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Analysis of Volatile N-nitrosamines in Red Swamp Crayfish *Procambarus clarkii*

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ABSTRACT

Recent studies on N-nitrosamines in seafood focus on compounds being formed during various processing methods without consideration of the N-nitrosamines already present in the fresh meats and seafood. The purpose of this study was to determine the concentration of N-nitrosamines in various compartments of commercially-available red swamp crayfish. Crayfish samples were purchased from a wholesale dealer. Samples were washed in tap water and boiled for 7-10 min at 100°C. Crayfish were dissected into shell, head (hepatopancreas and green gland) and tail meat. N-nitrosamines were extracted using standard solid-phase extraction protocol with Extrelut and Florisil cartridges. N-nitrosamines were identified and quantified using GC-FID. Results indicated the presence of N-nitrosodimethylamine (3.37±0.51 ng g^{-1}), N-nitrosodiethylamine (4.91±1.30 ng g^{-1}), N-nitrosopyrrolidine (48.94±2.01 ng g^{-1}) and N-nitrosopiperidine (13.60±2.02 ng g^{-1}), N-nitrosodipropylamine (12.52±2.55 ng g^{-1}), N-nitrosomethylethylamine (4.83±0.53 ng g^{-1}). This study has major implications since crayfish are consumed seasonally and in large amounts there may be a potential risk of exposure to N-nitrosamines.

Key words: N-nitrosamines, crayfish, *Procambarus clarkii*

INTRODUCTION

N-nitrosamines are a class of compounds formed endogenously or during food processing (Magee *et al*., 1976). They are a part of a larger group of compounds called N-nitroso compounds. N-nitrosamine structure consists of R_1R_2-N-N-O, where R_1 and R_2 are alkyl or acyl groups (Lijinsky, 1992; Rostkowska *et al*., 1998). N-nitrosamines have been shown to be carcinogenic to one or more animal species including rats (Lijinsky *et al*., 1988), murines, hamsters, rabbits, minks, dogs, pigs and monkeys. The National Center for Toxicological Research (NCTR) and the International Agency for Research on Cancer classifies several N-nitrosamine as posing a cancer risk to humans based on the carcinogenic activity in animal models (Abnet, 2007).

Food products that have been associated with N-nitrosamine contamination have been divided into five major categories: foods preserved with addition of nitrates or nitrites, foods preserved by smoking (Yurchenko and Molder, 2006; Griesenbeck *et al*., 2009), foods dried by combustion gas, pickled or salt-preserved food and food stored or grown in high humid conditions (Griesenbeck *et al*., 2009). Preformed N-nitrosamines are particularly a concern for meats that have been cured with nitrates and/or nitrites such as sausage (Biaudet *et al*., 1994),

bacon (Miller *et al*., 1989; Lijinsky, 1999), or seafood (Montesano and Bartsch, 1976; Yamamoto *et al*., 1984; Song and Hu, 1988). In seafood, N-nitrosamines can be formed as a result of the nitrosation reaction between secondary amines (dimethylamine, diethylamine, trimethylamine and trimethylamine oxide) and nitrate or nitrite from addition of additives or other environmental sources (Song and Hu, 1988; Yurchenko and Molder, 2006). Examples of volatile N-nitrosamine more commonly occurring in cooked food include NDMA (N-nitrosodimethylamine), NDEA (N-nitrosodiethylamine), NPYR (N-nitrosopyrrolidine) and NPIP (N-nitrosopiperidine) (Lijinsky, 1999).

The freshwater crayfish species *Procambarus clarkii* are harvested and consumed seasonally in high qualities in the southeastern region of the United States (Ahmadi *et al*., 2008). The demand and consumption of foods such as crayfish is a growing trend, with approximately 25% of Americans indicating that they consume Cajun cooking. Furthermore, crayfish have been found to be rich in omega-3 and omega-6 polyunsaturated fatty acids, thus attributing the prevention of cardiovascular disease (Ndem *et al*., 2008). According the USDA Continuing Survey of Food Intakes by Individuals, the mean consumption of crayfish is 0.012 g/person/day (EPA, 1980). However, this data is based on a complete year and does not consider the seasonal aspect of crayfish consumption. Furthermore, demand for domestic product has increased due to the effects of the earthquake in Japan. Consumption styles vary with individuals. Most individuals prefer the abdominal tail meat while others consume the contents of the head, foregut and midgut.

Environmentally, freshwater crayfish species have also been used as biological indicators of clean water, monitoring levels of heavy metals (Dickson *et al*., 1979; Martinez *et al*., 1993; Lind *et al*., 1995), PCBs (Khaniki *et al*., 2005), organochlorine pesticides (Fornstrom *et al*., 1997) and other toxic substances. Crayfish are efficient indicators of pollution because of its life span, high number of offspring, contact with both water and soil and position in the food web (Moss *et al*., 2010). In general, crustaceans accumulate heavy metals when there is an increase in the bioavailability of these metals in the water. In polluted water systems, crayfish are able to bioaccumulate a large amount of these metals in their tissues (Alikhan *et al*., 1990; Anderson *et al*., 1997; Bollinger *et al*., 1997).

Likewise, N-nitrosamines have the potential to bioaccumulate in various edible and non-edible tissues of crayfish. However, they can also be formed endogenously when the crayfish are exposed to nitrogenous compounds in the water system and during processing (Porte and Escartin, 1998). Crayfish and other another organisms may be exposed to N-nitrosamine precursors occurring in both drinking (Ukhun *et al*., 2005) and waste water (Nawrocki and Andrzejewski, 2011). Possible sources of N-nitrosamine precursors in drinking water include dimethylamine (Chen and Valentine, 2006), organic nitrogen (Gerecke and Sedlak, 2003) and other amines that could undergo oxidation during water treatment (Lee *et al*., 2007). These compounds can be present in natural waters (Nawrocki and Andrzejewski, 2011).

Though, much research on nitrosamines exist, toxicity data for nitrosamines accumulated in specific tissues of seafood are outdated and therefore, cannot be used to estimate the potential risk to target populations due to changes in industrialization and pollutants in water bodies. Recent studies on nitrosamines in seafood focus on compounds being formed during various processing methods without consideration of the nitrosamines already present in the fresh fish. Such studies do not include crayfish in sampling and fail to consider any changes in N-nitrosamine content after processing. Furthermore, determining the N-nitrosamine content in specific compartments is necessary to estimate hazardous risk due to differences in consumption styles. Without qualitative and quantitative information on the specific nitrosamines formed endogenously in red swamp

crayfish, tissue accumulation and nitrosamines levels formed during processing, a risk assessment for potential health hazards cannot be determined. Therefore, the purpose of this study was to isolate, identify and quantify the accumulation and formation of N-nitrosamines in specific compartments of red swamp crayfish obtained from southeastern United States.

MATERIALS AND METHODS

Sampling: Samples of live red swamp crayfish were purchased from various commercial wholesalers in southeastern United States.

Chemicals: A standard N-nitrosamine mixture containing N-nitrosomethylethylamine (NMEA), N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosodibutylamine (NDBA), N-nitrosodipropylamine (NDPA), N-nitrosopyrrolidine (NPYR), N-nitrosopiperidine (NPIP) was purchased from Sigma-Aldrich, St Louis, MO.

Processing methods: Live crayfish were processed according to Baek and Cadwallader (1996). Live crayfish were washed in tap water and boiled for 7-10 min at 100°C. After cooling, abdominal tail meat and shell were separated to prepare for extraction.

Crayfish dissection: Fresh whole crayfish were killed by placing in ice water for five minutes. Boiled and fresh samples were dissected, removing the tail meat, hepatopancreas and shell. Some samples were kept whole for analysis.

N-nitrosamine extraction: Samples were crushed using a mortar and pestle and homogenizer. Crayfish samples were prepared using a two-step solid-phase extraction with Extrelut and Florisil as described by Yurchenko and Molder (2006) with modifications. Approximately 6.0±1.0 g of sample was mixed with 6.0 mL of 0.1 N NaOH in a 100 mL beaker. A prepacked Extrelut (EMD Chemicals, MO) column was wetted with 20 mL hexane/dichloromethane 40:60 (v/v) and the sample eluted with two 20 mL portions of hexane/dichloromethane solution. The eluate was collected in a 50 mL concentrator flask and evaporated in a water bath at 60°C. In the second step, a 1 g prepacked Florisil cartridge (Phenomenex, CA) was wetted with 6 mL of dichloromethane/methanol 95:5 (v/v) and eluted with 6 mL of dichloromethane/methanol solution. The solution was evaporated to 0.5 mL using a Buchi Switzerland Rotavapor R-215 at 60°C and atmospheric pressure. The prepared solution was transferred to an amber sample vial. Extractions were performed in duplicate.

Chromatographic analysis: GC analysis was carried out using Hewlett Packard 5890 gas chromatograph equipped with flame ionization. (GC-FID) (Agilent Tehcnologies, Palo Atlo, CA). One half microliter of the extracted solution sample was injected into a Rtx-1 Crossbond® 100% dimethylsiloxane column (30 m×0.25 mm I.D.×0.1 film thickness (df)). For the gas chromatograph separation of N-nitrosamines, the oven program was started at 65°C (held for 1 min), set at 5°C min^{-1} from 60-100°C and held isothermally at 100°C for 2 min. Temperature went from 100-250°C at a rate of 15°C min^{-1} and held isothermally at 250°C for 2 min. The velocity of the helium carrier was 1 mL min^{-1}.

Statistical analysis: Data was analyzed in a 2×4 factorial design, with factor A being processing methods (boiled, raw) and factor B being body compartments (whole, tail meat, shell and head). Statistical analysis was conducted using SAS 9.1, (SAS, 2004) using analysis of the variance (ANOVA) to determine if interactions between factors A and B effect N-nitrosamine concentrations.

RESULTS

The mean concentration of N-nitrosamines in boiled crayfish is given in Table 1. The N-nitrosamine concentrations are given as whole body and various compartments including tail, hepatopancreas and shell. N-nitrosamines were not detected in any raw crayfish samples (Table 1). Total N-nitrosamine formation was highest in the hepatopancreas (147.90 ng g^{-1}). Concentrations in decreasing order were hepatopancreas>tail>shell>whole body. NDMA, NDEA and NPYR displayed similar trends in specific compartments. NPIP was not detected in the whole body and shell. Concentration of NPIP was also greatest in the hepatopancreas. NDPA was not detected in the hepatopancreas and the shell. The non-volatile N-nitrosamine NMEA was not detected in the tail meat. Furthermore, concentrations of NMEA in specific compartments were hepatopancreas>shell> whole body>tail.

Means comparisons (Tukey's studentized range test) between compartments for each N-nitrosamines are also shown in Table 1 (columns). The level of NDMA was highest in the hepatopancreas (4.68 ng g^{-1}) and lowest in the whole body (0.39 ng g^{-1}). The concentration of NDMA in the hepatopancreas was significantly ($p \leq 0.05$) higher compared to the whole body (0.39 ng g^{-1}), however, there were no significant differences ($p \leq 0.05$) among tail, head and shell (2.21-4.68 ng g^{-1}). NDEA was highest in the hepatopancreas (7.62 ng g^{-1}) though, there were no significant differences among the other compartments (0.62-7.62 ng g^{-1}). NPYR was highest in the hepatopancreas (112.78 ng g^{-1}) and significantly ($p \leq 0.05$) higher compared with the other three compartments (19.08-72.76). Tail concentrations (72.76 ng g^{-1}) were significantly ($p \leq 0.05$) higher than shell and whole body (19.08-33.57 ng g^{-1}) but lower than head (112.78 ng g^{-1}).

Table 1: N-nitrosamine concentration found in various sections of the fresh water crayfish

Body compartment	No. of samples	Concentrations of nitrosamines (ng g^{-1}) NDMA	NDEA	NPYR	NPIP	NDPA	NDBA	NMEA	SUM
Raw									
Whole	30	nd	nd	nd	nd	nd	nd	nd	nd
Tail	30	nd	nd	nd	nd	nd	nd	nd	nd
Hepatopancreas	30	nd	nd	nd	nd	nd	nd	nd	nd
Shell	30	nd	nd	nd	nd	nd	nd	nd	nd
Boiled									
Whole	28	0.39 ± 0.05^{bk}	0.62 ± 0.26^{ajk}	19.084 ± 1.70^{ci}	nd	9.78 ± 2.11^{aj}	nd	2.16 ± 0.32^{bk}	23.04
Tail	29	2.21 ± 1.06^{ab}	2.66 ± 0.07^{a}	72.76 ± 11.68^{bi}	7.36 ± 0.20^{a}	19.38 ± 6.60^{a}	nd	nd	101.14
Hepatopancreas	30	4.68 ± 0.73^{aj}	7.62 ± 2.21^{aj}	112.78 ± 16.61^{ai}	14.85 ± 2.22^{aj}	nd	nd	7.97 ± 1.13^{aj}	147.90
Shell	26	2.21 ± 1.03^{abj}	3.54 ± 1.80^{aj}	33.57 ± 4.75^{ci}	nd	nd	nd	4.40 ± 0.80^{bj}	44.13

Values are expressed as Means±SEM, ^{abcd}Means in the same column with same letter are not significantly different by Tukey's studentized range test ($p \leq 0.05$), ^{jkil}Means in the same row with same letter are not significantly different by Tukey's studentized range test ($p \leq 0.05$), NDMA: N-nitrosodimethylamine, NDEA: N-nitrosodiethylamine, NPYR: N-nitrosopyrrolidine, NPIP: N-nitrosopiperidine, NDPA: N-nitrosodipropylamine, NDBA: N-nitrosodibutylamine, NMEA: N-nitrosomethylethylamine, nd: Not detected, Detection limits (ng g^{-1}); NDMA: 0.25, NDEA: 0.78, NPIP: 1.56, NPYR: 1.56, NDPA: 1.56, NDBA: 3.12 and NMEA: 0.78

There were no significant ($p \leq 0.05$) differences among levels in shell (33.57 ng g^{-1}) and whole body (19.08 ng g^{-1}). NPIP was not detected in shell and whole body. The concentration of NPIP was highest in hepatopancreas (14.85 ng g^{-1}) but not significantly different from the tail (7.36 ng g^{-1}). NDPA was only detected in the tail and whole body, present at highest levels in the tail. There were no significant differences in NDPA in the two compartments. Tail meat did not contain NMEA. NMEA concentration was highest in hepatopancreas (7.97 ng g^{-1}). Level of NMEA in the hepatopancreas was significantly ($p \leq 0.05$) higher compared to the shell and whole body. In contrast, there were no significant differences between shell (4.40 ng g^{-1}) and whole body (2.16 ng g^{-1}).

The concentration of NDMA in the hepatopancreas was 12 fold higher than the whole body. In addition, whole body levels were approximately 6 fold lower than levels in the tail and shell. NDEA concentration in the head, tail and shell was approximately 12, 4 and 6 fold than the levels in the whole body, respectively. NPYR concentrations in the head, tail and shell were 6, 4 and 2 fold higher than the levels in the whole body. NDPA was nearly 10 fold higher in the tail than in the whole body. The levels of NMEA in the head and shell were approximately 3 and 4 fold higher than levels in the whole body.

Mean concentration of N-nitrosamines in various compartments (Table 2) in decreasing order were as follows: tail>hepatopancreas>shell>whole body. Concentrations of N-nitrosamines were significantly ($p \leq 0.05$) lower in whole body samples (9.98 ng g^{-1}) compared to the concentrations in the hepatopancreas (25.93 ng g^{-1}) and tail (42.50 ng g^{-1}).

The highest mean concentration of N-nitrosamines was in the hepatopancreas (42.50 ng g^{-1}) and the lowest in the whole body (9.98 ng g^{-1}). The concentration in the shell (17.23 ng g^{-1}) was higher compared to the whole body (9.98 ng g^{-1}), however, statistically ($p \leq 0.05$) it was not significant. The mean concentration in hepatopancreas (25.93 ng g^{-1}) was significantly ($p \leq 0.05$)lower compared with the tail meat (42.50 ng g^{-1}). In contrast, there were no significant differences in mean concentration in the hepatopancreas and the shell, despite the numerical difference of 8.6 ng g^{-1}. The concentration detected in the tail meat (42.50 ng g^{-1}) was more than

Table 2: Compartment effect on N-nitrosamine concentration

Compartment	Concentration (ng g^{-1})
Whole Body	9.98±1.28^{c}
Hepatopancreas	25.93±5.53^{b}
Tail meat	42.50±8.67^{a}
Shell	17.23±2.87^{bc}

Values are expressed as Means±SEM, ^{abc}Means with the same letter are not significantly different by Tukey's studentized range test ($p \leq 0.05$), n = 113

Table 3: Total mean concentrations of various N-nitrosamines found in crayfish samples

N-Nitrosamine	Concentration (ng g^{-1})
NDMA	3.37±0.51^{b}
NDEA	4.91±1.30^{b}
NPYR	48.94±2.01^{a}
NPIP	13.60±2.02^{b}
NDPA	12.52±2.55^{b}
NMEA	4.83±0.53^{b}

Values are expressed as Means±SEM, ^{abc}Means with the same letter are not significantly different by Tukey's studentized range test ($p \leq 0.05$), n = 113

twice the amount in the shell (17.23 ng g^{-1}). The concentration of N-nitrosamines in the hepatopancreas, tail meat and shell were approximately 3, 4 and 2 folds higher than the whole body, respectively.

The concentration of specific N-nitrosamines is shown in Table 3. N-nitrosopyrrolidine (NPYR) was detected in samples at a significantly ($p \leq 0.05$) higher concentration compared to the other six compounds assayed (3.37-13.60 ng g^{-1}). Though not significantly different, other N-nitrosamine concentrations varied from a low of 3.37±0.51 (NDMA) to a high of 13.60±2.02 (NPIP). N-nitrosodibutylamine was not detected in any samples analyzed.

Table 1 shows the trend analysis of seven N-nitrosamines in the various compartments of the crayfish. N-nitrosamine concentrations varied with compartment. However, NPYR was highest in all compartments. N-nitrosodibutylamine (NDBA) was not detected in any samples.

NPYR ranged from a low of 19.08 ng g^{-1} in whole body to a high of 112.78 ng g^{-1} in the hepatopancreas. NDEA was lowest in whole body (0.62 ng g^{-1}) and highest in the hepatopancreas (7.62 ng g^{-1}). NDMA ranged from a low of 0.39 ng g^{-1} in the whole body to a high of 4.68 ng g^{-1} in the hepatopancreas. NDPA was highest in the tail (19.38 ng g^{-1}) but not detected in the hepatopancreas and shell. NMEA ranged from a low of 2.16 ng g^{-1} in whole body to a high of 7.97 ng g^{-1} in hepatopancreas. NPIP was highest in the hepatopancreas (14.85 ng g^{-1}) and lowest in tail (7.36 ng g^{-1}). Total N-nitrosamine concentrations were highest in hepatopancreas (147.90 ng g^{-1}) and lowest in whole body (23.04 ng g^{-1}).

DISCUSSION

Our study quantified the amounts of various N-nitrosamines accumulated and formed in specific tissues of the fresh water red swamp crayfish. Specific tissues included the whole body, hepatopancreas, tail meat and shell. N-nitrosamines were not detected in raw samples of crayfish which implies the compounds were formed during processing. Formation of N-nitrosamines during heat processing of proteinaceous food has been well documented (Lijinsky, 1992; Biaudet *et al.*, 1994; Lijinsky, 1999; Abnet, 2007).

Results indicated highest mean concentrations of N-nitrosamines occurring in tail meat. (42.50 ng g^{-1}). This is a cause for concern since the tail is most commonly consumed as food. The sum of seven N-nitrosamines in the tail meat was much higher than concentration reported by many other researchers including sums ranging from 0.45-16.63 in kabobs (Ozel *et al.*, 2010), 0.65-6.69 in fish (Yurchenko and Molder, 2006). However, NDMA was detected in seafood at high levels up to 131.5 ng g^{-1} in a study analyzing a total of 695 Chinese foods (Song and Hu, 1988). No studies report N-nitrosamines in red swamp crayfish or any other crustacean. The mean concentration of N-nitrosamines was highest in tail meat, whereas the sum of the seven N-nitrosamines was highest in the hepatopancreas. This difference can be attributed to the difference in the total number (or percentages) of samples in which N-nitrosamines were detected. Overall, N-nitrosamines were detected in hepatopancreas samples than tail meat samples.

NPYR was detected at the highest concentration in all compartments, ranging from 19.08-112.78 ng g^{-1}. Likewise, mean concentrations were highest for NPYR (48.94 ng g^{-1}). Previous studies reported NPYR as occurring at highest levels in meat and fish when compared with other NAs. NDBA was not detected in any samples. Limited detection and low concentrations of NDBA have been documented in many studies (Yamamoto *et al.*, 1984; Yurchenko and Molder, 2006; Ozel *et al.*, 2010). Huang *et al.* (1981) indicated NDBA only being present in fried samples. This is a likely explanation for the lack of detection since the crayfish samples were boiled in our study.

Sum of seven NAs were highest in the hepatopancreas (147.9 ng g^{-1}). High levels in this compartment imply that protein structure contains higher levels of secondary and tertiary amines, including dimethylamine and trimethylamine which are commonly found in the seafood. NAs form at a higher rate from secondary and tertiary amines. Rate of formation also depends on the alkalinity of the secondary amines which decreases with aromaticity (Rostkowska *et al*., 1998). This could be a further explanation of the concentrations of NPYR, since its precursor, pyrrolidine, is a secondary aromatic amine (Hall, 1957).

Previous toxicokinetic studies have documented increased levels of trace metal accumulation in the hepatopancreas of the crayfish (Dickson *et al*., 1979; Martinez *et al*., 1993; Lind *et al*., 1995; Alcorlo *et al*., 2006) and in the liver of other species of fish (Mazloomi *et al*., 2008). Lind *et al*. (1995) reported an increase in cadmium load in the hepatopancreas, attributing the increase in accumulation to an increase in metallothionein, a cysteine containing protein which binds metals. Comparably, protein binding could possibly have a role in formation of NAs in this compartment. In addition, organophosphate compounds, such as terbufos and fenitrothion and their metabolites have been found in the hepatopancreas at high concentrations (Escartin and Porte, 1996; Fornstrom *et al*., 1997). Researchers attributed concentrations to the hepatopancreas being a key organ in the metabolism of toxicants due to the presence of a monooxygenase biotransformation system (Porte and Escartin, 1998).

The presence of NAs was detected in the processed shells of crayfish samples but not in the raw samples. Occurrence could be a result of denaturation, deacetylation and fractionation of the chitin and chitosan present in the exoskeleton (Goosen, 1996; Darryl *et al*., 2006). Chitosan is synthesized from chitin by heating in concentrated sodium hydroxide for a period of time and is commonly used as a biopesticide (Goosen, 1996). Other products of the reaction include glucosaminyl and amino acid residues (Goosen, 1996). Chitin may also be degraded through the action of chitinase-producing fungi *Trichoderma virens UKM1* (Abd-Aziz *et al*., 2008) and *Aeromonas* sp. (Jamialahmadi *et al*., 2011) producing monomeric N-acetyl-glucosamines. Darryl *et al*. (2006) indicated N-nitrosamine formation when glycol amines are fractionated with nitrous acid or by the addition of nitrates or nitrites. Whole body concentrations of NAs are lowest as compared to all other samples. This is due to a dilution effect during extraction, since N-nitrosamines are not distributed evenly through the organism.

In conclusion, our study indicated the presence of six volatile N-nitrosamines and one non-volatile N-nitrosamines, in both edible and non-edible portions of the freshwater red swamp crayfish *Procambarus clarkii*. The seasonality of consumption of crayfish and possible toxicological implications warrants further studies.

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