1990

Studies on Important Volatile Flavor Components in Louisiana Rangia Clam (Rangia Cuneata).

Uraiwan Tanchotikul
Louisiana State University and Agricultural & Mechanical College

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Studies on important volatile flavor components in Louisiana rangia clam (*Rangia cuneata*)

Tanchotikul, Uraiwan, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1990
STUDIES ON IMPORTANT VOLATILE FLAVOR COMPONENTS IN LOUISIANA RANGIA CLAM (RANGIA CUNEATA)

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Food Science

by

Uraiwan Tanchotikul
B.S., Prince of Songkhla University, 1983
M.S., Louisiana State University, 1986
May 1990
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LIST OF ABBREVIATIONS

a.m.u. atomic mass unit
approx. approximately
cm centimeter
°C degree Celsius
CLSA closed loop stripping analysis
DHS dynamic headspace sampling
DMS dimethyl sulfide
E entgegen; trans in geometric configuration
eV electron volt
FD flavor dilution factor
Fig. figure(s)
g gram(s)
H hydrogen
HRGC high resolution gas chromatography
HRGC/MS combined HRGC and MS
GC gas chromatograph or gas chromatography
GC/MS combined GC and MS
hr hour(s)
i.d. inside diameter
I.S.(IS) internal standard
Km kilometer(s)
L liter(s)
m meter(s)
MIB 2-methylisoborneol
<table>
<thead>
<tr>
<th>Symbol</th>
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<tr>
<td>min</td>
<td>minute(s)</td>
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<tr>
<td>mL</td>
<td>milliliter(s)</td>
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<tr>
<td>MLSA</td>
<td>multiple level standard addition</td>
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<tr>
<td>MS</td>
<td>mass spectrometry or mass spectrometer</td>
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<tr>
<td>MSD</td>
<td>mass selective detector</td>
</tr>
<tr>
<td>m/z</td>
<td>mass/charge ratio</td>
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<tr>
<td>N</td>
<td>nitrogen</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram(s)</td>
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<tr>
<td>PID</td>
<td>photo-ionization detector</td>
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<tr>
<td>ppb</td>
<td>parts per billion</td>
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<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>psi</td>
<td>pound per square inch</td>
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<tr>
<td>RI</td>
<td>retention index or retention indices</td>
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<tr>
<td>S</td>
<td>sulfur</td>
</tr>
<tr>
<td>SDE</td>
<td>simultaneous distillation and extraction</td>
</tr>
<tr>
<td>sec</td>
<td>second(s)</td>
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<tr>
<td>SIM</td>
<td>selective ion monitoring in mass spectrometry</td>
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<tr>
<td>TIC</td>
<td>total ion chromatogram</td>
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<td>TOC</td>
<td>threshold odor concentration</td>
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<tr>
<td>μL</td>
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<td>μg</td>
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<td>μm</td>
<td>micrometer(s)</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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<tr>
<td>V</td>
<td>volt(s)</td>
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<td>Z</td>
<td>Zusamen; cis in geometric configuration</td>
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ABSTRACT

Important flavor components in Louisiana brackish water clam (*Rangia cuneata*) were investigated in this dissertation research. Major achievements include: (1) positive identification of the major compound responsible for the objectionable musty/earthy off-flavor in the clam, (2) development of quantitative method to compare the levels of the off-flavor compound in clam samples before and after relaying, and (3) identification of desirable characteristic clam volatile flavor components.

The identification of the musty/earthy off-flavor of rangia clam was accomplished by using dynamic headspace sampling (DHS) coupled with high resolution gas chromatography/mass spectrometry (HRGC/MS) in selective ion monitoring (SIM) mode and HRGC aroma perception. Geosmin (trans-1,10-dimethyl-trans-9-decalol) was positively identified as a major compound responsible for this off-flavor based on gas chromatographic retention indices, electron ionization mass spectra, and odor characteristics with the authentic geosmin standard as the reference.

The quantification of geosmin was achieved by vacuum distillation followed by solvent extraction using an internal standard and multiple-level standard addition method in HRGC/SIM mass spectrometry. The concentrations
of geosmin in dredged and 2-week relayed rangia clam samples in different seasons of the year were determined and compared.

The volatile flavor components of rangia clam were extracted by dynamic headspace sampling (DHS) and simultaneous distillation and extraction (SDE). Twenty-two heterocyclic compounds were identified. The unique clam aromas were contributed by a combination of the heterocyclic S- and N-containing compounds and ketones. Volatile flavor compounds in other chemical classes such as aldehydes, ketones, alcohols, and terpenes were also detected. Most of these volatile flavor components were identified in the rangia clam for the first time.

These studies were the first series of comprehensive analytical flavor investigations on rangia clam. The information generated from this research may facilitate further research and development efforts leading to the ultimate commercialization of this abundant seafood source in the Gulf Coast. This information may also enhance current and future flavor research on other seafoods.
CHAPTER I

INTRODUCTION

Rangia clams (*Rangia cuneata*), approximately 50 billion in number (LaSalle and de la Cruz, 1985), can be found in the brackish waters along the Louisiana coast, especially in the periphery of Lake Pontchartrian and Lake Maurepas (Tarver, 1972). Their abundance provides a high potential for commercialization which can bring a tremendous economic opportunity to the Louisiana seafood industry. Louisiana rangia clams have been shown to meet the federal guidelines for human food safety (Andrews, 1988). However, a major problem associated with these clams must be solved before they can be marketed, that is, they often have a strong musty/earthy off-flavor which is objectionable to most American people. This off-flavor has been observed as more pronounced in steamed or boiled clams.

Musty/earthy off-flavor problems have been recognized in drinking water (Krasner et al., 1981), beans (Buttery et al., 1976), and many species of fish (Lovell and Sackey, 1973; Tabachek and Yurkowski, 1976). Several compounds contribute to musty/earthy off-flavor, such as 2-methylisoborneol (MIB), geosmin (trans-1,10-dimethyl-9-decalol), 2-methoxy-3-isopropylpyrazine, 3-methoxy-2-
isobutylpyrazine, 6-methoxy-2-isobutyl-pyrazine, 2-methylenebornane, and 2-methyl-2-bornene (Martin et al., 1987). However, the chemical nature of the musty/earthy off-flavor in Louisiana rangia clams has not been established. Before any effective off-flavor reduction systems can be implemented, it is necessary to know the chemical nature of the compound(s) responsible for the undesirable flavors.

Several off-flavor removal or reduction schemes have been proposed to include depuration and feeding the clam with non-geosmin algae (Rusch et al., 1988). Regardless of the types of the proposed systems, the efficiency of these approaches must be assessed by objective quantification of the off-flavor imparting compound(s) remaining in the sample after the treatment. Precise and accurate methods are needed for quantification.

Although rangia clams often contain musty/earthy off-flavor(s), they also have desirable cooked meaty/nutty clam flavors when steamed. Little data are available concerning volatile flavor compounds in clam meat. Information on the characteristic compounds contributing to the unique flavor of a particular food sample is highly important. It can be used as a significant parameter to monitor the flavor quality of a food product throughout its culturing, harvesting, formulation, processing, packaging, and storage.
The objectives of this study were: (1) to identify the compound(s) causing the musty/earthy off-flavor in Louisiana rangia clams (Chapter III), (2) to develop a quantitative method to determine the concentration of the compound(s) responsible for the off-flavor in different clam samples (Chapter IV), and (3) to identify the compounds contributing to the characteristic cooked clam meat flavor (Chapter V).
CHAPTER II

REVIEW OF LITERATURE

Food flavor

Flavor is an integrated response primarily of the aroma and taste sensations. It is considered as one of the most important palatability characteristics and asset in the successful marketing and consumption of food products (Charalambous, 1981). Without proper flavor quality, foods may not be consumed, although they may be nutritious, look good in appearance, and have a desirable texture (Reineccius, 1979). Aroma is perceived when a food releases its volatile compounds which travel to odor receptor sites on the olfactory epithelium in the nasal cavity, and the resulting interaction triggers a specific response in the brain (Cronin, 1982). Aroma components are generally considered among the most critical parameters for flavor quality of food (Jennings, 1981). A key step to understanding food flavor quality is to establish the chemical nature of those volatile components which act, either independently or in combination, to produce a characteristic aroma responses in food systems.
Analytical approach in food aroma extraction

Generally, the volatile aroma compounds in foods consist of various levels of compounds of different polarities, solubilities, and volatilities. In addition, the sample matrix may vary greatly in the contents of protein, lipids, and carbohydrates. Therefore, sample preparation, isolation, and concentration, can be critically affected by interactions of the sample matrix and the chemical properties of the volatile compounds in the sample. Two general approaches for sample preparation of food volatile aroma are headspace sampling and total volatile sampling.

Headspace sampling techniques are commonly used in flavor extraction because they are simple, produce less artifacts, and the aroma components sampled may be similar in the relative proportions to what one actually smells (Cronin, 1982). It is widely used in fresh or heat-sensitive foods because heating is generally not required and thermal degradation of the sample is thus minimized. Headspace sampling techniques include static and dynamic approaches. In static headspace sampling, the volatile flavors are withdrawn from the headspace in a closed vessel where the sample under study comes into equilibrium with its vapor at a pre-set temperature. However, this method usually fails to detect the trace components or the compounds that have low vapor pressures because of insufficient quantities in the extract. In dynamic
headspace sampling (DHS), the volatile flavors in the headspace of the food material in a closed system are purged with an inert gas to sweep the volatile compounds onto a porous polymer trap. The absorbed volatile compounds are either thermally desorbed or extracted with a suitable solvent prior to analysis. The sensitivity in headspace analysis can often be increased by elevating the sampling temperature to increase the vapor pressure of the analytes (Wyllie et al., 1978). However, raising the sampling temperature of a food sample also increases the amount of moisture trapped. Moisture in the trap must be removed before instrumental analysis to avoid poor chromatographic performance or shut down of the vacuum system in a mass spectrometer. Williams et al. (1988) suggested an off-line dry-purge procedure with helium gas purging through the trap and reduction of the trap's weight to its original weight, to remove the moisture from the trap. This off-line dry-purge procedure has been used successfully in DHS to analyze volatile flavors in many foods (Vejaphan et al., 1988; Tanchotikul and Hsieh, 1989; Matiella and Hsieh, 1990).

Volatile compounds in fish have been determined by using a dynamic headspace technique with solvent desorption of an activated carbon sorbent (Reinert et al., 1983) and a Tenax GC sorbent (Olafsdottir et al., 1985). Boiled crayfish flavor also has been investigated by DHS
(Vejaphan et al., 1988). In the latter study, different sample purging temperatures, 30, 50, and 70°C, were compared. More flavor volatiles were identified in the sample purged at 70°C than in that at lower temperature, however, longer off-line dry-purge has been found necessary (Vejaphan et al., 1988). Aroma compounds in crayfish waste (Tanchotikul and Hsieh, 1989) and crabmeat (Matiella and Hsieh, 1990) also have been analyzed by a combination of DHS and MS.

On the other hand, preparation of total volatile extracts is more laborious, time-consuming, and requires more careful handling to prevent artifacts. However, this approach generally gives a sufficient quantity of sample flavor extract for more comprehensive chemical and sensory analysis. The disadvantage of the distillation method is that the extraction and concentration of the extract before GC analysis may introduce solvent contaminants into the system. Use of high purity solvents is necessary to avoid such artifacts. Kawai and Ishida (1989) have studied the volatile components of dried squid (Loligo edulis) by using a distillation method at 120-180°C followed by dichloromethane extraction to prepare an aroma concentrate.

Distillation techniques such as simultaneous distillation and extraction (SDE) and vacuum distillation are frequently used to isolate food flavors. SDE
techniques generally combine steam distillation of the food volatiles with simultaneous extraction of the volatiles into a small quantity of a low-boiling water-immiscible organic solvents such as diethyl ether or pentane (Cronin, 1982). The SDE apparatus, designed by Likens and Nickerson (1964), is also called Likens Nickerson distillation extraction apparatus. SDE has been used to study seafood flavors such as cooked shrimp (Choi et al., 1983).

In vacuum distillation, vacuum is applied during the process so that the distillation temperature can be decreased to minimize thermal degradation of the sample (Kolor, 1979). Yurkowski and Tabachek (1974) and Lelana (1987) studied off-flavors in trout and channel catfish, respectively, by using vacuum distillation to extract the target compound before the GC analysis.

The flavor extract is usually very complex, thus gas chromatography is needed for separation prior to identification of the individual compound. Mass spectrometry is often the method of choice for structural identification of the volatile flavor constituents in food (Merritt and Robertson, 1982). Gas chromatography combined with mass spectrometry has been used to identify organic sulfur compounds in short-necked clams and eels (Ogata and Miyake, 1980). To obtain a better sensitivity for low concentration compounds, selective ion monitoring
mass spectrometry is often recommended (Lai, 1988).

Studies on the compounds causing musty/earthy off-flavors in seafoods

Occurrence of a musty/earthy odor is a world-wide problem affecting the utilization of aquatic resources (Persson, 1982). Musty/earthy off-flavors have affected rangia clam, shrimp (Lovell and Broce, 1985), and many species of fish such as channel catfish (Martin et al., 1987), rainbow trout (Yurkowski and Tabachek, 1974), lake herring (Yurkowski and Tabachek, 1980), and carp (Aschner et al., 1967). The off-flavors in catfish have been associated with the presence of MIB, 2-methylenebornane, 2-methyl-2-bornene (Martin et al., 1987), and geosmin (Lovell et al., 1986). This problem may be due to fish ingesting tainted feeds or food substances, or transfer of the off-flavor compounds to the fish by absorption (Lovell and Sackey, 1973). The source of these off-flavors has been attributed, in many instances, to the growth of some actinomycetes, especially *Streptomycyes* (Hansen, 1964; Dickson, 1968) and species of blue green algae in water (Lewis, 1966; Aschner et al., 1967). The problem also has been found to be related to the water temperatures, fish-stocking densities, and heavy-feeding regimes consistent with the establishment of an eutrophic environment (Brown and Boyd, 1982; Persson, 1982). The positive
identification of geosmin in catfish samples has been confirmed by comparing the mass spectra with that of a geosmin standard by ion-trap detector mass spectrometry (Lelana, 1987). A protein-geosmin conjugated has been prepared for development of antibodies against geosmin for geosmin quantification (Chung and Vercellotti, 1990). MIB from catfish flesh has been analyzed by microwave distillation (Martin et al., 1987) and an enzyme-linked immunosorbent assay (Chung et al., 1990). In order to reduce or prevent off-flavor problems in seafood species, it is necessary to know what compounds cause the off-flavor as well as the quantitative relationships with handling, processing, or environmental factors so that the off-flavors can be reduced effectively.

There are three general ways to reduce or eliminate the aforementioned off-flavor. The first is to control the culturing environment carefully. The second is control or removal of the source of the off-flavor; and thirdly, the off-flavor may be removed by either secondary processing or by purging the live fish already contaminated with the off-flavors (Iredale and York, 1976). The most widely recognized method is that of purging the off-flavors from the fish by transferring the live fish to an approved water environment for a sufficient period of time (Yurkowski and Tabachek, 1974). This method is called relaying. The other method, depuration, refers to a
procedure in which the fish or shellfish is purified in quality-controlled water (Becker, 1977). The efficiency of the system may be assessed by sensory panels and/or instrumental analysis. The latter is objective and more reproducible.

Quantitative analyses of target volatile flavor compounds

In general, volatile flavor components in food samples can be quantified by four methods: (1) absolute quantification without an internal standard, (2) quantification with internal standard, (3) standard addition method, and (4) quantification by isotope dilution (Lai, 1988). The choice of a particular quantification method is influenced by the type and sensitivity of instruments, interferences from sample matrix, and the number of samples to be analyzed (Willard et al., 1981). Absolute quantification without an internal standard is also called external standard method. In this method, the calibration curve of the target compound is first established by plotting the magnitude of the detector response of this compound versus its concentration in an external standard solution. Then, an unknown sample is analyzed and the concentration of the target compound is determined by use of the standard calibration curve. This method is most unreliable when compared with other methods. In the internal standard
method, the standard calibration curve is also established. However, it is the detector response ratio of analyte to internal standard versus the amount ratio of the analyte standard to the internal standard that is plotted instead of the absolute values. A known amount of internal standard must be added to the sample at the start of the analysis so that the ratio of the analyte to the internal standard is established. The purpose of using internal standards is to compensate for all procedural variations. The internal standard chosen should be similar to the analyte and minimally affected by other components of the sample. In addition, the signal of the internal standard should be readily measured and should not interfere with the response of the analyte(s).

Most of the external standard (only) method and internal standard (only) method may not be effective when a strong interference exists in the sample matrix, or the trace quantity of the analyte present is below detection limit. In this situation, standard addition methods may be applicable. In order to use the calibration curves, the instrument response must be a linear function of analyte concentration (Willard, 1981). Each sample is usually analyzed without and with the addition of analyte standards. The first analysis is on the sample only. In the second and further analyses, known amounts of the target compounds are added to the sample and the analysis
is repeated under the same conditions. Since the calibration is performed on the sample matrix, this method takes the matrix effect into consideration (Lai, 1988). Marsili et al. (1989) used a combination of an internal standard and a standard addition method for quantitative analysis of flavor components in orange juice. This method is reliable and is generally the method of greater precision when an isotope dilution method is not available.

In the isotope dilution method, a stable-isotope labeled analog of each target compound is used as an internal standard for quantification. Stable isotope analogues of target compounds have been considered as almost ideal analytical standards (Garland, et al., 1980). A stable isotope dilution mass spectrometry assay has been used to quantitate branched-chain amino acids in plasma (Haymond et al., 1980), organic compounds in turkey (Garland et al., 1980) and aroma compounds in bread crusts (Schieberle and Grosch, 1987).

Characteristic volatile flavors of seafoods

Mendelsohn and Brooke (1968) studied changes in the "head gas" components in soft-shell clam (Mya arenaria) meat as affected by radiation, processing, and storage. Dimethyl sulfide (DMS) was found to be the most major "head gas" component and the source of typical clam odor
as suggested by the odor of the effluent stream from a GC column. Therefore, dimethyl sulfide was used to monitor the effects of the treatments. Hydrogen sulfide, methyl mercaptan, dimethyl sulfide, and diethyl sulfide were identified in the clam meat (Mendelsohn and Brooke, 1968). Brooke et al. (1968) have studied the significance of dimethyl sulfide to the odor of soft-shell clam (Mya arenaria) and suggested that the concentrations of DMS found in clams were within the concentration range of pure DMS that was reported as a typical "clam-like" odor by the sensory panel. DMS can be formed from the degradation of methionine to methional and further to methyl mercaptan and dimethyl sulfide (Hughes, 1964). The effects of processing on juice flavor volatiles of ocean quahog clam (Arctica islandica) and surf clam (Spisula solidissima) have been studied by Flick et al. (1982) and Burnette et al. (1983). Only the gas chromatogram profiles, without the identification of individual volatile components, have been reported. Presence and/or absence of the peak has been used to determine the differences in flavor quality of different clam juice samples (Burnette et al., 1983).

The characteristic clam volatile flavors have not been studied in detail compared with flavors from other seafoods such as shrimp and krill. Kubota et al. (1989) have studied the volatiles in cooked small shrimp species by using SDE. Pyrazines and cyclic polysulfides such as
trithiolanes and dithiazines were the main components in boiled shrimp species. Various cyclic polysulfides and methylketone also have been considered important to impart an characteristic cooked shrimp flavor (Kubota et al., 1989). Free amino acids as precursors have been proposed for the formation of the cooked volatile flavors in small shrimp species. Methylketones, (5Z,8Z,11Z)- and (5E,8Z,11Z)-5,8,11-tetradecatrien-2-one, were considered as important components that contribute to cooked shrimp aroma. Kubota et al. (1982) also studied the odors of cooked antarctic krill. Pyrazines, carbonyl compounds, and some sulfur containing compounds were found to play an important roles in the characteristic krill flavors. 3-Methylthiobutanal contributed markedly to the cooked odor of krill because this compound has a low threshold value (Kubota et al., 1982).

Aroma perception of flavor components

Although GC/MS is a powerful technique for separation and identification of complex flavor extracts, GC/MS can not perceive the flavor quality of the food and it is not as sensitive as the human nose (Novotny et al., 1974; Teranishi, 1981; Zurer, 1987). Subjective flavor sensory panels often are used to compare flavor quality of food products. However, our nose and tongue can not evaluate the contribution of individual flavor components in a food
without certain analytical separation techniques. Generally, food flavor extracts consist of many compounds; however, only certain ones may contribute more to the characteristic flavor of that particular food than others in the system (Shell, 1986). For example, approximately 100 flavor compounds have been identified in muskmelon; however, (Z)-6-nonenal has been reported to be the most potent component responsible for the melon-like flavor (Schieverle et al., 1990).

Relative contribution of each volatile compound to the overall aroma in a flavor extract can be determined by perceiving the odor of the effluent from a GC column. Several techniques, such as charm odor analysis and aroma extract dilution analysis (AEDA), have been introduced (Zurer, 1987; Schieberle and Grosch, 1988). These are systematic approaches to evaluate the potent odorants of a food by determining CHARM values or flavor dilution factors (FD) of the volatiles. Relative contribution of the individual aroma components can be determined by ranking the FD values, since the FD factor of a flavor compound in a food is essentially the relative concentration of the component in that food divided by the odor detection threshold of the component (Schieberle and Grosch, 1988). By using these techniques, most potent odorants in a food system can be recognized.
Identification of Geosmin as the major musty/earthy off-flavor of Louisiana Brackish water clam (Rangia cuneata)

Uraiwan Tanchotikul and Thomas C.-Y. Hsieh

ABSTRACT

The major compound responsible for the musty/earthy off-flavor of Louisiana Rangia clam (Rangia cuneata) has been identified as geosmin (trans-1,10-dimethyl-trans-9-decalol). The positive identification was based on the matching of high resolution gas chromatographic retention indices, electron ionization mass spectra, and aroma characteristics of the sample with those of the authentic geosmin standard.
INTRODUCTION

Louisiana is a major source of blue crab, oyster, redfish, catfish, and crawfish as well as rangia clam \textit{(Rangia cuneata)}. This clam is a brackish water bivalve mollusc which populates in a low salinity, 0 to 18 ppt, waters \citep{LaSalle1985}. While rangia clams have been occasionally canned and eaten in New Jersey, Texas, North Carolina, and Mexico \citep{Rusch1989}, at present, Louisiana clams have not yet been commercialized. In Louisiana, they are used mainly as an animal food \citep{Remane1971} and their shells used in industry and for road construction \citep{Tarver1973}. Although, selected rangia clam samples have been shown to meet the federal safety guidelines for approved water quality and microbiological profiles for human food consumption \citep{Andrews1988}, they often contain undesirable earthy/musty off-flavors. These off-flavors have been the major marketing obstacle for Louisiana rangia clams. Sources of the musty/earthy off-flavor have been attributed to the growth of some actinomycetes, especially \textit{Streptomyces} \citep{Hansen1964, Dickson1968} and certain species of blue green algae \citep{Lewis1966, Aschner1967}. Musty/earthy off-flavors also have been observed in other seafoods, such as channel catfish \citep{Lovell1973}, rainbow trout \citep{Yurkowski1984} and
Tabachek, 1974) and shrimp (Lovell and Broce, 1985). Vacuum distillation/solvent extraction has been used to isolate geosmin from rainbow trout (Yurkowski and Tabachek, 1974) and shrimp (Lovell and Broce, 1985). 2-Methylisoborneol (MIB), and its dehydration products, 2-methylenebornane and 2-methyl-2-bornene, which impart a musty odor in channel catfish, have been analyzed by microwave distillation and GC/MS (Martin et al., 1988). However, the compound(s) causing the musty/earthy off-flavor in rangia clams has not been investigated. Identification of such compound(s) may be useful in designing off-flavor reduction or prevention systems.

The objective of this study was to identify the chemical structure of the major compound(s) responsible for the musty/earthy off-flavor in rangia clam.

MATERIALS AND METHODS

Materials

Louisiana rangia clams (Rangia cuneata) were dredged from brackish waters in St. Bernard Parish, LA. The clams were placed in plastic bags and transported on ice within 4 hr to the LSU Food Science Department in Baton Rouge, LA. After overnight storage in a refrigerator, live clams were washed with tap water to remove foreign substances from the shell and steamed for approx. 45 min until the
shells were opened. Steamed clam meat was removed from the shell manually and vacuum packed in polyethylene bags (size 20.3 cm x 30.5 cm, Koch Supplies, Inc., Kansas City, MO). The clams were stored frozen at -20°C before analysis. Immediately before analysis, the clam meat of approximately 60 clams (100g) was thawed and homogenized in a grinder (Model Galaxie, Oster Corp., Milwaukee, WI). Standard geosmin (trans-1,10-dimethyl-trans-9-decalol) and 2-methylisoborneol (MIB) were obtained from Dr. R. T. Lovell of Auburn University. This standard was originally obtained from A. R. Hochstetler (Givaudin Corp., Clifton, NJ) and was synthesized by the procedure described by Marshall and Hochstetler (1968). The standard MIB was synthesized at the USDA Southern Regional Research Center, New Orleans, LA according to the procedure of Wood and Snoeyink (1977). Standard hydrocarbons were purchased from PolyScience Corp., Niles, IL.

Dynamic Headspace Analysis (DHS)

A 7g aliquot of clam sample was placed in a headspace sampling tube (15.2 cm x 1.6 cm i.d.) of a DHS system consisting of a Tekmar model 4200 Automatic Heated Sampler and a model 4000 Dynamic Headspace Concentrator (Tekmar Co., Cincinnati, OH). Ultra high purity helium (99.999%, Linde Div., Union Carbide Corp., Danbury, CT), passing through an oxygen scrubber (Am. Sci. Prod., McGaw Park,
IL) and a hydrocarbon trap (Supelco, Inc., Bellefonte, PA), was used to purge the volatile compounds from the clam sample. The sample was purged without heating for 2 min to avoid oxidation, and then heated without purging for 5 min to a temperature of 60°C. The headspace volatile components were then purged at 40 mL/min from the sample onto a porous polymer Tenax TA (0.24g, 60-80 mesh, Chrompack, Raritan, NJ) trap. After a 30-min sample-purge time, 40 min of on-line dry-purge (bypassing the sample) to remove the trapped moisture was carried out. Ten sample tubes, in series, were used for a total sample size of 70g to concentrate the volatile flavor components into the trap. Additional off-line dry-purge also was performed to remove the residual moisture in the trap by purging helium gas through the trap at 60 mL/min until the trap's weight was reduced to its original weight. This off-line dry-purge procedure was carried out according to the procedure described by Williams et al. (1988).

**Gas chromatography**

The trapped volatile compounds were flash-desorbed at 185°C for 15 min to transfer them from the trap to the GC column in the splitless mode. The column head pressure was increased from 15 psi to 30 psi to facilitate the transfer of volatiles from the trap to the GC column. During this trap desorption time, the injector part of the
GC column was gently bent into a U-shape and lowered into a dry ice/ethanol bath (-70°C) to cryogenically focus the volatiles prior to chromatography. At the end of desorption, the column head pressure was adjusted back to 15 psi so that the MSD would not be subjected to excessive pressure during its active data acquisition period. Separation of the components was achieved by high resolution gas chromatography on a fused silica capillary column bonded with polyethylene glycol (60m length x 0.25 mm i.d. x 0.25 µm film thickness; Supelcowax 10, Supelco, Inc., Bellefonte, PA) installed in an HP 5792 GC. The oven temperature was programmed from 60°C to 175°C at a rate of 2°C/min and maintained at 175°C for 15 min. The carrier gas flow rate and linear velocity were 0.75 mL/min and 25.5 cm/sec, respectively.

**Gas chromatography/mass spectrometry (GC/MS)**

Mass spectra were acquired by using a GC/mass selective detector (MSD) (HP 5970 B) system with the following conditions: capillary direct interface temperature, 195°C; ion source temperature, 200°C, electron ionization voltage, 70 eV, and electron multiplier, 1800 V. Full MS scans over a mass range of mass/charge (m/z) 40-290 were used for an initial observation. To increase the MS sensitivity in the final analyses, the following ions: m/z 55, 57, 69, 83, 95, 97, 107, 108, 110, 111, 112, 125, 126,
135, 149, 150, 168, and 182 were monitored in a selective ion monitoring mode. A combination of these ions and additional GC retention data allowed unequivocal identification.

Chromatography-coupled aroma perception analysis

The effluent from the GC column was split into 2 equal streams by a splitter (Vitreous silica outlet splitter, Scientific Glass Engineering, Inc., Austin, TX). One stream was directed to a hydrogen flame-ionization detector and the other was directed to a heated sniffing port (200°C) with a make-up flow (30 mL/min) of moistened breathing air. The aroma in the effluent at the end of the sniffing port was perceived by two panelists (Hsieh and Tanchotikul) who were familiar with the sniffing techniques and the musty/earthy off-flavors of Louisiana rangia clams.

Chromatography and identification of compounds

Positive identification was confirmed by matching chromatographic retention indices (RI), calculated according to Van den Dool and Kratz (1963), mass spectra with those of the authentic standards, and aroma characteristics under the same experimental conditions for the unknown and the standard compounds.
RESULTS & DISCUSSION

A total ion chromatogram (TIC) of the headspace volatile of clam sample and a TIC of standard mixture of hydrocarbons, MIB, and geosmin from direct injection, are shown in Fig. 1. Geosmin was positively identified for the first time as the major compound responsible to the musty/earthy off-flavor of Louisiana rangia clam. The mass spectra at the retention times 54.129 and 54.025 min of the sample and geosmin standard, respectively, are essentially identical and their retention times matched within experimental error (Fig. 2).

The odor characteristics also were investigated by HRGC-coupled aroma perception technique at the GC sniffing port. Only one section at approx. 54 min in retention time during the chromatographic elution of the clam headspace components gave the musty-earthy off-flavor observed in the clam. The aroma characteristic and the retention time also matched with those obtained from standard geosmin. This sniffing data confirmed the positive identification of geosmin as the major musty/earthy off-flavor in Louisiana rangia clam. Although the geosmin peak was not the most abundant peak in the whole chromatogram, it was the only one perceived to have the musty/earthy note above the sniffing threshold.
Geosmin and MIB have been reported as the two major odorous compounds imparting the musty-earthy off-flavors in fish and shrimp (Tabachek and Yurkowski, 1976; Persson, 1982; Martin et al., 1987; Lovell and Broce, 1985). It has been proposed that the steric configuration of the hydroxyl and methyl groups of geosmin, a saturated tertiary alcohol, may be important to interact with receptors in the nose, causing the earthy odor (Krasner et al., 1981).

MIB was not detected in this study according to mass spectrometry and sniffing evaluation. It is possible that only geosmin contributes to the off-flavor in the clam samples studied or that the dynamic headspace sampling and detection procedure may not be sensitive enough to detect other minor compounds that could contribute to other musty/earthy off-flavors. The selective ion monitoring mass spectrometry used is a sensitive method suitable for detection of the trace organic components. Identification of other minor off-flavor compounds in the rangia clam may require the use of a larger scale extraction procedure such as simultaneous steam distillation and solvent extraction. In addition, nondestructive detectors such as a photo-ionization detector (PID) may be more useful than a flame ionization detector in obtaining the sensory data since splitting of the GC effluent, which reduced detection sensitivity, can be avoided and the probability
of perceiving higher threshold compounds may be increased. Geosmin now can be used as a marker to determine the off-flavor of rangia clam and the effectiveness of geosmin reduction systems. The basic information generated in this study may facilitate further research needed to improve the flavor quality of the abundant Louisiana rangia clam resource.
Fig. 1 - (A) Total ion chromatogram of volatile components in steamed Rangia clam. (B) Total ion chromatogram of a standard mixture of MIB, geosmin, and straight-chain hydrocarbons as retention time markers.
Fig. 2 - (A) Mass spectrum at retention time 54.129 min of sample in Fig. 1(A). (B) Mass spectrum at retention time 54.025 min of the standard solution in Fig. 1(B).
Figure A: Mass-Spectrum of Musty/Earthy Off-Flavor in Rangia Clam

Figure B: Mass-Spectrum of Standard Geosmin
CHAPTER IV

Methodology for Quantification of Geosmin and Comparison of Levels in Rangia Clam (Rangia cuneata) Samples*

Uraiwan Tanchotikul and Thomas C.-Y. Hsieh

ABSTRACT

The concentration of geosmin in freshly dredged and 2-week relayed rangia clam (Rangia cuneata) samples at different seasons of the year was determined by vacuum distillation/solvent extraction using an internal standard and multiple-level standard addition method in gas chromatography/selective ion monitoring mass spectrometry. Most coefficients of variation were lower than 10%. On a dry-weight basis, the geosmin level in dredged clams was highest (343 ng/g) in August and averaged 51.6 ng/g in other months. A 52% reduction of geosmin was achieved after a two week relaying during the period of May-June. However, only 1% reduction of geosmin was observed during February-March.

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INTRODUCTION

Approximately fifty billion rangia clams (*Rangia cuneata*) were estimated to exist in the Louisiana coastal area, the largest source of such clams along the Gulf of Mexico coast (Hoese, 1973). Although selected clam samples have been shown to meet federal guidelines for approved water and microbiological profiles for human food safety (Andrews, 1988), they have a strong musty/earthy off-flavor that limits their marketability. The major compound responsible for the musty/earthy flavor has been identified as geosmin (trans-1,10-dimethyl-trans-9-decalol) by Hsieh et al. (1988). Common purification methods of molluscan shellfish to reduce microbiological and/or chemical contamination include relaying and depuration. Relaying refers to the process when the shellfish is relocated from the growing area to the approved waters. Depuration is when the shellfish is cleansed in quality-controlled water (Becker, 1977).

To aid further research and development efforts, a need existed for developing a quantitative method to objectively determine the levels of geosmin in clam samples. This method would facilitate proper timing for dredging, and to assess the efficiency of various geosmin removal or reduction systems, such as relaying and depuration. This objective method is necessary since
reproducible subjective sensory evaluations of a trained panel are often difficult to achieve.

Closed loop stripping analysis (CLSA) (Hwang et al., 1984) and solvent extraction (Johnsen and Kuan, 1987) have been used for quantitative analysis of geosmin in water. Vacuum distillation/ solvent extraction has been used to determine the levels of geosmin in fish samples (Yurkowski and Tabachek, 1974; Lelana, 1987). However, none of these studies used an internal standard coupled with multiple-level standard addition (IS/MLSA). IS/MLSA methods have been reported to give more accurate quantification by considering sample matrix effects as well as instrumental and handling variations (Willard, et al., 1981; Lai, 1988; Marsili, et al., 1989).

The objectives of this study were (1) to develop a quantitative method using IS/MLSA to determine the amounts of geosmin in clam samples and (2) to determine the amounts of geosmin in clam samples before and after a relaying procedure.

MATERIALS & METHODS

Clam samples and standards

Louisiana rangia clams were collected by dredging, in bushel quantities, from the marshes in the St. Bernard Parish area during 4 periods beginning April 1987 through
March 1988. The first half of the clams were placed in 0.5 m² relay baskets at a pile depth of less than 7 cm (approx. 2 layers of clams). The baskets were lowered into approved waters of Lake Borgne, Louisiana (approx. 15 Km south east of New Orleans) at 2-6 m below the surface of the water for 2 weeks. The other half was placed in plastic bags and transported on ice within 4 hr to the LSU Food Science Department in Baton Rouge, LA according to recommended procedures of the American Public Health Association (APHA, 1984) for transport of molluscan shellfish. After 2 weeks of relaying, the first half of the clam samples were transported to LSU in a similar manner. After overnight storage at 4°C, the clams were washed with water to remove attached sediment from the shell, and steamed for approximately 25 min until the shells were open. Steamed clam tissues were manually removed from the shell and vacuum packed in polyethylene bags (size 20.3 cm x 30.5 cm, Koch Supplies, Inc., Kansas City, MO) and stored at -40°C until analyzed. An authentic geosmin standard was obtained from USDA Southern Regional Research Center (New Orleans, LA). Cyclo-octanemethanol was purchased from Aldrich Chemical Company (Milwaukee, WI) and used as an internal standard (IS).

Duplicate analyses were performed at the original and each of the three levels of geosmin addition to each clam sample. Samples were ground using a grinder (model
Galaxie, Oster Corp., Milwaukee, WI). Aliquots of each sample were prepared by vortexing vigorously, using a Maxi Mix ii (Thermolyne Corp., Dubuque, IA) for 2 min. To each 5g aliquot of ground clam tissue the following were added: 3.50 mL of HPLC grade water (Mallinckrodt Inc., Paris, KY) and 0.50 mL of a methanol solution containing 7200 ng/mL cyclooctanemethanol and standard geosmin at various concentrations: 0, 65, 130 and 195 ng/mL. To keep the ratio of the amount of the added analyte and the original amount of the analyte in the sample within a narrow and comparable range throughout the study, in period 2 (August), the amount of sample aliquot used for distillation was 1g instead of 5g. A total of 8 analyses were performed on each clam sample.

In addition to cyclooctanemethanol, several other compounds including 1-chlorododecane, methyl dodecanoate, 3-cyclohexyl-1-propanol, and undecanol were evaluated in the initial stage of this study. Cyclooctanemethanol was found to have less chromatographic and mass spectrometric interferences under the conditions tested and gave a better overall reproducibility.

Vacuum distillation and solvent extraction

The samples were vacuum-distilled and hexane-extracted before analysis by gas chromatography/selective ion monitoring mass spectrometry (GC/SIMMS). Vacuum
distillation was conducted at -85 kPa for 45 min using the device shown in Fig. 3. The sample flask was heated in a water bath by increasing the temperature from 60°C to 90°C at a rate of 2°C/min. Two collection tubes in tandem were used to collect the distillate to minimize loss during distillation. Dry ice in an ethanol bath was used to cool the distillate collection tubes. The geosmin and the internal standard in the distillate were extracted twice by using 0.20 mL of redistilled n-hexane for each extraction in a 10-mL Mixxor (Xydex Corp., Bedford, MA). This device consisted of a graduated glass mixing chamber, into which was fitted a glass piston containing an axial channel.

Gas chromatography/Mass spectrometry analysis
An aliquot of 4 μL of the hexane extract from each distillation/extraction was injected into an HP 5792 GC (Hewlett-Packard Co., Palo Alto, CA) in the splitless mode. Separation of components was achieved on a fused silica capillary column Supelcowax 10 (60 m x 0.25 mm i.d. x 0.25 μm film thickness; Supelco, Inc., Bellefonte, PA). The linear velocity of the helium carrier gas was 25.8 cm/sec and the oven temperature was programmed from 120°C to 175°C at 6°C/min and from 175°C to 195°C at 4°C/min and set at 195°C for 15 min. The GC was directly coupled to an HP 5970B mass selective detector operating in the
electron ionization (EI) mode with the ion source temperature set at 200°C, ionization energy at 70 eV and the electron multiplier voltage at 2400 V. Ions, m/z (mass/charge) 81, 83, 111, 112, 125, and 126, were monitored in the selective ion monitoring mode for better MS sensitivity than that of MS scanning over the full mass range. Ion abundance ratios were used to ensure correct peaks for peak area integration.

To calculate the original amount of geosmin in a clam sample, the following formula was used (Lai, 1988).

\[
C = \frac{Q_i \times A}{W \times (B_i - A)}
\]

where: 
- \(C\) = concentration (ng/g) of the geosmin originally present in the sample,
- \(Q_i\) = concentration (ng/g) of the geosmin added to the sample at addition level \(i\),
- \(W\) = weight (g) of sample aliquot,
- \(A\) = ratio of peak area of ion m/z 112 of geosmin over peak area of ion m/z 111 of cyclooctanemethanol from the sample with no geosmin addition,
- \(B_i\) = ratio of peak area of ion m/z 112 over peak area of ion m/z 111 from the sample aliquot with addition of geosmin at level \(i\),
- \(i\) = addition level 1, 2, or 3.

A graphic representation of this calculation is shown in
Fig. 4. Thus, three values of the amount of geosmin in each sample were obtained from the calculation of data from three additional levels of standard.

The coefficient of variation was used to demonstrate the reproducibility of the procedure. To indicate significance in geosmin reduction, differences between the means before and after relaying were determined by the least significant difference method (LSD) using the Statistical Analysis System (SAS Institute, Inc., Cary, NC).

Determination of Recovery

Five-g aliquots of period 3 relayed clam sample (geosmin concentration was 51.4 ppb, dry weight, 75% moisture content, Table 1) were spiked with 0.5 mL of 195 ng/mL geosmin for a total geosmin of 161.75 ng and used in the determination of geosmin recovery. The vacuum distillation was done in the same manner as mentioned above except that 0.5 mL of 7200 ng/mL of the IS in methanol was added to the distillate before solvent extraction. The peak area ratio of ion m/z 112 and ion m/z 111 in each aliquot was determined by GC/MS as mentioned in the Materials and Methods section.

An external standard calibration curve of the ratio of peak area of ion m/z 112 and ion m/z 111 vs. ratio of the amounts of geosmin and IS was obtained by analyzing a
series of mixtures containing various concentrations (0.195, 0.390, and 0.780 ng/μL) of geosmin and a constant concentration (7.2 ng/μL) of the IS.

RESULTS & DISCUSSION

Quantification of geosmin in clam tissue by IS/MLSA

A method of vacuum distillation/solvent extraction with IS/MLSA was developed for the quantification of geosmin in rangia clams. In this study, geosmin and the internal standard were added during sample tissue preparation before distillation/extraction, so that the variations from distillation to the end of analysis could be precisely analyzed. Yurkowski and Tabachek (1974) and Lelana (1987) also used vacuum distillation methods to determine geosmin level in trout and channel catfish, respectively. However, internal standards were added to the distillate after distillation (Yurkowski and Tabachek, 1974; Lelana, 1987). Buttery et al. (1976) used vacuum steam distillation/continuous hexane extraction and silica gel chromatography to isolate geosmin from dry beans for GC/MS analysis. In this study, a Mixxor was used to extract geosmin from the vacuum distillate. Six piston movements have been reported to be equivalent to 40 shakes in a separatory funnel (Peleg and Vromen, 1983). The apparatus provided highly efficient extraction, especially
when a small amount of solvent was used (Parliment, 1987).

Fig. 5 (A and B) shows a typical selective ion chromatogram of the hexane extract from clam distillate. The single ion chromatograms of ions m/z 112 for geosmin and m/z 111 for cyclooctanemethanol were used to determine the peak areas because they provide better signal to noise ratios than those of other ions. A graphic representation using the standard addition method is shown in Fig. 4.

The X-axis shows the concentrations of the analyte (geosmin). The unknown sample concentration is determined by the point at which the extrapolating peak area-concentration line intersects the X-axis. Since the sample analyte and standard analyte are measured within the same analysis under identical conditions, a multiple level standard addition method gives more accurate results than those obtained from external standard calibration curves by analyzing samples and standards separately (Willard et al., 1981). Schieberle and Grosch (1987) used a heavy isotope-labeled standard for quantification of volatile flavor compounds in bread. Due to the unavailability of such counterparts for geosmin, isotope dilution mass spectrometry was not possible in this study.

In this study, most of the coefficients of variation were less than 10%, except the dredged sample from period 2 (15.0%), which might have been affected by the high initial level of the geosmin present. The average
recovery rate of this analytical procedure was determined to be $97.9 \pm 6.3\%$ ($n = 2$). Based on the recovery rate and a practical MS detection limit of 20 pg geosmin with a signal to noise ratio of 2:1 in the selective ion monitoring (SIM) mode, the sensitivity of this analytical procedure (IS/MSLA using GC/MS) for geosmin in clam tissue can be expected to be approximately 2 ppb (ng/g dry weight).

Comparison of geosmin levels in clam samples

Table 1 shows the geosmin levels in dredged and relayed clam samples in different periods of the year. Only in period 1 (May-June), was the amount of geosmin reduced significantly ($p < 0.01$) by 52% after 2 week of relaying. In period 2 (August), the reduction of geosmin by relaying was only 17%. It was possible that the initial amount of geosmin in clams (343 ng/g dry weight) in period 2 might have been too high, or the water in Lake Borgne also might have an excessive geosmin level (not determined) for efficient purification. In studies of geosmin removal in rainbow trout and catfish (Yurkowski and Tabachek, 1974; Lovell and Sackey, 1973), it was reported that removal was dependent primarily on the initial levels of the compounds present in the fish. In periods 3 (Nov.-Dec.) and 4 (Feb.-Mar.), the amounts of geosmin in the dredged and relayed clam were not significantly different. The water
temperature might be critical for effective purification especially, since the water temperature was generally low during those two periods of the year. Hopkins et al. (1972) reported that the metabolic rate of rangia clam was reduced considerably when the water temperature was less than 18°C or more than 27°C. Detailed temperature readings at the relay site during the relaying periods were not available for correlation. Other environmental and weather conditions also may affect the efficiency of relaying in open waters. Several studies have been reported on the exposure period of fish for muddy-musty flavor removal (Maligalig et al., 1973; Lovell and Sackey, 1973; Iredale and York, 1976). However, it is difficult to compare these studies directly due to lack of comparable parameters (Iredale and York, 1976). From the present investigation, the relaying process of the clam in Lake Borne was not sufficiently efficient to remove all the geosmin in a 14-day period. The objective analytical method developed in this study could be used to correlate the effects of controlled environmental conditions on the reduction of geosmin in rangia clam.

Geosmin has been reported to have a threshold odor concentration (TOC) at about 6 ppb in rainbow trout (Yurkowski and Tabachek, 1974; Persson, 1980). However, a TOC has not been determined for geosmin in rangia clams. It may not be necessary to remove all of the geosmin since
it may be feasible to blend the geosmin containing rangia clam into various food products.
Fig. 3 - Vacuum distillation apparatus: (A) sample flask, 300 mL; (B) glass transfer line, 0.7 cm i.d. x 15 cm L.; (C, D) distillate collection tubes, 50 mL; (E) vacuum gauge and (F) vacuum pump.
Fig. 4 - A graphic representation of peak area ratios 

\[ \left( \frac{m/z \ 112}{m/z \ 111} \right) \times 10 \] vs. concentration of geosmin. On the X-axis, positive values indicate various levels of geosmin added to sample and the negative value indicates the concentration of geosmin originally present in the sample. Only the variables pertaining to addition level 1 are illustrated here. Symbols are explained in the Materials and Methods section.
2.0

0

Concentration of geosmin (ng/g)

Area Ratio x 10

-32.5

0

32.5

65

97.5

B1-A

Q1

B1

A

C

Concentration of geosmin (ng/g)
Fig. 5 - Typical selective ion chromatograms from capillary gas chromatography/selective ion monitoring mass spectrometry of ions m/z 112 and m/z 111 of a standard solution (A, B) containing standard geosmin and cyclooctanemethanol (IS); and a clam sample (C, D) with IS added only.
Table 1 - Concentrations of geosmin in dredged and relayed rangia clam samples

<table>
<thead>
<tr>
<th>Period</th>
<th>Sampling dates</th>
<th>Geosmin concentration (ng/g dry weight)</th>
<th>% Geosmin reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dredged clam</td>
<td>Relayed clam</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>05/20/87</td>
<td>06/03/87</td>
<td>50.9 6.0 24.5 9.0 52 *</td>
</tr>
<tr>
<td>2</td>
<td>08/11/87</td>
<td>08/24/87</td>
<td>343 15.0 284 7.2 17</td>
</tr>
<tr>
<td>3</td>
<td>11/30/87</td>
<td>12/14/87</td>
<td>57.8 6.8 51.4 6.8 11</td>
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<td>03/08/88</td>
<td>46.1 7.6 45.8 1.3 1</td>
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</table>

* Reduction of geosmin was significant (p < 0.01) based on LSD statistical analysis.

% cv = Percent coefficient of variation.
CHAPTER V

Analysis of Volatile Flavor Components in Rangia Clam by Dynamic Headspace Sampling and Simultaneous Distillation and Extraction*

Uraiwan Tanchotikul and Thomas C.-Y. Hsieh.

ABSTRACT

The volatile flavor components of rangia clam (Rangia cuneata) were extracted by dynamic headspace sampling (DHS) and simultaneous distillation/extraction (SDE) techniques, and analyzed by gas chromatography/mass spectrometry (GC/MS) and GC-coupled aroma perception. Twenty-two heterocyclic aroma compounds were identified. Volatile flavor compounds in other chemical classes, such as aldehydes, alcohols, ketones, and terpenes, also were identified. The unique clam aromas were contributed by a combination of S-, and N-containing compounds and ketones. In general, both sampling methods (DHS and SDE) complemented each other in the compounds detected in the clam sample. However, SDE gave more information on the characteristic steamed clam flavors.

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INTRODUCTION

Louisiana is a major seafood producing state including shrimp, crab, crayfish, and oyster. Less known is the abundance of rangia clam (*Rangia cuneata*), approximately 50 billion, in Louisiana brackish waters (Hoese, 1973). At present, these clams have a musty/earthy off-flavor problem that limits their commercialization for food use. The compound responsible for this off-flavor has been identified as geosmin (trans-1,10-dimethyl-trans-9-decalol) by Hsieh et al. (1988). Several off-flavor reduction approaches including relaying, depuration, and feeding the clams with non-geosmin producing algae have been proposed (Rusch et al., 1988). Despite the off-flavor, initial observations by Hsieh (1988) indicated that these clams also contained desirable sweet, nutty, and good clam meat flavors.

Although crustacean shellfish aroma components, especially in shrimp (Choi et al., 1983; Kubota et al., 1986; Kubota et al., 1989) and krill (Kubota et al., 1980; Kubota et al., 1982), have been studied extensively, very little information has been reported on molluscan volatile flavor compounds. Mendelsohn and Brooke (1968) studied the effects of radiation, processing, and storage on the soft-shell clam (*Mya arenaria*) meat components. A total of 13 compounds were identified. Flick et al. (1982) and
Burnette et al. (1983) used a direct sampling/packed column gas chromatographic method to compare the effects of processing on volatile flavors in ocean quahog (*Artica islandica*) and surf clam (*Spisula solidissima*) juice. Eleven compounds were identified in the clam juice (Flick et al., 1982).

The objectives of this study were (1) to extract the volatile flavor components in rangia clam by dynamic headspace sampling (DHS) and simultaneous steam distillation/solvent extraction (SDE) and (2) to separate and identify the aroma components by capillary column gas chromatography/mass spectrometry.

**MATERIALS & METHODS**

**Clam samples and standards**

Rangia clams (*Rangia cuneata*), dredged from brackish waters in St. Bernard Parish (approx. 15 Km south east of New Orleans), LA, were placed in plastic bags and transported on ice within 4 hr to the LSU Food Science Department in Baton Rouge, LA. The live clams were washed to remove foreign substance from the shell and steamed until the shells were opened. Total steaming time was about 20 min. Steamed clams were sensorially evaluated by the authors who were very familiar with flavor quality of rangia clam to contain desirable nutty and cooked meaty
notes in addition to the musty/earthy off-flavor. Steamed clam meat was manually removed from the shell and vacuum packed in polyethylene bags (size 20.3 cm x 30.5 cm, Koch Supplies, Inc., Kansas City, MO) and stored frozen at -20°C until analyzed. Before analysis, the clam meat was thawed and ground in a grinder (model Galaxie, Oster Corp., Milwaukee, WI) and divided into 2 parts for flavor extraction by SDE and DHS.

Standard flavor compounds were purchased from Aldrich chemical Co., Inc (Milwaukee, WI), Pfaltz and Bauer, Inc. (Waterbury, CT), American Tokyo Kasei, Inc. (Portland, OR), and Fluka Chemical Corp. (Ronkonkoma, NY). Some standards were generous gifts from Flavor & Fragrances, Aldrich Chemical Co., Inc. Standard geosmin (trans-1,10-dimethyl-trans-9-decalol) was obtained from Dr. Peter Johnsen, USDA Southern Regional Research Center (New Orleans, LA).

Simultaneous distillation and extraction techniques (SDE)
A Likens-Nickerson type SDE head (Cat. No. K-523010-0000, Kontes, Vineland, NJ) was used for preparation of the SDE extract. A 5-L round bottom flask was used as the sample flask to contain 350g of the clam meat sample, 2 L of boiled distilled water and a 2-mL aqueous solution of 25 μg/mL of collidine (2,4,6-trimethylpyridine) as an internal standard (IS). A 100-mL round bottom flask
containing 85 mL redistilled diethyl ether was attached to the solvent arm of the SDE head. The contents in the sample and the solvent flasks were heated to a boil and the distillation/extraction was continued for 4 hr. The aqueous layer in the extract was removed as ice after freezing of the extract at -20°C for 4 hr. The volume of the extract was reduced to 10 mL by evaporating the solvent under a gentle nitrogen stream. The residual moisture in the extract was removed by drying the extract over 3g of anhydrous sodium sulfate. The volume of the extract was further reduced to approx. 0.2 mL under a nitrogen stream. To increase the concentration of the extracted volatile compounds from the clam, the above procedure was carried out twice and the extracts were combined for further analysis.

Dynamic headspace analysis (DHS)

A 3.5g aliquot of the clam sample was placed in a headspace sampling tube (15.2 cm length x 1.6 cm i.d.) and spiked with 5 μl aqueous solution of 20 ng/μl collidine internal standard. The sample tube was installed in a stainless steel tee (part No. 14-1025-016, Tekmar Co., Cincinnati, OH) and was heated in a 60°C water bath. Ultra high purity helium (99.999%, Linde Div., Union Carbide Corp., Danbury, CT) passing through an oxygen scrubber (Am. Sci. Prod., McGaw Park, IL) and a
hydrocarbon trap (Supelco, Inc., Bellefonte, PA) was used to purge the volatile compounds from the sample and trap them onto a Tenax TA (0.24g, 60-80 mesh, Chrompack, Raritan, NJ) trap. The sample was purged without heating the sample for 2 min to avoid oxidation and purged at 60°C for 45 min with a flow rate of 40 mL/min for trapping of the volatile compounds. Ten sample tubes were used for a total sample size of 35g to concentrate the aroma components in the trap for each analysis. A dry purge procedure to remove trapped moisture was carried out by purging the trap with helium at 60 mL/min (Williams et al., 1988). The trapped volatile compounds were flash-desorbed at 185°C for 10 min to transfer the compounds from the trap to the GC column in the splitless mode. The GC septum purge vent and split vent also were closed to avoid the loss of volatile compounds. The carrier gas head pressure was increased from 12 psi to 30 psi to facilitate the transfer of volatiles from the trap to the injector part of the GC column which was cryogenically focused in liquid nitrogen bath. At the end of desorption, the carrier gas head pressure was adjusted back to 12 psi. Separation of the components was achieved by high resolution gas chromatography (HP 5792).
Chromatography and Identification of Compounds

A Hewlett Packard (Palo Alto, CA) gas chromatograph/mass selective detector (5792 GC/5970B MSD) was used for separation of the compounds and acquisition of the electron ionization mass spectra from the SDE and DHS extracts. Five $\mu$l of the extract from SDE was injected in the splitless mode into a fused silica capillary column bonded with polyethylene glycol (Supelcowax 10, 60 m length x 0.25 mm i.d. x 0.25 $\mu$m film thickness; Supelco, Inc., Bellefonte, PA). To achieve optimal chromatographic separation, two different GC oven temperature programs were used for the two flavor extracts. For DHS analysis, the column temperature was programmed to stay at 40°C for 5 min, increased from 40°C to 175°C at a rate of 2°C/min, and maintained at 175°C for 30 min. For the SDE extract, the column temperature was set to 40°C, increased from 40°C to 195°C at a rate of 1°C/min, and maintained at 195°C for 30 min. The GC/MSD conditions were as follows: helium carrier gas flow rate, 0.76 mL/min; linear velocity, 25.7 cm/sec; injector temperature, 155°C; capillary direct MS interface temperature, 195°C; ion source temperature, 200°C; ionization voltage, 70 eV; mass range, mass/charge 33-300 a.m.u.; and electron multiplier voltage, 2000 V.

Positive identifications were confirmed by matching retention indices (RI), calculated according to Van den
Dool and Kratz (1963), and mass spectra with those of authentic standards under comparable conditions. Tentative identifications were based mainly on mass spectra of the unknowns with those in the Wiley/NBS mass spectral library (Hewlett Packard Co., 1988) and the literature (Eight Peak Index of Mass Spectra, 1974; Boelens et al., 1974, Tang et al., 1983; Hwang et al., 1986).

**Chromatography-coupled aroma perception analysis**

The SDE extract also was used in GC-coupled aroma perception analysis. Chromatographic conditions were the same as those used in GC/MS analysis. A photoionization detector (PID, HNU, Inc., Newton, MA) installed in a Hewlett-Packard 5793 gas chromatograph was used to monitor the effluent in an essentially non-destructive manner. The PID conditions were as follows: UV lamp energy, 10.2 eV; PID body temperature, 220°C; PID make up gas, 30 ml helium/min; sniffing port temperature, 200°C. The aroma of the GC effluent was perceived by two analysts familiar with the rangia clam flavor and the GC aroma perception techniques.
RESULTS & DISCUSSION

Clam volatile compounds extracted by DHS versus SDE

Typical MS total ion chromatograms of the volatile flavor compounds in steamed rangia clam by DHS and SDE techniques are shown in Fig. 6 and 7, respectively. Compounds identified are listed in Table 2. Both qualitative and quantitative differences in the two extracts were observed. Fifty-two and seventy-five volatile flavor compounds were identified in DHS and SDE analysis, respectively. Twenty-nine compounds were found in both extracts. The volatile compounds in the vapor phase above a food can often be sampled by DHS. However, since the ability of this technique depends on the vapor pressures of the compounds, the difficulty occurs in detecting important higher boiling aroma components. Volatile aldehydes, ketones, and terpenes eluted in the early part of the chromatograms were more abundant by DHS than by SDE. In DHS, the volatile compounds were trapped inside the tenax trap; thus, the overall aroma could not be evaluated for comparison with the flavors of steamed clam before extraction. A few highly volatile compounds were found at higher concentrations in DHS than in SDE. Dimethyl sulfide, reported by Mendelsohn and Brooke (1968) to contribute to a clam (Mya arenaria) flavor significantly, was detected in the DHS but not in the SDE
Possible loss of very volatile flavor components during solvent evaporation, as well as interference of the solvent front during GC elution, might have contributed to the difficulty of detection of the highly volatile compounds in the SDE extract. However, many important heterocyclic aroma components were identified only in the SDE extract. The SDE extract exhibited very desirable cooked meaty, roasted nutty, and refreshing green plant aromas as observed in the freshly steamed rangia clam. The SDE method affords an extract that can be used for more detailed investigation, an advantage lacking in the DHS approach.

**Important volatile flavor components in rangia clam**

Twenty-two heterocyclic nitrogen-, oxygen-, and/or sulfur-containing compounds, including 6 pyrazines, 3 thiophenes, 2 furans, 5 thiazoles, 4 trithiolanes, 1 dithiazine, and 1 pyrrole were identified in rangia clam. Many of these compounds have been reported as important volatile compounds contributing to the aromas of cooked shrimp (Kubota et al., 1989). Among the possible contributors to the desirable roasted nutty/meaty aromas observed in steamed rangia clam, six alkylpyrazines were identified. Certain alkylpyrazines can be formed by the reaction of protein-carbohydrate degradation products with...
lipid oxidation products (Huang et al., 1987). Dimethylpyrazines were the major pyrazines present in the cooked clam. The concentration of 2,5- and 2,6-dimethylpyrazines found by SDE were 17.7 and 14.1 ng/g sample, respectively. Both 2,5- and 2,6-dimethylpyrazines were reported to be the major products in the acetol-ammonium acetate reaction system (Rizzi, 1988). Alkylpyrazines also have been reported as important aroma components in boiled crayfish tailmeat and hepatopancreas (Vejaphan et al., 1988; Hsieh et al., 1989). One dithiazine and four alkyltrithiolanes, including 3,5-dimethyl-1,2,4-trithiolanes and 3,5,5-trimethyl-1,2,4-trithiolanes, were found in the clam sample. Trithiolanes and dithiazines can be produced from aldehydes, ammonia, and hydrogen sulfide, which are normally generated from the thermal degradation of sugars, lipids, and amino acids in the cooked foods (Boelens et al., 1974; Kawai et al., 1985). The average concentration of dimethyltrithiolanes detected in the SDE clam extract sample was approximately 5 times higher than that of trimethyltrithiolanes. Only one dithiazine, 2,4,6-trimethylidihydro-1,3,5-derivative, was detected in the clam sample. This compound has been considered an important flavor in crustaceans (Kubota et al., 1980; Choi et al., 1983).

Various cyclic polysulfides have been reported to contribute to characteristic cooked shrimp flavors (Kubota
et al., 1989). Methylthiophene, and 2- and 3-thiophenecarboxaldehydes, thermal degradation products of sulfur-containing amino acids (Vernin and Parkanyi 1982), were detected in the SDE extract. Methyl thiophene and 2-thiophene carboxaldehyde have been found in krill (Kubota et al., 1982), crab (Matiella and Hsieh, 1990), and crayfish (Vejaphan et al., 1988). 3-Thiophenecarboxaldehyde has not been reported previously in crustaceans and mullusks, and was identified in clam for the first time. This compound may play an important role in rangia clam flavor.

A series of C5-C10 unsaturated aldehydes and C6-C10 dienals, oxidation products of polyunsaturated fatty acids, were identified in clam tissue. Volatile aldehydes, ketones, alcohols, and hydrocarbons may be generated from degradation products of linolenate and linoleate hydroperoxides (Grosch, 1982; Selka and Frankel, 1987) and act as the precursors for volatile components in clam sample. Geosmin was also identified in the SDE extract (Table 2). Aliphatic aldehydes were found to be the major components in the steamed clam flavor. Among them, pentanal had the highest concentration, 296 and 109 ng/g sample by DHS and SDE respectively. Aldehydes may react with Maillard reaction intermediates to form heterocyclic compounds (Boelens et al., 1974). Twenty-one ketones were identified in the clam sample. It was not
clear why isomers of 5,8,11-tetradecatrien-2-one, reported by Kubota et al. (1989) as having a shrimp, crab, and clam-like aroma, were not found in the clam sample. Several terpenes, generally derived from essential oils of plants, also were detected, and may have originated from algae via the food chain.

Sensory evaluation of rangia clam by GC-Aroma Perception

Sniffing of GC effluent of the clam extract was conducted to perceive the characteristic aroma components. Clam odors perceived were classified into 4 major groups: (1) green/woody, (2) floral/fruity, (3) nutty, and (4) clam meat odor. The compounds responsible for refreshing green/woody notes were mainly aldehydes, some alcohols, and terpenes. Sweet floral and fruity notes were mainly contributed by ketones. For example, in the chromatogram area of RI between 1290-1350, heptanone, cyclohexanone, 3-hydroxy-2-butanone, and 6-methyl-5-hepten-2-one contributed to sweet floral fruity notes in the clam sample. Alkylpyrazines, pyrrole, and benzaldehyde were found to give strong unique nutty aromas in the SDE extract. Desirable clam meat aromas were observed in two elution periods of RI between 1600-1660 and 1700-1760. In the RI 1600-1660 region, 3,5-dimethyl-1,2,4-trithiolane, 2-acetyl-3-methylpyrazine, acetyl pyrazine, and 2-acetylsulfide were identified. 3,5,5-Trimethyl-1,2,4-
trithiolane, thiophenecarboxaldehyde, dodecanal, trimethyl cycloheptadien-1-one, 3-methyl-3-decen-2-one and dihydro trimethyl dithiazine were detected, among others, in the RI region between 1700 and 1760.

It is possible that the desirable roasted nutty and cooked meaty aromas may be important and critical in making rangia clams attractive to potential consumers despite the presence of musty/earthy off-flavors. No single compound could be judged as solely responsible for any of the observed roasted nutty/meaty clam flavors. However, it is possible that a mixture of the above mentioned compounds at proper concentration ratios may resemble to some extent the cooked clam meat flavor complex. Chromatography-coupled aroma perception analysis on serially diluted samples (Schieberle et al., 1990) may be used to further assess relative importance of these flavor compounds in steamed rangia clam.
Table 2: Volatile components identified in rangia clam as determined by SDE/GC/MS and DHS/GC/MS

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<th>Conc. ng/g DHS</th>
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**Heterocyclic compounds**

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* = Tentative identification by matching a sample spectrum with literature reference spectra.
a = Previously identified in clam by Mendelsohn and Brooke (1968).
b = Previously identified in clam by Flick et al. (1982).
c = Identified for the first time in clam.
d = Concentration was difficult to determine due to coelution and lack of standards.
Fig 6. (A-B) - Total ion chromatogram of volatile flavor components in the dynamic headspace of rangia clam. Peak numbers correspond to those shown in Table 2.
Fig 7. (A-C) - Total ion chromatogram of volatile flavor components from the simultaneous distillation and extraction of rangia clam. Peak numbers correspond to those shown in Table 2.
CHAPTER VI

SIGNIFICANT FINDINGS AND CONCLUDING REMARKS

Although off-flavor problems in aquatic food resources have been recognized for a long time, many of these off-flavors have not been completely solved. This affects the utilization of the resources tremendously. For example, the abundant Louisiana rangia clams have not been commercialized. Off-flavor problems in the channel catfish aquaculture industry are costing over 750 million dollars every year (Jensen, 1988).

Significant findings of this research include the following (1) positive identification of geosmin as the major musty/earthy off-flavor component in rangia clam, (2) analytical method development and quantification of geosmin on rangia clams before and after relaying, and (3) comprehensive investigation and identification of important and desirable cooked rangia clam aromas. By employing state-of-the-art analytical flavor research techniques, the studies in this research obtained a vast amount of specific flavor information in rangia clam. The findings of this research will eventually benefit the Louisiana seafood industry and ultimately increase the employment opportunities to Louisiana coastal regions. The study may also contribute to reduction of the off-
flavor problems in other aquaculture industries.

By using a combination of modern analytical techniques, such as high resolution gas chromatography, selective ion monitoring mass spectrometry, and aroma perception analysis, geosmin was identified as the major compound responsible for the musty/earthy off-flavor of rangia clam (Chapter III). Selective ion monitoring mass spectrometry provides a better sensitivity than full mass range scanning in detecting compounds present in trace (ppb) quantities. After the compound responsible for the off-flavor had been identified in this study, researchers in LSU Civil Engineering Department have initiated engineering approaches to remove geosmin in water and clam to eliminate the off-flavor.

Quantification of the geosmin was necessary to objectively evaluate the efficiency of the geosmin removal or reduction system. Further efforts were then directed toward development of a vacuum distillation/solvent extraction using an internal standard and multiple-level standard addition method (Chapter IV). Although this technique provides accurate results, it is not a rapid method because the number of analyses per sample is quite high (8) and the procedure is quite labor intensive. A heavy isotope-labeled geosmin standard, if available in the future, would serve as a suitable internal standard for the assay.
The geosmin level in dredged clams was highest in August and 52% reduction was achieved by relaying during the period of May-June. A depuration/feeding system of rangia clams to remove geosmin has been proposed (Rusch et al., 1988). In this system, the water quality is controlled in an attempt to purge the geosmin from live clams on a green algae diet which has not been reported to contain or produce geosmin. The evaluation of this system is currently underway. A subjective sensory evaluation is necessary to correlate results with the objective method to accurately study the threshold odor concentration (TOC). A detailed investigation to correlate all the environmental parameters with the variations of the geosmin levels in the rangia clam is not within the scope of this dissertation research. However, an objective and reproducible quantification method such as the one developed in this study should be used to determine the efficiency of any geosmin reduction systems.

Currently, there is no active program on removal of geosmin by a food processing approach to obtain the shelf-stabilized clam tissue that can be incorporated into clam flavor emphasized products such as chowder and clam powder. Several natural seafood flavor ingredients, such as clam based powder, shrimp based powder, have been commercially prepared by using an appropriate food processing procedure. Certain flavor components must be
used as objective markers to examine the flavor quality of the products. The characteristic clam volatile flavor was studied by using 2 different flavor extraction methods, DHS and SDE. The GC-aroma perception results were correlated with the compounds identified to indicate the significance of each compound contributing to the clam flavor (Chapter V). It was found that the unique clam aromas could be attributed to a combination of sulfur- and nitrogen- containing compounds and ketones. Additional investigations and experiments such as flavor dilution analysis, may help to determine the relative importance of these compounds.

Overall, the critical flavor components, both undesirable off-flavors and desirable cooked aromas, in the Louisiana brackish rangia clam (Rangia cuneata) were studied. It is hoped that information obtained will contribute to future research efforts leading to the utilization of these abundant clams. In addition, this information may also enhance efforts in current and future seafood flavor research and development.
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Appendix A. Map of Louisiana indicating major sources of rangia clams

1 LAKE PONTCHARTRIAN
2 LAKE MAUREPAS
3 LAKE BORGNE
4 St. BERNARD PARISH AREA
Appendix B. Chemical structures of compounds causing the musty/earthy off-flavor

Geosmin (trans-1,10-dimethyl-trans-9-decalol)

MIB (2-methylisoborneol)

2-methyl-2-bornene

2-methylenebornane
Appendix B. (continued)

3-isopropyl-2-methoxypyrazine

2-isobutyl-3-methoxypyrazine

2-isobutyl-6-methoxypyrazine
Appendix C.  Chemical structures of heterocyclic compounds and nitrogen- and sulfur-containing compounds

- Thiophene
- Thiazole
- Furan
- Pyrrole
Appendix C. (continued)

Pyrazine

2-acetylpyrazine

2-thiophenecarboxaldehyde

3,5-dimethyl-1,2,4-trithiolane

2,4,6-trimethyldihydro-1,3,5-dithiazine (thialdine)
Appendix D. Calculation of retention indices: the retention indices for the unknown and the standards were calculated according to van den Dool and Kratz (1963) as follows:

\[
RI = 100 \times \frac{(T_A - T_Z)}{(T_{Z+1} - T_Z)} + 100Z
\]

where:
- \( T_A \) = retention time of compound A
- \( T_Z \) = retention time of hydrocarbon Z eluted just before compound A
- \( T_{Z+1} \) = retention time of hydrocarbon Z+1 eluted just after compound A
- \( Z \) = number of carbon atoms in hydrocarbon Z
Appendix E. Letter of permission from Journal of Food Science to print Chapter II

INSTITUTE OF FOOD TECHNOLOGISTS
221 North LaSalle Street
Chicago, Illinois 60601
(312) 782-8424
Fax: (312) 782-8348
John B. Klis, Director of Publications

April 3, 1990

Miss Uraiwan Tanchotikul
Food Science Department
Louisiana State University
Baton Rouge, LA 70803

Dear Miss Tanchotikul:

Permission is hereby granted to you to use the material requested in your letter of March 28, 1990, for the purpose stated, provided this is done in good taste and appearance and provided further that a credit line be included substantially as follows:


Very sincerely yours,

John B. Klis
Director of Publications

FOOD TECHNOLOGY
JOURNAL OF FOOD SCIENCE
VITA

The author was born in Phattalung, Thailand, on November 6, 1960. She entered Saipanya High School and graduated in March, 1979. In March 1983, she graduated from Prince of Songkhla University with a Bachelor of Science degree in Chemistry.

The author was accepted to the Graduate School of Louisiana State University in the Department of Food Science in August, 1984. She worked as a graduate research assistant in the department of Food Science. She conducted research under Dr. J. Samuel Godber's supervision. Her thesis was entitled "The Relationship between End-point Temperature and Oxidative Stability in a Restructured Beef Roast Utilizing Surimi as a Low Temperature Binder." She received the master of science degree from Louisiana State University in December, 1986.

She continued her Ph.D. program in the same department in January, 1987 under the supervision of Dr. Thomas C.-Y. Hsieh in analytical flavor research area.

She is a member of the Institute of Food Technologists (IFT), Gamma Sigma Delta Honor Society of Agriculture, American Chemical Society (ACS) and Chinese American Food Society. She was awarded a Mid-South Section IFT Scholarship, a national IFT certificate of Merit in Recognition of Outstanding Scholastic Ability, and an IFT
Gulf Coast Section Traveling Scholarship to the 49th IFT Annual Meeting in 1989.

She is currently a candidate for the degree of Doctor of Philosophy in Food Science.

Publications:


Presentations:


Candidate: Miss Uraiwan Tanchotikul

Major Field: Food Science

Title of Dissertation: Studies on Important Volatile Flavor Components in Louisiana Rangia Clam (Rangia cuneata)

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Date of Examination:

April 20, 1990