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Determination of semicarbazide in marine crustaceans by ultra-performance liquid chromatography tandem mass spectrometry with an improved pre-treatment method

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ABSTRACT

An improved liquid chromatography-mass spectrometry method was developed for the determination of SEM in the marine crustaceans. First, the optimization of amount of hydrochloric acid added was made to ensure complete reaction in the SEM derivatization system. Secondly, five adsorbents (PSA, C_{18}, Florisil, Al_2O_3 and MWCNT) were compared for the purifying effect of impurities in detecting SEM, the most suitable adsorbent was PSA. This method performed additional PSA clean-up procedure gives good linear range and met the FDA guide on Analytical Procedures and Methods Validation in LOD, LOQ and mean recovery. The chromatogram of spiked samples also showed less endogenous interference and gave clearer effect. Finally, by using this improved LC-MS/MS method, 12 different marine crustaceans were analyzed. Distribution of SEM in tissues was found from testing muscle, viscera, whole body and shell of each crustacean. SEM in different tissues showed the lowest SEM content distribution in muscle and highest SEM content in shells. It can be inferred that SEM, as an endogenous substance commonly found in marine crustaceans, come from shells.

KEYWORDS

Semicarbazide; Detection; Crustacean; Adsorbent; Distributione.

INTRODUCTION

Semicarbazide (SEM), an amine small molecule, is considered as metabolite of the banned drug nitrofurazone (NFZ). While the nitrofuran antibiotics have been prohibited using in food-producing species within the European Union since 1995[1] for the carcinogenicity and teratogenicity[2]. Because the parent compound is rapidly degraded in vivo[3,4], analysis of the residue in food has centered on the detection of the tissue-bound, side-chain metabolite. SEM in animal-derived food was usually considered originating from metabolism of NFZ used in animal breeding. At present, many countries detect SEM to judge illegal use of NFZ in the animal breeding process to achieve the purpose of controlling NFZ.

Research shows many crustacean marine products can produce precursors of SEM themselves by the impact of environment, even in the case of not feeding NFZ[5]. To study the possibility of natural occurrence of SEM in wild shrimp, Robert McCracken etc.[6] collected and analysed samples from 29 sites across Bangladesh to test meat and exoskeleton combined and found SEM concentrations were approximately 100 times higher in the exoskeleton. It was a dear indication of the SEM was unrelated to sampling location which strongly suggesting natural occurrence. Between 2002 and 2003, nearly 300 notifications concerning nitrofuran residues were reported in the European Union Rapid Alert System for Food and Feed (RASFF). Many of them related to positive findings of SEM were in poultry, shrimps and other aquaculture products. Hundreds of tonnes of foodstuffs were affected and consequently destroyed[7].

Various advanced methods have been published for the determination of SEM by enzyme immunoassay, liquid chromatography electrospray ioniastion tandem mass spectrometry (LC-ESI/MS/MS) and liquid chromatography electrospray ionisation mass spectrometry (LC–MS), and matrix have been not only honey, poultry, shrimps but also egg powder, and carrageenan[4, 8-14]. Currently, there is no report on the detection for different testing tissues in crustacean marine products. Analysis methods from former articles can basically fit the testing requirements of nitrofuran metabolites in marine products, but in the detection of shells and whole bodies they can not achieve better result. Uniquely among the tissues, compositions of shrimp and crab shells were largely in chitin and $CaCO_3$. Presence of $CaCO_3$ consumed HC1 and decreased acidity of the reaction system, it would affected the separation of SEM from protein and derivative efficiencies, resulted in lower values in detecting of shrimp and crab shells by standard methods. Additionally, shells are usually have more complex components as pigments and fat, if appropriate and effective purified ways were not went, it will cause serious loss of analyte. So it is necessary to establish an analytical method for the crustacean in SEM. Dispersive solid phase extraction method is a fast, stable pre-treatment method, but it has not been reported using for the detection of SEM.

In this study, a sensitive and fast method, in which optimization of the amount of hydrochloric acid was in pre-processing stage and SPA was used as effective adsorbent for SPE coupled with UPLC-MS/MS, was developed for determination of SEM in marine crustaceans. With this method, marine crustaceans in different origins were detected, naturally occurring SEM was proved and distribution in crustaceans was found.

MATERIALS AND METHODOLOGY

Raw material

Fresh marine samples purchased were selected and delivered the very day alive to the laboratory. All of them were got from fishing rather than breeding. The detailed information was in TABLE 2. Before the experiment, the materials were washed, and frozen two hours to death. And then were spared

and crushed by the viscera, muscle, shell and whole body to get four test parts for the respective material.

Reagents

Semicarbazide (SEM-HCl, remove hydrochloric acid when calculating the mass) and its internal standard substance (SCA-HCl-^{13}C-^{15}N2) were obtained from Sigma-Aldrich (Helsinki, Finland) and 2-nitrobenzaldehyde was from Aldrich Chemicals (Milwaukee, IL, USA). Liquid chromatography-grade solvents (JT. Baker etc.) were used throughout the study. All other chemicals used were analytical grade. Compact ultra-pure water purification system (18.2 MΩ, Millipore, USA) was used for all solution preparations. Absorbent N-Propylethylenediamine (PSA), C_{18}, Florisil, neutral alumina (Al_2O_3), multiwalled carbon nanotube (MWCNT) were from Agela (Agela etc., Beijing).

Stock solution of SEM (1.0 g·L^{-1}) was prepared by dissolving 14.9mg of SEM-HCl in 10 mL methanol. The stock solution of SEM was stored in a refrigerator at -20℃ in an amber container when not in use. Both solutions were deoxygenated by bubbling nitrogen through the solution before use. Working standard solutions of SEM was prepared daily by making appropriate dilutions (10 and 100 mg·L^{-1}) of the SEM stock solution (1.0 g·L^{-1}) with the carrier solution. All solutions should be stored in dark place due to the instability of very dilute SEM under UV radiation. Stock solution of internal standard (1.0 g·L^{-1}) was made in accordance with SEM stock solution, and diluted to 100 mg·L^{-1} in water for use.

Instruments

ACQUITY ultra-high performance liquid chromatography-tandem mass spectrometry (coupled with electrospray ion source, Quattro Premier XE) (Waters company, United States); T18 homogenizer and MS2 Vortex mixer (IKA company, Germany); nitrogen drying apparatus (United States Organomatio company); centrifuge 5810 high speed centrifuge (Eppendorf company, Germany); oscillator (Changzhou Guohua electric appliance Co., Ltd, China); BPG-9140A precise blasting drying oven (shanghai Yiheng Technology Co., Ltd. China).

UPLC-MS/MS settings

Separation was conducted on an ACQUITY UPLC BEH C_{18} column (2.1 mm×50 mm, 1.7 μm, Waters, Milford, MA) and column temperature was 40 ℃; the injection volume was 10μL and flow rate was 0.2 m L·min^{-1}; eluent A acetonitrile and eluent B 0.1% formic acid aqueous solution (containing 2 mM ammonium acetate) were in a gradient condition, the gradient program totally run time 6 min and started at 90% of acetonitrile and decreased to 90% over 3 min, returned to initial conditions in 2 min, the column was then allowed to stabilise for 1 min at the initial condition before proceeding to the next injection.

Mass spectrometric detection was conducted by a Quattro Premier XE triple-quadrupole instrument (Waters Technology, Milford, MA) equipped with an electrospray positive ionization (ESI) interface. MassLynx software (version 4.1) from Waters was used for both system control and data acquisition. The mass spectrometer was operated in the ESI+ mode, and the capillary voltage was set at 3.0 kV, the extractor at 3.0V, the source block temperature at 120℃ and the desolvation temperature at 380℃. High purity nitrogen (600 L·h^{-1}) was used as both the drying and ESI nebulizing gas, while argon (50 L·h^{-1}) was used for collision-induced dissociation. Multiple reaction transitions mode (MRM) for the transitions 209>166 as quantification transition and 209>192 for confirmation, for the internal standard the transition 212>168 was used.

Sample preparation

Tissues weighed were put into 50 mL centrifuge tubes. Calibration standards were prepared by addition of standard solutions to 50 ml tubes. Internal standard (50 μL of a 100 solution) was added

equally to all samples, controls and standards. Control tissues from nitrofuran-free shrimps were fortified with standard (100 μL of a 100 ng·mL^{-1} solution) to act as recovery control samples. Blank tissues were also included in every analytical run. All tubes were added hydrochloric acid (0.2 or 0.5 M) to proper reaction acidity and 2-nitrobenzaldehyde (150 μL, 50 mM in dimethyl sulfoxide). After vortex mixing, tubes were incubated overnight (approximately 16 h) in Shaker Incubator at 37 ℃. All tubes were then adjusted to pH 7.4±0.2 with 0.1 M disodium hydrogen phosphate. Liquid-liquid extraction was carried out using ethyl acetate (2×5 mL, mixing for 1 min and centrifugation at 6000 rpm for 5 min at 4 ℃) and the organic phase evaporated to dryness at 50 ℃ under nitrogen. Residues were re-dissolved in initial mobile phase (A: B =90:10 v/v; 1ml). Absorbents were chosen for purification and then transferred to HPLC micro vials through a 0.45μm syringe filter. LC-MS /MS analysis was carried out using the conditions described above.

RESULT AND DISSCUSS

Effects of hydrochloric acid addition on SEM detection

SEM exists in the forms of being free and bound (bound to a protein) in marine organisms. Detection method was used primarily 5 steps by acid hydrolysis, derivatization, extraction, concentration and determination[15]. Among them, the acidity would affect SEM separated from protein and derivation. Uniquely among the tissues, compositions of shrimp and crab shells were consisting largely in chitin and $CaCO_3$. Especially the crab shells were containing high components of $CaCO_3$. Presence of $CaCO_3$ consumed HC1 and decreased acidity of the reaction system, it would affected the separation of SEM from protein and derivative efficiencies, resulted in lower values in detecting of shrimp and crab shells by standard methods. Meanwhile internal standard derivatives also need acidic conditions. Within the standard detection, best acidic conditions ware arriving through the most suitable volumes of HCl added.

After optimization and experiments, the best suitable acid volumes for SEM were determined respectively: shrimp shell (2. 0 g): add 7mL 0. 5mol·L^{-1} HCl solution; whole (2. 0 g): 3mL 0. 5mol·L^{-1} HCl; the crab shell (2. 0 g): 8mL 0. 5mol·L^{-1} solution and whole (2. 0 g): adding 6mL 0.5mol·L^{-1} HCI solution (Figure 1). These acidic conditions ensured that the SEM from shells and whole could completely dissociate from binding proteins and SEM-derivatization reaction went smoothly.

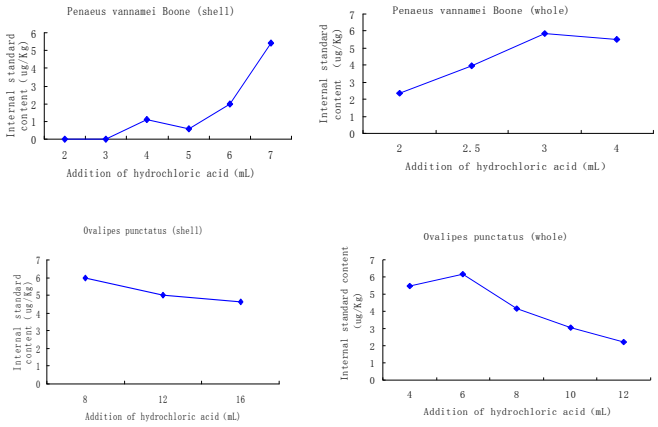

Figure 1 : The effect on detection of SCA-HCL-^{13}C-$^{15}N_2$ with the different contents hydrochloric acid

Optimation of absorbents

Shells and whole body also contain lipids, pigments and other impurities. After dissolved by final solution, the solution was cloudy which need to be purified before filter and injection. Certain

amounts of PSA, C_{18}, Florisil, Al_2O_3 and MWCNT were weighed to purify spiked level 2.5 μg / kg of SEM in shrimp (*Parapenaeopsis hardwickii*) whole body to investigate purifying effects of different adsorbents by comparing the SEM recoveries. The results were shown in Figure 2: MWCNT had huge specific surface area and structure which can effectively remove pigments and other interferences, but the adsorption of SEM was also strong; Florisil as strong polar sorbent, can not effectively remove lipid impurities; the PSA, C_{18}, Al_2O_3 on the SEM had higher recovery rates.

For better purification effects and recoveries, dosages of these three kinds of adsorbents were optimized. Weighed each 25, 50, 75, 100, and 125 mg to purify spiked level of 2.5 μg / kg SEM in shrimp whole body and observed decontamination liquid color, transparency, and calculated the signal to noise ratio. As Figure 3, despite Al_2O_3 and C_{18} had good recoveries and removed lipid impurities, the purification effect was not obviously have a good improvement, even increased the amount of adsorbents, cleaning fluid is still cloudy; PSA, as a weak anion exchanger, had not only good purification effect to remove fatty acids, sterols, organic carbohydrates, pigments and other impurities which making purified liquid colorless and transparent, but also a high recovery rate.

When PSA amount is less than 100 mg, purifying effect increased significantly with the amount of PSA but leveled off when more than 100 mg. By choosing 100 mg of PSA as an adsorbent, balance was got among reducing the sample matrix impurities (pigments, carbohydrates, lipids and other substances), better purification effect and SEM recoveries. Compared with previous method, this method performed additional PSA clean-up procedure, the chromatogram of spiked samples showed less endogenous interference and gives more clearer effect.

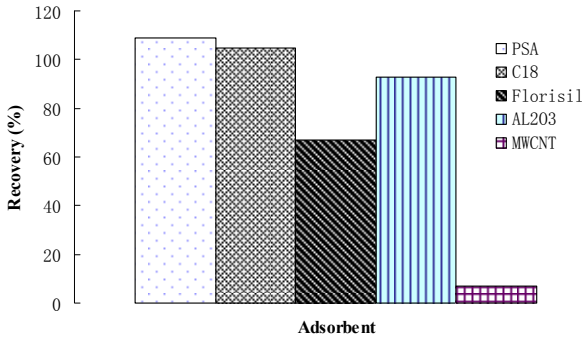

Figure 2 : Influence of different sorbents on the recoveries of SEM in 2.5 μg/kg shrimp whole body

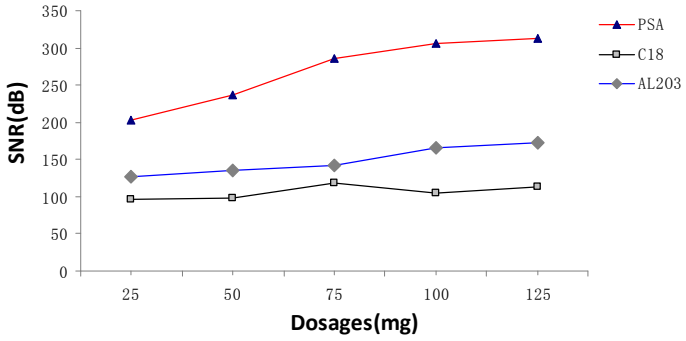

Figure 3 : Influence of different dosages on the SNR of SEM in 2.5 μg/kg shrimp whole body

Method validation

The linearity of standard curve was established by the analysis of standard solutions at six concentrations (0. 5、1.0、2.0、5.0、10.0、20.0μg/L). To each sample 5.00μg/kg was added. The

linear range of SEM was at 0.5～20.0 μg/L with linear regression equation: y=1.54138x+0.76985 and correlation coefficients 0.9975.

Limit of detection (LOD) and limit of quantitation (LOQ) are considered as the analyte minimum concentrations that can be confidently identified and quantified by the method. LOD and LOQ were calculated on the basis of signal to noise ratio S/N = 3 for LOD and S/N = 10 for LOQ) on the chromatograms of 20 shrimp samples. The method LOD was 0.1μg/kg and LOQ was 0.3μg/kg, respectively. The concentration of LOQ meet the FDA guide on Analytical Procedures and Methods Validation, which was defined as the concentration that could be determined with 80-120% accuracy and not higher than 20% precision.

Blank matrix with replicates at three concentration levels (0.5, 1.0, 5.0μg /kg), six determinations were carried out at negative shrimp matrix. Precision were assessed by performing replicate analysis of spiked samples against calibration curves. Mean recovery, intra-day and inter-day precision of the method are reported in TABLE 1. The result showed good recovery in shrimp muscle, and the recovery of SEM was not substantially affected by the concentrations. As indicated by the results, the RSD values were in 97.8%～106.5%, and the accuracy of the method did not deviate from 100 % by more than 5%.

The recovery was determined by experiments using spiked blank matrix with replicates at three concentration levels (0.5, 1.0, 5.0μg /kg). Six determinations were carried out at negative shrimp matrix. Precision were assessed by performing replicate analysis of spiked samples against calibration curves. Mean recovery, intra-day and inter-day precision of the method are reported in TABLE 1. The result showed good recovery in shrimp muscle, and the recovery of SEM was not substantially affected by the concentrations. As indicated by the results, the RSD values were in 97.8%～106.5%, and the accuracy of the method did not deviate from 100 % by more than 5%.

TABLE 1 : Average recoveries and relative standard deviation for SEM (n=6)

Compound	Added（μg/kg）	Average found（μg/kg）	Average recovery（%）	RSD (%)
SEM	0.5	0.508	103.3	2.77
	1.0	1.046	106.5	7.08
	5.0	4.893	97.8	3.57

Content and distribution of SEM in marine crustaceans

By using the improved method above, determination of SEM was applied to the 12 typical marine crustaceans. In order to understand the distribution of SEM in tissues, muscle, viscera, whole body and shell of each crustacean were tested.

TABLE 2 showed that, SEM was detected in many marine crustaceans: contents of SEM in shells ranged within 0~28.55μg/Kg; muscles were detected at 0~22.55 μg/Kg. Contents of SEM were also different in tissues: SEM levels were highest in shells, next were whole bodies, muscle may have the least amount. But in *Macrobrachium nipponense* it could as high as 17.9±0.38μg/Kg; viscera was hard to detect SEM, all samples were not detected except *Portunus trituberculatus.* SEM in different tissues showed the lowest SEM content distribution in muscle and highest SEM contents in shells. It can be inferred excessive SEM in shrimp, crabs from shells.

Samples from Zhoushan Islands were caught from East Ocean where were known as the natural water bodies. But samples taken in the region, whether in shells, in visceral tissue or muscle, still had varying degrees of SEM, and distribution of SEM in bodies was consistent with products in Nanjing and other places. This SEM present in the samples could not be caused by nitrofurazone drug, because nitrofurans would not exist in natural waters. All samples measured were from different types and their

sampling locations distributed so widely, but there was one feature, many of them had SEM detection in shells. Therefore, it can be inferred, SEM should be endogenous substances naturally present in the marine crustaceans; nitrofurazone was not the only source SEM in marine products. This conclusion was consistent with the studies[5, 6, 16].

TABLE 2 : Detection results of semicarbazide in crustacean (X±SD, n=3)

Sample	Location	results (μg/Kg)			
		Muscle	Viscera	Whole	Shell
Oratosquilla oratoria	Location 1 a)	ND b)	ND	0.713±0.16	1.655±0.018
Parapenaeopsis hardwickii	Location 1	ND	ND	ND	ND
Palaemon gravieri	Location 1	ND	ND	0.763±0.141	1.215±0.226
Solenocera melantho	Location 1	ND	ND	ND	ND
Ovalipes punctatus	Location 1	ND	ND	ND	ND
Portunus sanguinolentus	Location 1	ND	ND	0.51±0.11	2.80±0.26
Portunus trituberculatus	Location 1	ND	0.72±0.014	0.87±0.23	2.719±0.17
Charybdis feriatus	Location 1	ND	ND	ND	1.07±0.26
Penaeus vannamei Boone	Location 1	ND	ND	1.06±0.67	2.5±0.2
Macrobrachium rosenbergii	Location 2	2.12±0.07	--	14.7±3.11	23.51±2.55
Procambarus clarkii	Location 2	2.2±0.35	--	8.25±0.78	14.13±1.9
Macrobrachium nipponense	Location 3	17.9±0.38	--	22.55±3.41	28.55±2.96

a): location 1: zhoushan free market of agricultural products in zhejiang; location 2: nanjing free market of agricultural products in jiangsu; location 3: taihu lake in jiangsu; b): nd means not detected

CONCLUSIONS

In conclusion, an improved method was set up for determination of SEM in marine crustaceans in this work. Optimization for adding HCl in shells and whole bodies supported a stable condition for derivatization system of SEM; the PSA was chosen and applied as an effective SPE adsorbent for enrichment and clean-up of SEM. through this method and on the basis of previous studies, we expanded the study samples, confirmed the existence of endogenous SEM. Also we studied SEM distribution in bodies: the lowest SEM content distribution in muscle and highest SEM contents in shells. By comparing the samples from different regions, the conclusions were more reliable.

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