly, 2±0.02g of minced muscle was washed by a pre-extraction step with 8 mL of methanol–water (50:50; v/v) and 8 mL of methanol-water (75:25; v/v) to remove free SEM.

To calculate the concentration present in the samples, blank *P. Vanmamei* samples were spiked with a series of standard SEM concentrations before extraction. The samples of this matrix-matched calibration curve were then analyzed.

2.4. Determination for SEM in Prawns

A triple quadrupole tandem mass spectrometer (TSQ Quantum ultra, Thermo Fisher Scientific, USA) was connected to an HPLC instrument (Thermo Fisher Scientific). The LC column was CAPCELL PAK C18 column (100mm×2.0mm I.D., 5 μm) and was operated using a gradient mode. The composition of the mobile phase was a combination of two eluents A (methanol) and B (2mM ammonium acetate in water with 0.1% formic acid). The gradient profile started with 17% A for 2 min and increased to 85% for 6.5 min and then decreased to 17% for last 1.5 min. The injection volume was 25μL, a flow of 0.3mL/min. The column temperature was maintained at 30℃throughout the analysis. This gradient profile gave acceptable resolution and reduced sample carry-over. Electrospray ionisation was used as in positive ion mode $[M+H]^+$. Spray voltage was set at 2.8 KV, capillary temperature at 320℃, vaporizer temperature at 300℃, shealth gas (N_2) pressure at 10psi, and aux gas (N_2) pressure at 5psi. Collision pressure was set at 1.5mTorr. Data were collected in the multiple reactions monitoring (MRM) mode in *Table 1*.

Table1. *the parent and product ions of SEM for MRM mode.*

Derivatives	Parent ion /m/z	Product ions /m/z	Collison energy /eV
NPSEM	209.0	192.0 (qualitative ion)/ 166.0	12/11
NPSEM-$^{13}C^{15}N$	212.0	168.0	11

2.5. Qualitative Analysis for SEM by Higher Resolution LC-MS/MS

Accurate-mass (AM) Orbitrap mass spectrometer features much higher resolution and enables the assigned masses of identified fragments with high accuracy [12] and it can eliminate interferences of geometric isomers or fragments with similar mass in coextraction substances that may result in negative results [13~15]. Detection was carried out by using AM Orbitrap mass spectrometer LC-MS/MS (Thermo Fisher Scientific, USA) which combines high-performance quadrupole precursor selection,. The LC column was Hypersil GOLDTM C18 column (50mm × 2.1mm I.D., 1.9 μm). The sheath gas (N_2)was 35 psi, and auxiliary gas (N_2) was 10 psi, capillary temperature was 320℃. The composition of the mobile phase was methanol/water. The gradient profile started with 30% methanol for 2 min and increased to 100% methanol at 2.01s and ended with 30% methanol for last 2 min. The total ion scan was at m/z 150–500. Resolution Settings (FWHM) was set at 70000 (T-SIM) and 17500 (ddms^2), respectively.

2.6. Animal Experiment

The carrying-egg prawns were bought from the professional aquafarm, where the culturing process was under standard conditions of National Aquaculture Quality Safety Management Regulations [16]. The carrying-egg prawns were transported to the laboratory in net cages, and cultured in a cement pool (2m×7m×0.8m). Temperature was kept at 25~27℃. Larvae were hatched out three days later, and the healthy and vigorous larvae were equally held in three aquariums with aerated, as three reduplicates. The larvae were fed with artificial diet (composed with milk powder, cod liver oil and hairtail muscle), soya-bean milk, commercialized spirulina power and *artemia salina* three times per day. The aquariums were cleaned twice daily to remove excess feed and wastes. We collected the larvae samples at 1, 4, 6, 9, 12, 15 days after hatching from every aquarium for analyzing the total SEM. The 15th day's larvae samples developed into postlarvae. All the inputs (i.e., feed and cultured water) were permitted to use after detecting no residue of SEM or NFZ.

3. Results and Discussions

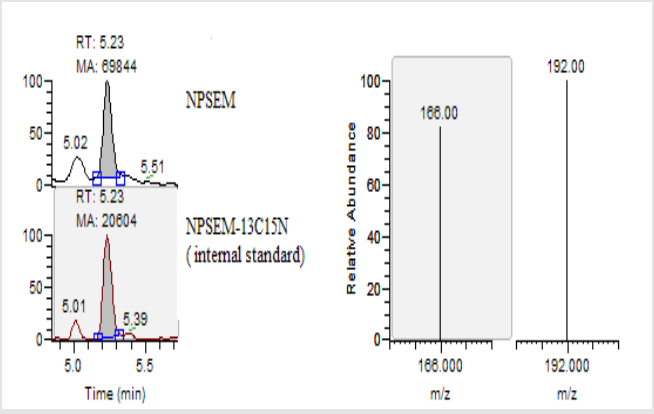
3.1. Determination for SEM in Prawns

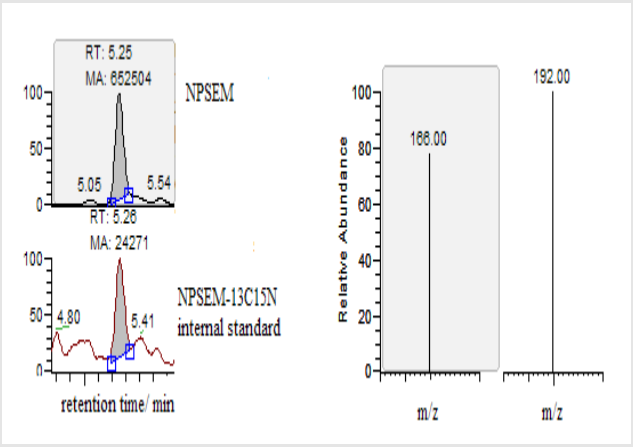
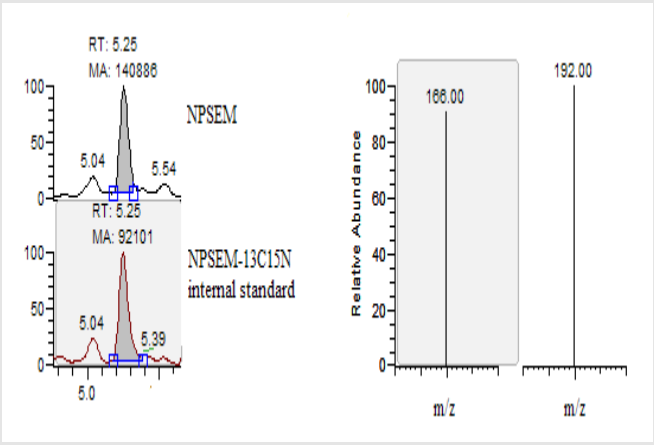
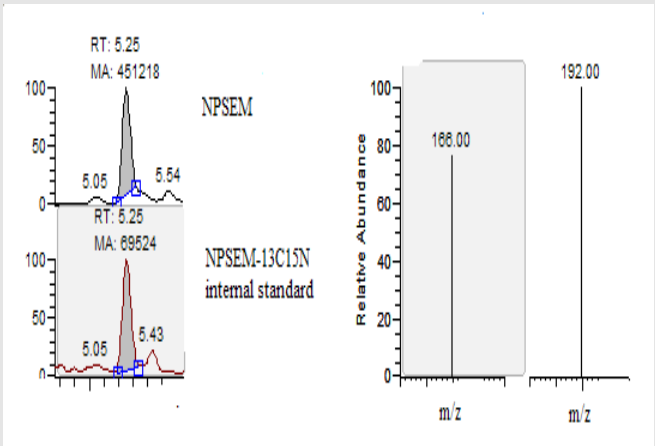
3.1.1. Analysis of SEM

The ions m/z 166 and m/z 192 of SEM were optimized. A linear response was gained by spiking blank *Penaeus vanmamei* muscle over the range of 0.5~500 μg/kg of SEM standard (correlation R_2> 0.9990). Day-to-day variation was in an acceptable range (Coefficient of variation 6.4~12.3). The recovery ranged from 80% to 112%. The limits of detection (LOD) and limits of quantitation (LOQ) were 0.5 μg/kg and 1 μg/kg, respectively. According to the qualitative rule by the European commission 2002/657/EC[17], it is namely the ±2.5% tolerance for relative retention time of the analyte to that of the corresponding standard and observation of the tolerances set by EU criteria of peak area ratios from the controlled transitions reactions. The results were shown in *table 2*.

All the *M. Nipponense* prawn's samples contained SEM, and every part (i.e., muscle, head and shell) was detected out SEM (*Table 2*). Comparing the concentrations of total SEM in the three parts of prawns, a consistent relationship was observed; that was shell > head > muscle. The result was similar with the previous studies by Poucke et al. and McCraken et al., who both reported that SEM was mainly in the shell of *M. Rosenbergii* prawns [7~8]. The lowest concentration of total SEM was 2.30μg/kg in muscle part.

Table 2. *The result of SEM in the M. Nipponese prawn.*

Tissues	Total icon chromatorgraphy and MS spectrum	Retention time/min	Product ions /m/z	Concentration of total SEM/μg/kg
Standard SEM		5.23 (SEM) 5.23 (NPSEM -$^{13}C^{15}N$)	166.00, 192.00	10μg/kg, Added in blank prawns

Tissues	Total icon chromatorgraphy and MS spectrum	Retention time/min	Product ions /m/z	Concentration of total SEM/μg/kg
Shell of *M.Nipponese* prawn		5.25 (SEM) 5.26 (NPSEM -$^{13}C^{15}N$)	166.00, 192.00	46.6~370
Muscle of *M. Nipponese* prawn		5.25 (SEM) 5.25 (NPSEM -$^{13}C^{15}N$)	166.00, 192.00	1.84~21.3
Head of *M. Nipponese* prawn		5.25 (SEM) 5.25 (NPSEM -$^{13}C^{15}N$)	166.00, 192.00	28.6~124

3.1.2. Total and Tissue-Bound of SEM

We selected 12 prawns for determining background concentration of total and tissue-bound SEM in the muscle.

Table 3. *the concentration of SEM in the muscle of M. Nipponese prawns.*

Sample number	1	2	3	4	5	6	7	8	9	10	11	12
Tissue-bound SEM/μg/kg	1.17	1.18	<0.5	0.66	0.57	0.62	<0.5	1.38	<0.5	<0.5	<0.5	0.82
MRPL by Commission Decision 2003/181/EC	Over	Over	/	/	/	/	/	Over	/	/	/	/
Total SEM /μg/kg	7.95	9.29	4.03	3.25	6.25	2.5	3.15	3.13	2.34	2.22	1.84	5.94
Detection limitation by Chinese current regulation	Over	Over	Over	Over	Over	Over	Over	Over	Over	Over	Over	Over

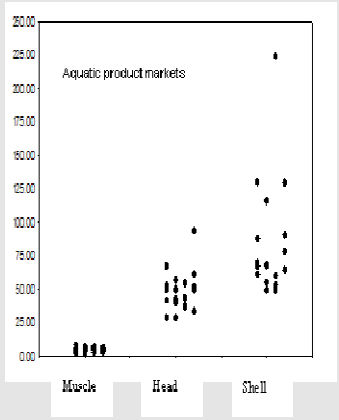
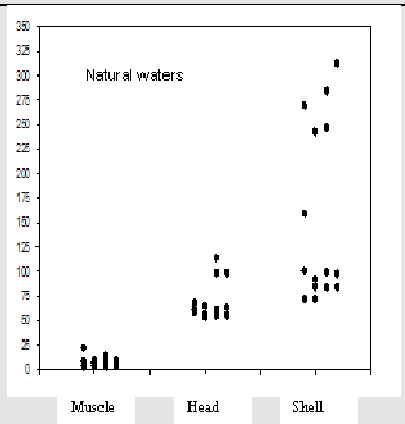
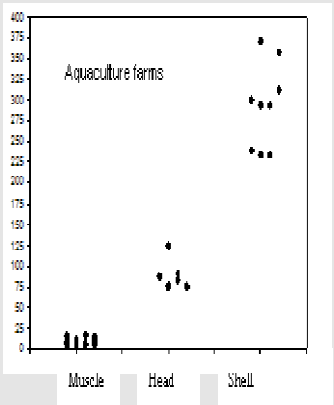
Note: 1~8 collected from natural waters, 8~12 collected from the aquatic product markets

The total SEM in muscle was ranged from 1.84-9.29μg/kg and the tissue-bound SEM ranged from<0.5~1.38μg/kg. Even the concentration less than 0.5μg/kg, is also positive. Three samples are over than MRPL by Commission Decision 2003/181/EC, and all samples are over than detection limitation by Chinese current regulation. There were increasing cases of positive SEM tests in freshwater *M. Nipponense* prawns in China, while not reported in European countries yet. Based on the results in this study, a possible explanation for this discrepancy could be the fact that Chinese official laboratories employed the total SEM as the marker of related nitrofurazone-contaminate in the edible tissue (muscle) of prawns, while most European Community Laboratories employed the tissue-bound SEM as the marker.

3.1.3. Distribution of SEM

The distribution of total SEM in the *M. Nipponese* prawn from aquatic product markets, natural waters and aquaculture farms resources was shown in *table 4*.

Table 4. *the distribution of total SEM in the M. Nipponese prawns from different resources.*

Sample resource	Aquatic product markets	Natural waters	Aquaculture farms
The distribution of SEM /μg/kg			

The concentration of SEM in meat and head of wild-caught prawns from natural waters was similar with those from aquatic product markets, and aquaculture farms, and the concentration of SEM in shell in wild-caught prawns was discrete.

3.1.4. Qualitative Analysis for SEM

Wild-caught prawns' samples were used to the re-analysis of SEM by AM Orbitrap mass spectrometer LC-MS/MS. Our study showed that it was a precise and reliable method for confirming the mass of compounds. The limit of detection (LOD) of 0.2 μg/kg for SEM by AM with the higher resolution was gained. After the optimization of conditions by SEM standard (1 μg/mL), the results showed that the mass of parent ion at m/z 209.06714 for $[M+H]^+$ in selected ion monitoring (T-SIM) at 70000 FWHM, and the mass of the product ions at m/z 192.04036 and m/z 166.06110 in MS/MS of Isolated Ion Monitoring ($ddms^2$) at 17500 FWHM. The mass accuracy of both parent and product ions was less than 2 ppm.

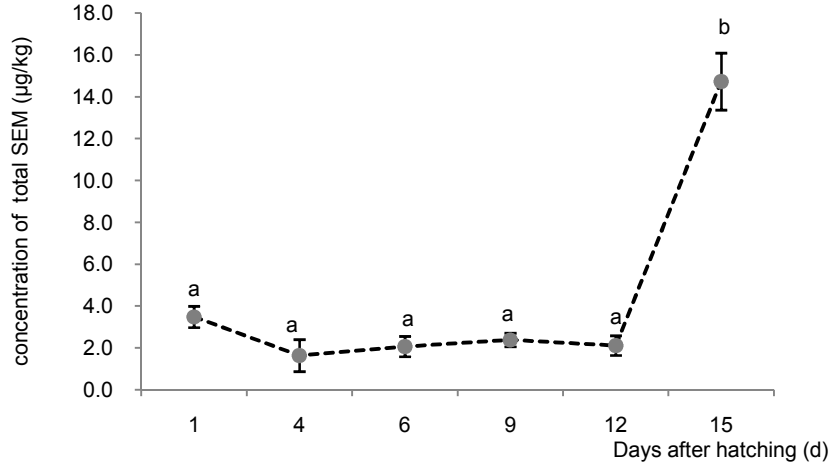
The results showed that the parent ions and the product ions of prawn's samples were both in the incredible range (table 5). The retention time and the ion mass/charge ratio were consistent with the SEM standard. The results of the re-analysis can further confirm the occurrence of SEM in wild-caught prawns.

Table 5. *The accurate-mass of parent ion and product ions of SEM in samples.*

	The mass of parent ion $[M+H]^+$	The mass of product ions	
Accurate-mass of SEM	m/z 209.06714	m/z 192.04036	m/z 166.06110
Mass incredible ranges	m/z 209.06672-209.06756	m/z 192.03998-192.04074	m/z 166.06076-166.06143
Prawns samples	m/z 209.06686-209.06721	m/z 192.04006-192.04036	m/z 166.06085-166.06123
Deviation of mass ΔM	0.24-1.82 ppm	0.18-1.51 ppm	0.00 -1.56 ppm
Matching results	positive	positive	positive

3.2. Investigation of Source of SEM in M. Nipponense

In order to explore the source of SEM in prawns, we investigated the developmental variation of SEM in the *M. Nipponense* prawns larva under non-NFZ condition. It was observed that SEM occurred in freshly hatched *M. Nipponense* larva with a level of 3.48 ± 0.51 μg/kg, and the level of SEM slightly decreased in the 4th day after hatching and maintained this level until the 12th and significantly increased to 14.6 ± 1.36 μg/kg at15thday (*Figure 1*).



Different letters indicate significant difference between days after hatching.

Fig. 1. *Total SEM concentration in the prawn larva after hatching.*

The finding of SEM in *M. Nipponese* larvae obviously had transmitted from parent prawns. It was noted that artemia salina was one of the feeds during the experiment. Although we determined a very trace level of SEM in artemia salina, the level of SEM in prawns showed no significant change before the metamorphosing into postlarvae period (12d after hatching), while the level of SEM appeared a sudden increase nearly 7 times after metamorphosing (15d after hatching).The sudden increase of SEM rather than a continuous increase was seemingly not associated with the feed of artemia salina. The larvae metamorphosed into postlarvae during the 12th to 15th day, meanwhile, SEM level in larva increased abruptly on the 15th day. The SEM suggestively has some connection with the metamorphosing development. Poucke et al, Kennedy et al and Yu et al suggested that SEM was present as a natural component in shell of the *M. Rosenbergii* prawns tested [8~9,18]. Based on the current studies and previous reports, the increase of SEM observed in our study may be the result of natural shell development and shell-related behaviors of the prawns after metamorphosing into postlarvae period.

4. Conclusions

Our study has observed the natural increase of semicarbazide during *M. Nipponense's* larva stage. It is the first time to clearly demonstrate SEM in *M. Nipponense* due to the internal source. This study can provide valuable information for further research on the natural occurrence of SEM in crustaceans. Moreover, the results of this study suggested that neither the tissue-bound SEM nor total SEM should be used as the marker of nitrofurazone any longer. An appropriate marker needs to be explored for monitoring nitrofurazone abuse. Whether the natural occurrence of SEM in crustaceans will exert a potential negative effect on human health or not, needs to be further explored.

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