Petroleum Research Atlantic Canada – Project 76

Project Title: SPME: Exploring Solid Phase Microextraction for Hydrocarbon Tainting of Shellfish

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Purpose of report:
The objective of this project was to explore the possibility of SPME (solid phase microextraction) for rapid and simple determination of hydrocarbons and/or other contaminants in sentinel mussels and other marine life from the Scotian Shelf monitoring program.

Acknowledgement of all funding sources: Petroleum Research Atlantic Canada

Date of the Report: September 29, 2004

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Media Summary / Abstract

Key components of project included design and testing of temperature controlled block for holding sample vials under stable and draft-free conditions; design of a support to hold the SPME holder in a stable upright position on the GC injection port; evaluation of the optimum conditions for the 30 μm polydimethylsiloxane (PDMS) SPME fiber to absorb target hydrocarbons; and most importantly, comparison of hydrocarbons recovered from shellfish using SPME vs traditional extraction methodology.

The project is significant to the Atlantic Canadian oil and gas industry because the development of a simple method which could provide accurate results quickly on shellfish hydrocarbon tainting without the use of organic solvents and would be a major improvement in monitoring of environmental hydrocarbon tainting.

The key outcomes of SPME as applied in this current study gave promising results for determination of hydrocarbons in water but did not for the measurement of hydrocarbon content in actual shellfish samples.

The conclusion of the study was unsatisfactory because by the time the SPME methodology was finally in determined it was too late to allow more than a few preliminary and frustrating trials with shellfish at a moderate temperature. Only with another two months could the hoped-for results have been demonstrated.

Suggested recommendations for future R&D include the following. Separation of hydrocarbons between water and animal lipids such as cellular triglycerides or even shellfish phospholipids at a moderate temperature could be a crucial factor. In addition, several more suitable selective fiber coatings or tissue treatments remain to be examined but because of the basic simplicity of the concept were not high priority.

Recommendations for practical application to industry from our limited explorations with very dilute aqueous solutions of hydrocarbons suggest that this technology could be applicable to contaminated waters if they were free of gross contamination with lipids.

Technical Summary

Analyses of monitoring shellfish deployed in ocean areas associated with petroleum and natural gas production off Nova Scotia was initially contracted to this laboratory in 1995. A method was developed for hydrocarbons that prevented contamination or loss of hydrocarbons, and was selective for an acceptable range of alkanes (C10- C25). This method took experienced technicians up to two days of work per sample, and required total lipid extraction and careful partitioning of the hydrocarbons by chromatography.

A faster analytical method for the sentinel shellfish was considered desirable, partly for economy, and partly to cope with the plethora of urgent samples generated by emergencies. Such a novel method appeared in the early 1990's in the guise of SPME (solid phase microextraction). With the experience of contract work as backup it appeared logical for the CIFT to explore SPME for recovery of contaminating alkane hydrocarbons from marine organisms with a particular objective of faster turnaround of sentinel samples. Basically SPME was a micro adaption of the very old technology of steam distillation for the purpose with dilute aqueous solutions of hydrocarbons recovery of volatile organic materials from dilute aqueous solutions. Our hypothesis that the lipids naturally present in the sample were "fixing" (tying up) the trace hydrocarbons is independently supported by our belated discovery of a 2000 paper from Denmark (Haar et al., 2000). However this came to our attention too late to be useful.

For SPME the objective was to absorb some of the organic molecules from the vapor phase above the warmed water phase in a small glass vial, using as an absorbant a bundle of silica fibers coated with a non-volatile organic polymer resistant to water. The fibers could then be inserted into the heated (250ºC) injection port of a gas chromatograph (GC) where the volatiles would immediately desorb and be separated on passage through the GC column.

Temperature control was vital to obtain reproducible results since the hydrocarbon materials to be absorbed and recovered (C10-C16) could include a boiling point range of 174 to 287ºC. At the same time it was felt necessary to include magnetic stirring capability since the volatiles should be liberated from the largest possible surface area of the sample and some mincing of muscle tissue to perhaps 1-2 mm size was forseen as necessary. The heating block design was in fact excellent for the purpose.

A popular fiber coating of dimethylpolysiloxane (DPMS) was selected for method development as it appeared to be the best available absorbant for hydrocarbons and was in use by others conducting SPME research.

The Figure series are attached as Apppendix A to this report. The fiber could be cleaned and did not itself add spurious hydrocarbon peaks to the analysis (Fig. 4). Halifax city water, distilled in the laboratory, could contribute at that time, three low level components which did not interfere with the hydrocarbons of interest for the SPME of water solutions.

Rock crab muscle (sample arrived wet with seawater from Scotian Shelf) showed (Fig. 8) a very feeble response to SPME for reasons not understood at the time. About 6 trace peaks were however seen in the appropriate locations for C14-C18 hydrocarbons. These hydrocarbons were shown to be present by extracting the same muscle with the usual recovery procedure and the anticipated hydrocarbons were recovered (Fig. 9). We feel retention of hydrocarbons by lipids could be the problem at the low temperatures used for the SPME. A similar result was obtained with blue mussels.

Other chemical classes of volatile materials could be present in fresh marine shellfish but this problem was not part of the initial exploration of temperature of operation for the critical recovery of hydrocarbons.

This work was necessarily stopped when the funds and time were exhausted. All spiked samples of hydrocarbons in plain water could be successfully recovered. The non-recovery of normal levels from tissue of rock crab and mussel blended with additional water was unexpected although possibly hinted at by the failure of steam distillation for low-level diesel oil contamination of lobster in 1975 (Ackman and Noble, 1973, Paradis and Ackman, 1975). Danish work suggests that adding a detergent to the homogenate to promote lipid emulsification might be useful to release hydrocarbons (Haahr et al., 2000).

Tentatively we have concluded that the natural presence of lipids in the muscle tissue of rock crab and of common mussel meat could be a limiting factor. These cellular lipids could "lock up"the hydrocarbons at the moderate temperature used in these trials. Although boiling in the range 174-287ºC, these hydrocarbons were recovered from water alone exactly as expected. Lipids in the organic animal tissues could be the factor preventing the hydrocarbon recovery under this fixed set of circumstances and do not invalidate the concept.

Bibliography for the Technical Summary

Ackman, R.G. and D. Noble, Steam Distillation: A Simple Technique for Recovery of Petroleum Hydrocarbons from Tainted Fish, Journal Fisheries Research Board of Canada, Vol. 30, No. 5: 711-714, 1973.

Paradis, M. and R.G. Ackman, Differentiation Between Natural Hydrocarbons and Low Level Diesel Oil Contamination in Cooked Lobster Meat, Journal Fisheries Research Board of Canada, Vol. 32, No. 2: 316-320, 1975.

Haahr, A.-M., et al., Flavour Release of Aldehydes and Diacetyl in Oil/Water Systems, Food Chemistry, 71: 355-362, 2000.

Introduction

This report is the culmination of more than a decade of participation in the use of mussels for monitoring off-shore petroleum exploitation off Nova Scotia, work that the Canadian Institute of Fisheries Technology has handled, partly on contract, and partly in training graduate students in environmental problems or food tainting. In combination with sensory evaluation, primarily for public relations purposes, isolation of C12-C21 hydrocarbons currently is by slow and multi-step handling. This is labor-intensive and as a consequence inapplicable in emergency situations.

A visiting scientist (M. Linder) experimented with SPME (solid phase microextraction) for natural flavors in scallops and his methods and results were published (1). He was able to access the GLC-MS aspect of SPME elsewhere in Halifax. With new and dedicated equipment the SPME technology could be the solution to the need for fast monitoring of hydrocarbon tainting of shellfish from local or offshore contamination in the Atlantic Provinces. There are far-reaching possibilities in food flavor research as well as in environmental concerns, since mussels are hardy and increasingly popular in the restaurant seafood sector. Unfortunately they can be tainted (inedible for flavor reasons, not necessarily dangerous) if grown in proximity to human activities. The same would apply to petroleum tainting of farmed salmon, an area where the CIFT established a reputation with projects that have educated several graduate students and were executed with the help of several visiting scientists (2-5).

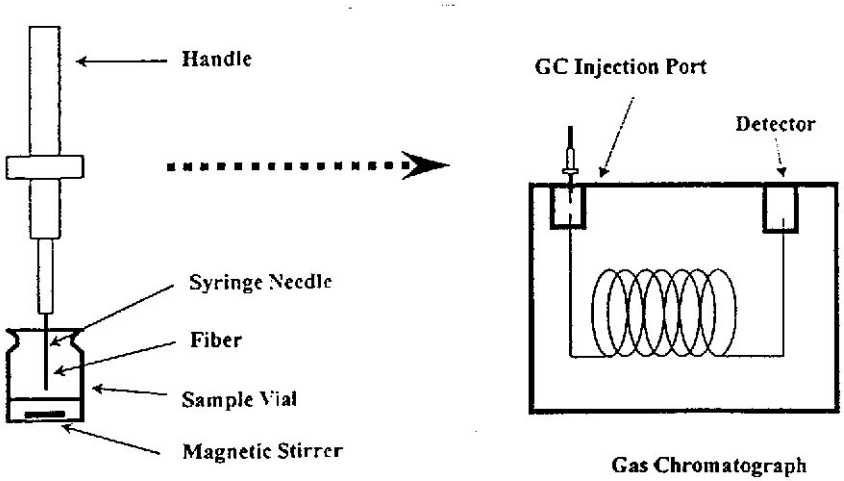
The objective of this project was to explore the possibility of SPME (solid phase microextraction) for rapid and simple determination of hydrocarbons and/or other contaminants in sentinel mussels and other marine life from the Scotian Shelf monitoring program. The standardized procedure currently in use for such analyses requires two to three days of solvent extraction of total lipid-type solubles, partitioning among liquid phases to clean up the lipids and prepare an unsaponifiable fraction, and a final separation of non-polar materials (i.e., hydrocarbons) from moderately polar materials such as fatty alcohols or sterols. This key step is conducted on a simple and commercially available silica column (Sep-Pak), but all are conducted with care to avoid loss of volatile hydrocarbons in the range C10-C25 for n-alkanes, but including some isoprenoids such as pristane and phytane, and even naphthalenes and methylnaphthalenes. This mixture can be traced in large part to a background of water-soluble hydrocarbons generated by marine alga and bacteria. However the profile of these materials had also been found to be reliable as an indicator of water-soluble spills of natural gas or light petroleum materials. They are all basically taken up and stored by animals in their lipids. Our work in this direction is described for fish in several years of publications (2-4), and particularly for mussels (5). The latter publication is attached to this report as Appendix B.

SPME as a technique began to appear in the literature about 1990 and by 2000 became established when Supelco recognized the market potential of SPME and began to supply most of the components for applications, with one exception which became important for our work on this project, accurate temperature control.

Most flavor researchers were dissatisfied with complex systems for "purge and trap" at one end of the spectrum, and with simple steam distillation at the other end. In fact the application of the latter technology to tainted fish was published by the grant holder as early as 1973 (6). It was useful for fingerprint identifications of hydrocarbon fuel contamination but was not judged applicable in this newer era of complex environmental concerns.

The main factor in luring people to SPME (7) for determining environmental contaminents was the apparent straightforward operation and simplicity (Figure 1).

Figure 1. SPME setup and SPME volatile collection device (left) with syringe needle inserted (right) into the injection port of a gas chromatograph (from 7).



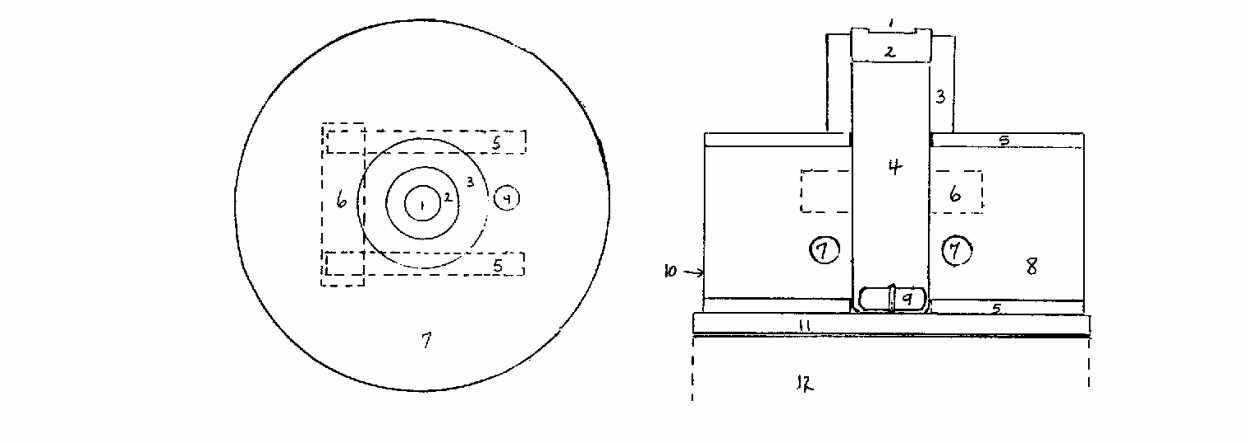
In brief, a special hollow needle is inserted through a rubber (Teflon-faced) septum into the headspace of a sample vial containing the sample, usually homogenized in water, and a cluster of silica fibers attached to a metal rod and coated with a suitable organic polymer is passed through the needle into that headspace to collect molecules of any volatiles present. After a predetermined time the fiber is retracted and the needle inserted into a specially modified injection port of a gas chromatograph. Suitable heating immediately desorbs the volatiles to be swept into the GC column.

The majority of papers on SPME that were published before 2000 were exploratory. The papers on SPME that were published after about 2000 were in food journals and with the aid of GC-MS announced new lists of simple and complex flavor molecules for vegetables, wines, herbs and spices or flavoring compounds. Up to 40 compounds would be listed, including unknowns, but with scant interest in quantitation which we regarded as essential for our work. One such paper from France (8) was for cooked mussels, processing which defeats the purpose of our work.

In the case at hand, volatile hydrocarbons over an aqueous headspace, variable partial vapor pressures would be encountered, requiring tight temperature control.

To satisfy this requirement for our work a considerable delay followed from the fact that no suitable heating pad, block, jacket or other system was commercially available. Accordingly a suitable small heating block housing the bottom part of the glass sample container and protecting the upper part from drafts was fabricated in the Department of Engineering shop (Figure 2). Auxiliary equipment for the GC injection system to support the syringe was also developed.

Figure 2. Top and side views of temperature controlled block designed for holding sample vials under stable and draft-free conditions. Designed and fabricated in the CIFT of Dalhousie University.



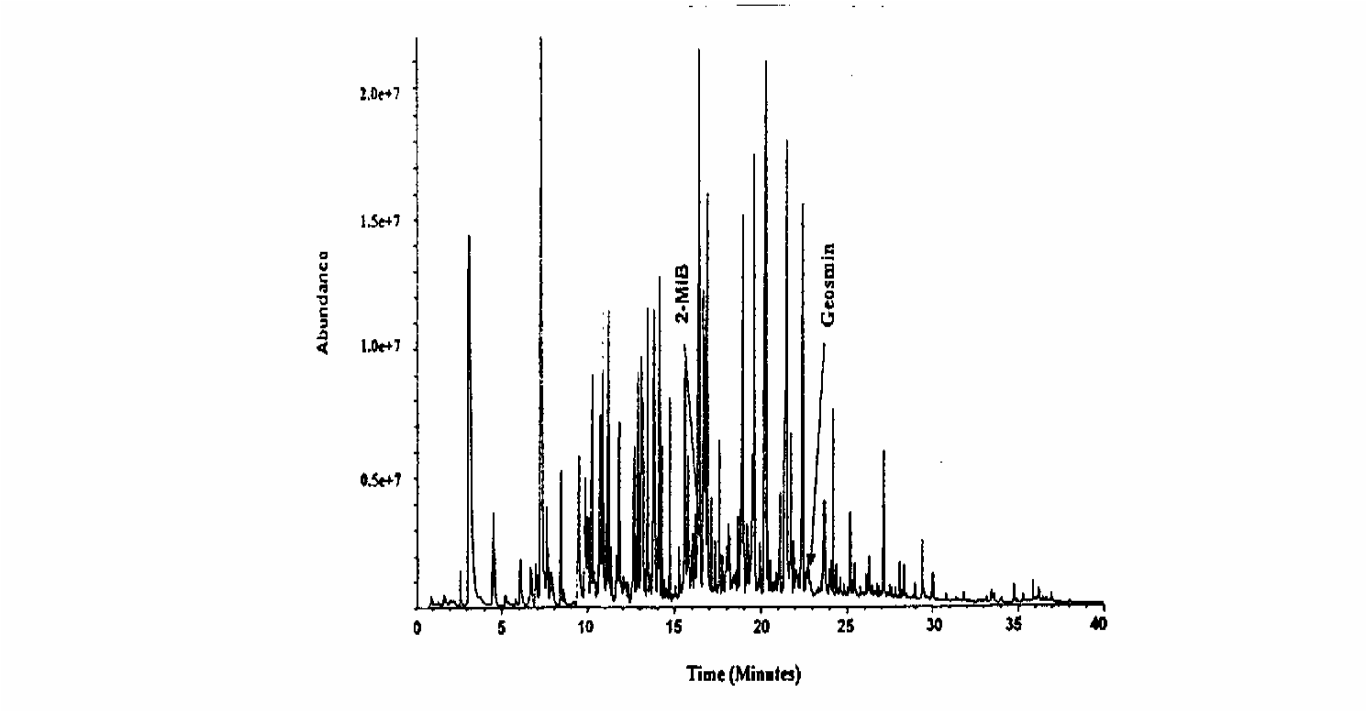
Top view: 1,PTFE/silicone septum; 2, phenolic screw cap; 3, Teflon collar; 4, thermometer well; 5, 80 watt electric heater; 6, 5 amp thermostat.

Side view: 1,PTFE/silicone septum; 2, phenolic screw cap; 3, Teflon collar; 4, SPME vial; 5, Teflon layer; 6, 5 amp thermostat; 7, 80 watt cartridge heaters;8, glass wool insulation; 9, stir bar; 10, aluminum casing; 11, thermal insulation pad; 12, magnetic stirrer top plate (ceramic).

About the time this unit was completed the laboratory mass spectrophotometer attached to a GC unit expired of old age. The early experiments with mussel tissue in water revealed that an unexpectedly large number of volatiles were captured. This should have been forseen but only one prior SPME paper (7) included fish volatiles. The chromatogram on recovery of the natural freshwater problem contaminents geosmin and 2-methylisoborneol (2MIB) indicated (Figure 3) what might be expected, especially as these are from freshwater fish from notoriously contaminated water systems in the southern USA. On the other hand research surveys of local seawater samples near Halifax and in an isolated area with mussel farms, suggested that biogenic hydrocarbons were to be expected as a substantial part of seawater solubles or particles (9). Our view was that the mussels could be “cleaner” in respect to total volatiles than fish and as long as the background hydrocarbon levels anticipated were at least comparable in magnitude to

many of the other mussel volatiles, the desired hydrocarbon peaks could be selected and quantified with new developments and GC-MS equipment. NSERC Grant applications were put in train towards acquiring that GC-MS equipment, a normally lengthy process.

Figure 3. Total ion chromatogram of an MD-SPME trace of volatiles from an off-flavor catfish (from 7).



This report is based on the funded work, which was carried out irregularly by a highly qualified technician when high priority contract work did not interfere. We have reached a limited conclusion, that the levels of a specific hydrocarbon mixture known from our other technology to resemble that naturally present in mussels were recoverable from very dilute water solutions. The same hydrocarbons could not be successfully desorbed from the animal sample matrix, and absorbed on fibers and quantified, under the particular temperature/time procedure developed for the hydrocarbons in water, with no oil or other lipid present. That was in fact proof, that in SPME, absorption on the fibers and desorption in the gas chromatograph were practical. However, the separation of hydrocarbons between water and animal lipids such as triglyceride oils or even phospholipids at a moderate temperature could be a crucial factor, but no funding or time remained for exploring this matter. In addition, some more suitable selective fiber coatings or tissue treatments remain to be examined because of the basic simplicity of the concept.

Objectives

The objective of this project was to explore the possibility of applying SPME (solid phase microextraction) for rapid and simple determination of hydrocarbons and/or other contaminants in sentinel mussels and other marine life from the Scotian Shelf monitoring program.

Results and Discussion

Special Equipment

Heating Block

Heating of the sample must be consistent to provide reproducible analyte release into the headspace. To attain this a heated block was designed and fabricated (Fig.2). This consists of two 80 watt cartridge heaters controlled by a thermostat and powered by one dedicated rheostat. The block is aluminum jacketed to prevent heat loss, and covered with a Teflon layer to prevent radiant heat transfer to the headspace from the surrounding top surface of the block. The block has 2 wells respectively for a thermometer and for one of the standard 22 ml clear glass SPME vials (Supelco, Sigma-Aldrich, Oakville, ON, Cat.No. 27170). The vials have polyphenol screw-cap tops fitted with replaceable single-use Teflon-coated silicone rubber septa.

The depth of the well for the vial is such that the top of the liquid is visible so that the vortex area can be plainly viewed to allow correct depth of insertion of the SPME fiber. The glass bottom of the vial is immediately above a variable-speed magnetic stir plate where the heating block sits on a thermal insulation pad on.

The block temperature was adjusted to 51EC and the temperature monitored over the work day via the thermometer in the well of the block, initially with no stirring and then with the magnetic stir plate set to the speed normally used with samples. There was no difference in the heating of the block whether the mixer was on or not. Next, the temperature of the water in the SPME vial was compared by thermometer to the temperature of the block. The water temperature was consistently the same as the block temperature. Finally, the temperature of the headspace compared to the block temperature was monitored. The headspace was consistently 10EC cooler. A Teflon ring was then designed to slide down over the vial to shield the headspace from air currents and prevent heat loss. With this improvement the headspace was now consistently only 5EC cooler. Following this design multiple sample units could provide material as rapidly as available GC or GC-MS would be able to handle.

Stir Bars

Vortexing of sample must be consistent. The use of small Teflon-coated magnetic stir bars was adequate for pure hydrocarbon standards in distilled water, however the introduction of actual mussel and crab homogenate necessitated sourcing the largest magnetic stir bar (Fisher Cat. No. 14-511-62, 13 mm x 9 mm) that could enter the

opening of the SPME vial to force movement in the viscous sample. Even with this modification it was necessary to dilute the mussel tissue homogenate with distilled water (wt:wt, 1:1.5) and the crab tissue homogenate with distilled water (wt:wt, 1:4) to cause stirring with an effective vortex . The SPME fiber could then be exposed in the headspace of the vortex as it had been with the pure standards in distilled water.

SPME Holder Support

A support was designed to hold the SPME holder in a stable upright position on the GC injection port. It is an aluminum cylinder open at both ends which fits like a glove over the injection port septum cap of the particular GC unit employed.

SPME Fiber

For volatile capture and transfer a 30 Φm fiber polydimethylsiloxane (PDMS) (Supelco, Sigma-Aldrich, Oakville, ON) was used for this project. This coated fiber is actually designed to trap hydrocarbons in the range of C5 to C30 (molecular weight 80 - 500). Absorptive fibers such as PDMS have greater capacity and linear concentration ranges than adsorptive fibers and are good for semi-volatile components. A thinner coating film (30 Φm vs. 100 Φm) will give a quicker desorption of heavier components. The fiber is designed to retract into the steel sleeve of the SPME holder when it is not being exposed to headspace and until it is placed in the injection port of the gas chromatograph. The holder has an adjustable needle gauge (0 - 5 cm) that controls the depth of fiber positioning in the vortex region of the sample headspace or in the gas chromatograph injection port.

Gas Chromatograph

A Varian model 3400 equipped with a SPI splitless, temperature programmable, injection port and a flame ionization detector (Varian Canada Inc., Missauga, ON) was used.

Integrator

An LCI 100 laboratory computing integrator (Perkin Elmer Canada Inc., Woodbridge, ON) was used.

Analytical GC Column

DB-1MS dimethylpolysiloxane, 60m, 0.25 mm ID, 0.25 Φm coating (Chromatographic Specialties Inc., Oakville, ON).

GC Injection Port Liner:

Two different types were tried:

(1) Inlet liner for SPME designed for the Varian SPI 1093 injection port as advertised by Supelco (Sigma-Aldrich, Oakville,ON) (530 Φm).

This liner was advertised in the Supelco catalog as a "SPME" liner for this injection port. It has a narrow area in which the trapped analytes on the SPME fiber can be desorbed. Initially no peaks were seen when headspace analyses were run on the gas chromatograph. After verifying that there were no leaks in the injection port the technical assistance person at Varian Canada was contacted. The column must seal with the polyimide coating on the exterior of the column adhering to the inner surface of the injection port glass liner to enable introduction of the sample into the column when the SPI 1093 is used and the internal dimensions of the Supelco liner could not permit this.

(2) Varian SPI 1093 Glass insert (0.8 mm ID)

This liner has internal dimensions to permit sealing of the analytical column to the liner as well as to provide a small space in which the trapped analytes on the SPME fiber can be desorbed. This injection port inlet liner was then used for the rest of the project.

Materials

Distilled Water

Initially the headspace of water from a traditional laboratory glass still distillation system was analysed. This distilled water provided acceptable chromatography blanks with minimal baseline disturbance except for a few reproducible sharp peaks.

Standard Hydrocarbons (with abbreviations) were:
Decane (C10) (Matheson Coleman and Bell, East Rutherford, NJ)
Dodecane (C12) (Fluka, Sigma-Aldrich Canada Ltd., Oakville, ON)
Tetradecane (C14) (Fluka Sigma-Aldrich Canada Ltd., Oakville, ON)
Hexadecane (C16) (Alltech Associates, Deerfield, IL)
Pristane (Pris) (Sigma-Aldrich Canada Ltd., Oakville ON)
Ethylbenzene (EB) (Fluka,Sigma-Aldrich Canada Ltd., Oakville, ON)
1-Methylnaphthalene (1-MN) (Fluka, Sigma-Aldrich Canada Ltd., Oakville, ON)

All hydrocarbon standards had been analysed by gas chromatography previously for purity.

Operating Conditions

Routine Conditions for SPME Fiber Exposure to Headspace:

- 16 ml distilled water in the 22 ml SPME vial, water containing single or a standard mixture of hydrocarbons or a tissue homogenate, and a stir bar
- Heating block 51EC, vortex 30 minutes at speed setting 8 to equilibrate between the aqueous phase and the headspace volatiles in the headspace
- Expose 30 Φm PDMS fiber at barrel gauge depth of 1.4 cm for 20 minutes while the SPME vial is held at 51EC and the sample is being mixed with the magnetic stir bar
- Retract the SPME fiber into the steel sleeve
- Transfer SPME holder containing the exposed fiber to Varian 3400 gas chromatograph (GC), place it in the SPME support, and expose the fiber to the barrel gauge depth of

3.6 cm in the hot (250EC) injection port, thus positioning the fiber in the center of the injection port, and simultaneously start the GC run

Gas Chromatograph Program

Helium carrier 17 psi, injection port 250EC; detector 280EC, oven: 45EC for 15 minutes followed by a 13EC / minute ramp to 280EC. This temperature was held for 36 minutes.

Methodology Procedures

All new fibers were placed in the GC injection port (250EC) with flowing gas for 3 hours to condition. The GC was then run through two complete cycles to ensure that any impurities from the conditioning of the fiber were eluted from the system. The fiber was left in the injection port of the GC and a blank run of the fiber/GC system acquired. That in Figure 4 is a typical example.

The headspace of the distilled water to be used for this project was analysed. A typical resulting chromatogram is shown as Figure 5.

An n-alkane mixture dissolved in dichloromethane that was used for identification of component hydrocarbons in organic solvent extractions of saponified mussel flesh matrix samples routinely analysed in this laboratory (occasionally also crab meats) was injected on the GC to ensure that the chromatographic system was functioning properly (Figure 6).

Previous work on SPME in this laboratory was based on a 100 Φm PDMS fiber. An old standard solution of hydrocarbons (now used for qualitative results), dissolved in distilled water at a concentration used at that time, was run as a SPME sample to observe the sensitivity of the new 30 Φm PDMS fiber. The integrator attenuation was set at 32 (Figure 7). The C12, C14, C16, Pris and 1-MN are approximately 0.125 ppm, while the C10 and EB are approximately 0.375 ppm. Our improved temperature control for fiber exposure eventually allowed more sensitivity to be implemented for routine evaluation of absorption on this particular fiber. This study was necessary to improve the sensitivity of the method and achieve a substantial increase in response over previous work, and the ability to use more dilute standard solutions was achieved. The routine GC integrator attenuation for all work on this project was then standardized at 4. Figure 7 is the only figure at the 32 attenuation setting. This type of result was obtained repeatedly and provided evidence that SPME was applicable to aqueous solutions of hydrocarbons.

Initial Trials with Animal Samples

A homogenized rock crab flesh sample was analysed for hydrocarbon content by the routine method followed at this laboratory (saponification followed by separation and recovery of the non-saponifiables into an organic solvent) (Figure 9) and compared with

the chromatogram of the same sample obtained by SPME headspace (Figure 8). The procedure followed for the headspace of the crab homogenate involved gradually increasing the amount of distilled water which was added to the homgenized tissue until an acceptable viscosity was attained enabling thorough mixing of the mixture with the magnetic mixer (crab homogenate (3.2 g) in distilled water (12.8 g)). The initial crab homogenate was very viscous and would not mix well enough to have a vortex in which to expose the SPME fiber until this 4 : 1 ratio of water : homogenate was reached. The routine conditions described above for headspace sampling and analysis were then followed. There is no similarity in the chromatograms of the same rock crab sample hydrocarbon profile produced by the two different methods.

The crab sample in the SPME vial was spiked with 0.1 ml stock standard solution of a hydrocarbon mixture in distilled water providing C12, C14, C16, Pris at 0.0125 ppm, EB, 1-MN at 0.025 ppm and C10 at 0.0375 ppm in the homogenate. A headspace of this sample/standard mixture was analysed following the routine SPME procedure (Figure 10). A headspace of the same concentration of standard mixture (Figure 10A) analysed by GC is provided for comparison.

The response normally seen from headspace exposure of this concentration of standard mixture in distilled water to the 30 Φm PDMS fiber did not occur. It seems that when the matrix of the sample includes lipids, that prevents release of the hydrocarbons into the headspace. An alternative is that some volatile component in the matrix (not detected by GC-FID) absorbs preferentially on the PDMS fiber, thereby preventing the absorption of the hydrocarbons. While considering this unexpected development it was therefore decided to focus on the rate of absorption of hydrocarbons on the fiber since this information was not available.

Accordingly the next investigation undertaken was to determine the optimal exposure time of the SPME fiber to the headspace of a hydrocarbon standard mixture in distilled water. For this optimization study of volatile absorbance on the 30 Φm PDMS fiber a standard mixture was prepared at a concentration of C12, C14, C16, Pris 0.048 ppm, EB, 1-MN 0.096 ppm and C10 0.144 ppm.

The concentrations were varied so that the peak sizes on the GC were of similar size since the different hydrocarbons absorb to varying degrees on the PDMS fiber. All conditions so far described were held constant except for the time the PDMS 30 Φm fiber was exposed to the headspace of the standard mixture. This varied as follows: 5,10, 15, 20, 25 minutes. All exposures were done in duplicate on virgin standard aliquots. A chromatogram of the headspace of this hydrocarbon mixture at the optimal exposure time (20 minutes) is shown in Figure 11.

Optimization of Headspace Sampling Times

Area counts, average of two analyses
Sampling time

Hydrocarbon	5 min	10min	15 min	20 min	25 min
EB	810314	714988	679864	553012	492165
C10	199886	137788	74185	50246	28983
C12	342324	358975	268910	278502	171982
1-MN	2050935	1994478	2041714	2032350	2028899
C14	524568	690764	668376	801348	776462
C16	370136	453942	508650	612984	613962
Pris	360426	624889	644670	773701	1169736

Shorter exposure times of the fiber to the headspace of the standard mixture in distilled water gave larger area counts for C10, C12 and EB. Longer exposure times gave larger area counts for C14, C16 and Pris. 1-MN area counts were not affected by changes in exposure time, an important observation. The maximum area counts for EB and C10 were at the 5 minute interval, C12 at the 10 minute interval, C14 and C16 at the 20 minute exposure, while Pris area counts increased as time of exposure increased. These results are shown in Table 1. The 20 minute headspace sampling time at 51EC was chosen for all future development work towards routine applications.

A difficulty encountered was the inability to dissolve longer chain n-alkanes such as docosane (C22) in the distilled water. These are routinely observed in GC analyses of organic solvent extractions of saponified mussels. If an excessive amount (0.5 mg / 16 ml) of pure solid standard C22 was put in distilled water, and the headspace obtained was analysed as per the routine procedure, a peak matching the retention time of C22 was seen on the GC (Figure 12). However the C22 was clearly not dissolved and this peak perhaps represented only the minimal amount of volatiles from the C22 which did go into solution.

An investigation of a carrier substance that would dissolve the longer chain hydrocarbons (LCH) (C20, C22, C25) and itself be soluble in distilled water was undertaken. The LCH were soluble in benzene, however benzene is not miscible with water. They were soluble in acetone and isopropyl alcohol but immediately precipitated out when distilled water was added. They were insoluble in methanol and ethanol at room temperature. They were soluble in 62EC methyl ethyl ketone and remained in solution as it cooled to room temperature. A headspace of methyl ethyl ketone (MEK) diluted in distilled water at a concentration of 0.2 ppm was analysed by GC . The headspace of the MEK contained many peaks which would interfere with the hydrocarbon chromatography (Figure 13). Modern technology finds that many “chemically pure” solvents contain these unsuspected minor components that do not interfere with solvent applications such as extractions or GLC solutions.

The long-chain hydrocarbons seen in authentic natural marine animal tissue non-saponifiables, extracted and concentrated in hexane, and analysed by GC, must have been dissolved in the lipid component of the tissue matrix and were released upon saponification. We have not yet determined a way to create a standard mixture with which to spike distilled water or tissues to mimic all of the hydrocarbons observed in natural samples.

Two homogenized mussel samples were analysed for hydrocarbon content by the routine method followed at this laboratory (saponification followed by recovery of the non-saponifiables into an organic solvent) (Figures 16 and 18) and compared with the chromatograms of the same samples obtained by SPME headspace following the SPME procedure described previously (Figures 14 and 17). The mussel homogenate was far less viscous than the crab so mussel homogenate (6.4 g) in distilled water (9.6 g), a ratio of 1.5 parts distilled water:1 part mussel homogenate enabled thorough mixing by the magnetic stirrer. The chromatograms of the headspace of the two samples differ from the chromatograms of the hydrocarbon profiles obtained using the saponification and recovery of the non-saponifiables into organic solvent for the hydocarbon profiles. However the mussel sample No. 1 shown in Figure 14 indicates that some progress was being made with real animal tissues at this point.

Mussel sample No. 2 was interesting in that the large unidentified group of components recovered following the saponification procedure and included in reports filed at the time

(Fig. 18) was also similarly detected by SPME (Fig. 17). There must have been contamination of this mussel sample, possibly by drilling mud or some similar narrow distillate band of hydrocarbons.

The mussel sample No. 1 in the SPME vial was spiked with a hydrocarbon mixture of 0.1 ml stock standard solution in distilled water providing C12, C14, C16, Pris at 0.0125 ppm, EB, 1-MN at 0.025 ppm and C10 at 0.0375 ppm in the homogenate. A SPME headspace sampling of this sample/standard mixture was analysed according to the routine procedure (Figure 15). A headspace of the same concentration of standard mixture (Figure 10A) analysed by GC is provided for comparison.

In the spiked sample the responses normally seen from headspace exposure of this concentration of standard mixture in distilled water to the 30 Φm PDMS fiber did not occur. This exactly repeated the previous observations with crab tissue. This verifies that the matrix of the sample prevents release of the hydrocarbons into the headspace or possibly some component in the matrix (not detected by GC-FID) absorbs preferentially on the PDMS fiber thereby preventing the absorption of the hydrocarbons. Time factors prevented any exploration of higher temperatures, the most likely factor in the release of hydrocarbons from the tissue.

Saponification of the tissue to improve the hydrocarbon recovery was planned. A blank consisting of the amount of potassium hydroxide (KOH) to be used with sample matrix was put in distilled water and a headspace of this (routine method) was analysed

by GC (Figure 19). There were more peaks observed in the headspace of this "blank" than seen in the sample matrix . No supplier providing a "pure" grade of KOH could be sourced so this line of investigation was terminated. In fact a similar observation had been made independently with common salt (NaCL). It was shown (unpublished results) to freely absorb a variety of volatiles that could only be dislodged by baking at 600EC. This salt then gave clean baselines.

Acid digestion with H_2SO_4 was considered, however upon consultation with the technical assistance representative at Supelco we learned that the fiber would be destroyed by acid volatiles. If lipids in the animal tissues are in fact retaining hydrocarbons the next phase of investigation should be to use higher temperatures. Concurently some attempt to hydrolyse the lipids and/or disperse them, perhaps with non-volatile surfactants, could be tried. It might be fast-acting and preferable to hydrolysis, which is usually slow.

The results described were exploratory research findings. The need to stabilize temperatures to provide good replications or test serial changes in technology required time that limited productivity, but also often enabled it to mesh efficiently with other laboratory activities.

Technology Transfer

The final conclusion on the lipid as a problem hindering SPME with animal samples was only interpreted in April of 2004, when funds/time were exhausted. An oral presentation of it as an analytical tool for hydrocarbons dissolved in water is being considered.

Recommendations

This work should be supported as a graduate student project. Owing to the retirement of Dr. R.G. Ackman in June, 2004, a renewed PRAC application should come from Dr. Sue Budge, now director of the appropriate CIFT laboratory. With access to a new GC-MS it could be focused more on the distribution of hydrocarbons in fluids in mussel tissue, perhaps with emphasis on gills, and a view to recovery by gentle centrifugation or other means, rather than examination of the whole animal.

Networking

The CIFT was prepared to propose to Jacques Whitford (via S. Belford) that a routine sample of mussels, from offshore monitoring be the subject of duplicate analyses by two methods. However this objective could not be realized.

Funding

No other funding was provided for this study and no proposals are currently under review.

Conclusion

SPME is a technology still in its infancy. Despite the novelty of this application, papers appear sporadically, but now almost every month, in food journals describing applications in flavor profiling. Several are listed in Appendix C for background information, and SPME has now achieved mention in a book on flavor volatiles (R. Marsili). In fact this person prepared an ACS conference report for Supelco in 1998 (Appendix D). In view of the glowing titles of chapters, including one from Health Canada, progress over the last decade should have been more rapid. Apparently other laboratories also found technical practicality hard to achieve.

The work we have conducted was of a preliminary nature and regrettably was interrupted several times by the commercial requirements of our self-supporting Institute. In fact the total volume of work is about the same as that done for many M.Sc. level thesis projects in our Food Science Department.

A GC-MS is mandatory for this work, and is now standard equipment in pollution laboratories. To illustrate the difficulties in working with shellfish, reference (10) states that the human panelists could detect 42 odors in fresh oysters by "sniffing". Mussels of course continuously ingest unicellular plant cells for food, and also detritus from larger plants near the shore. To illustrate how these algal materials provide background volatiles from the mussel note that the common red algae *Palmaria palmata* yielded over 20 volatile chemicals by dynamic purge and trap analysis (11).

Our opinion is that the fiber absorption technology itself for hydrocarbons is satisfactory. It is the release of the desired range of hydrocarbons from the cellular tissue matrix that was the unexpected and critical obstacle to SPME use with shellfish. The few additional compounds from habitat water are not a serious problem since in practice, with routine environmental samples, GC-MS sorts out desired compounds in such situations.

In fact our limited explorations with very dilute aqueous solutions of hydrocarbons suggest that this technology could be applicable to contaminated waters if they were free of gross contamination with lipids. It is only recently that a probable source of the natural background of hydrocarbons dissolved in seawater has been identified in the North Atlantic. The common copepod *Calanus finmarchicus* has been shown to release DOC (dissolved organic matter) by either sloppy feeding or by leakage from fecal pellets (12). This can include the stable pristane and phytane from the reduction of the phytol in chlorophyll by this animal. These two hydrocarbons are a frequent feature of hydrocarbons in mussel lipids.

I am pleased to have Anne Timmins sign this report in appreciation of her patience in this frustrating but rewarding exploration.

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Listing of Appendices

Appendix A

List of Figures

Figure 4. Blank PDMS 30um fiber analysed by gas chromatography.

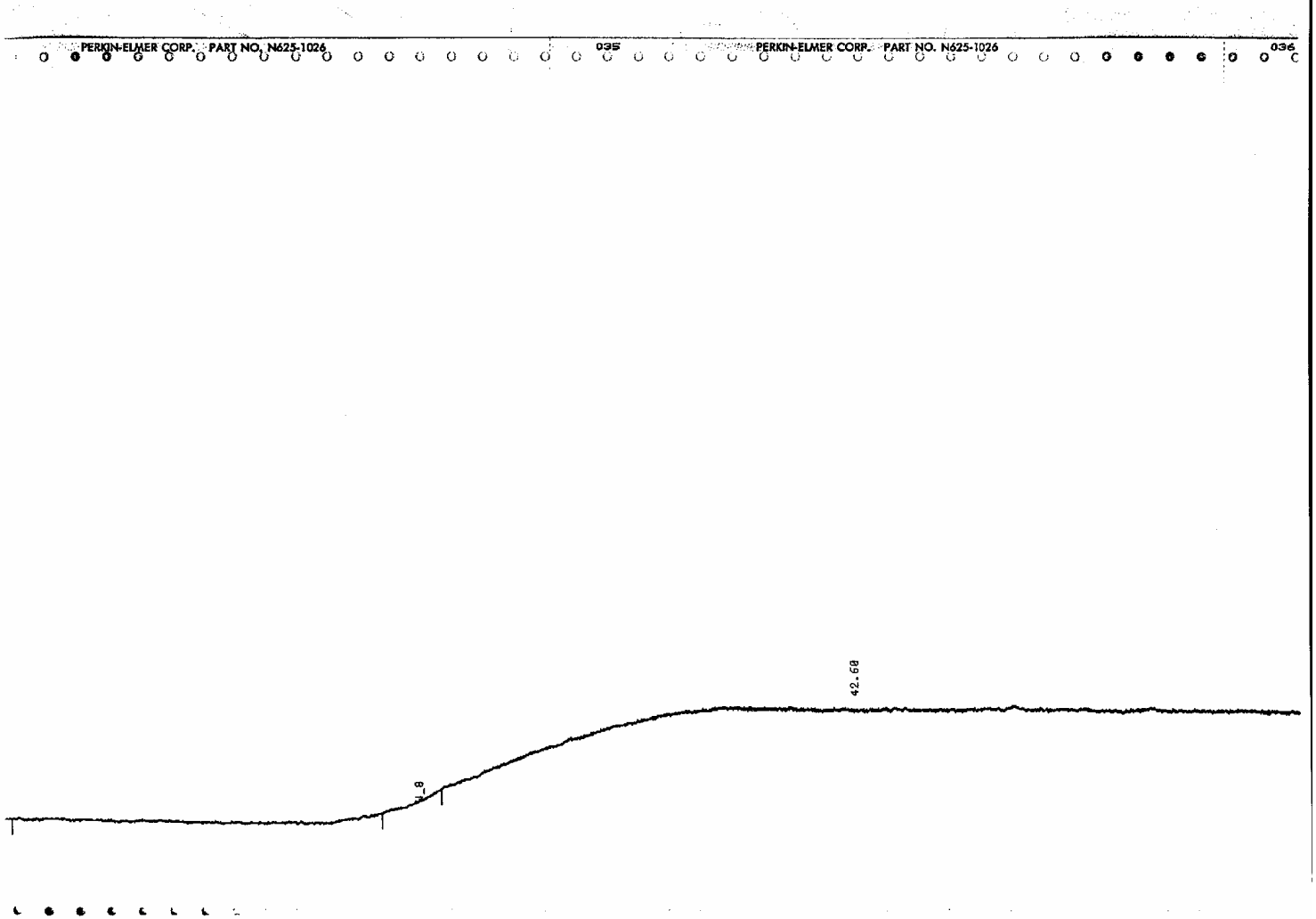


Figure 5. Headspace of vortexed distilled water analysed by gas chromatography.

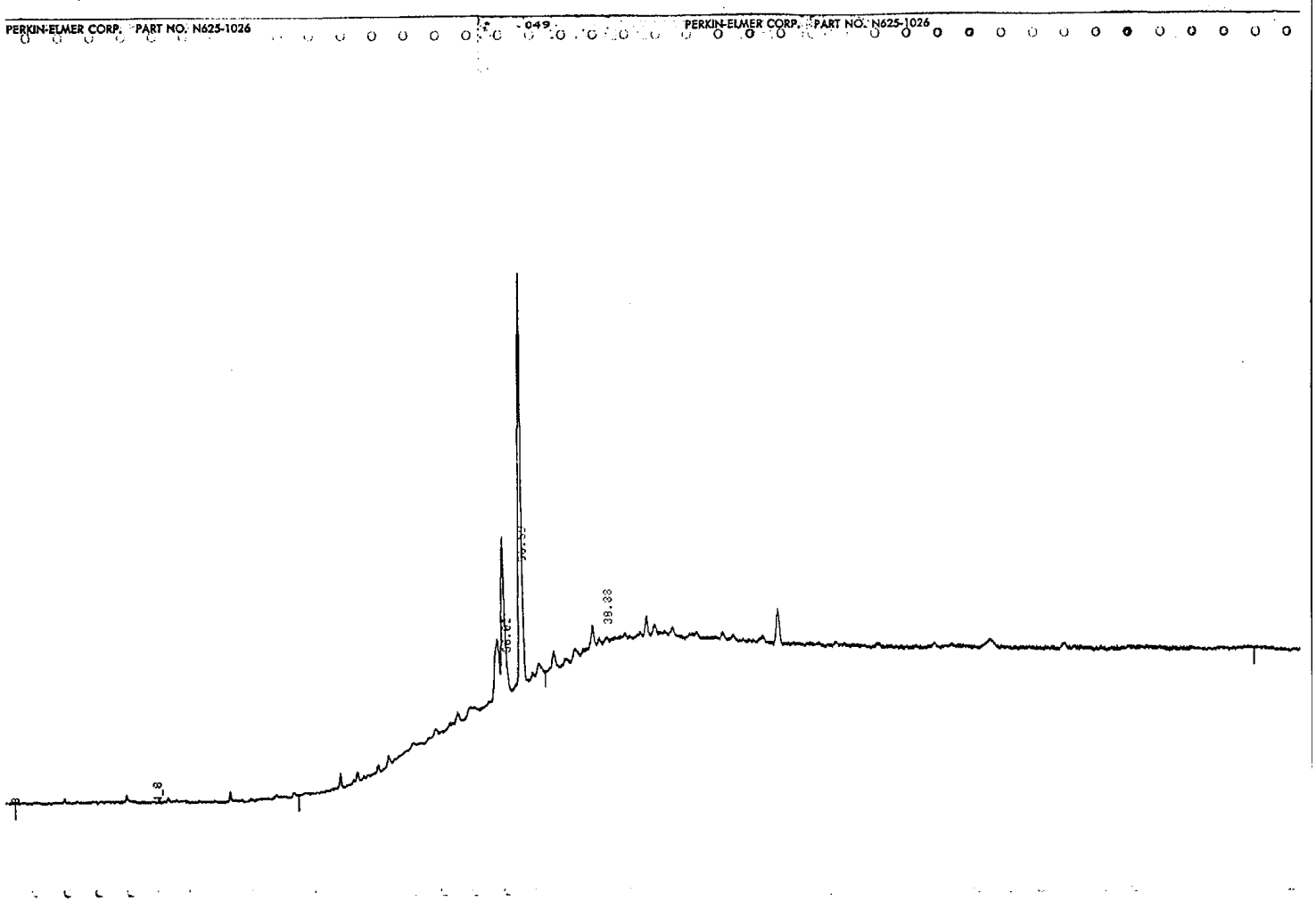
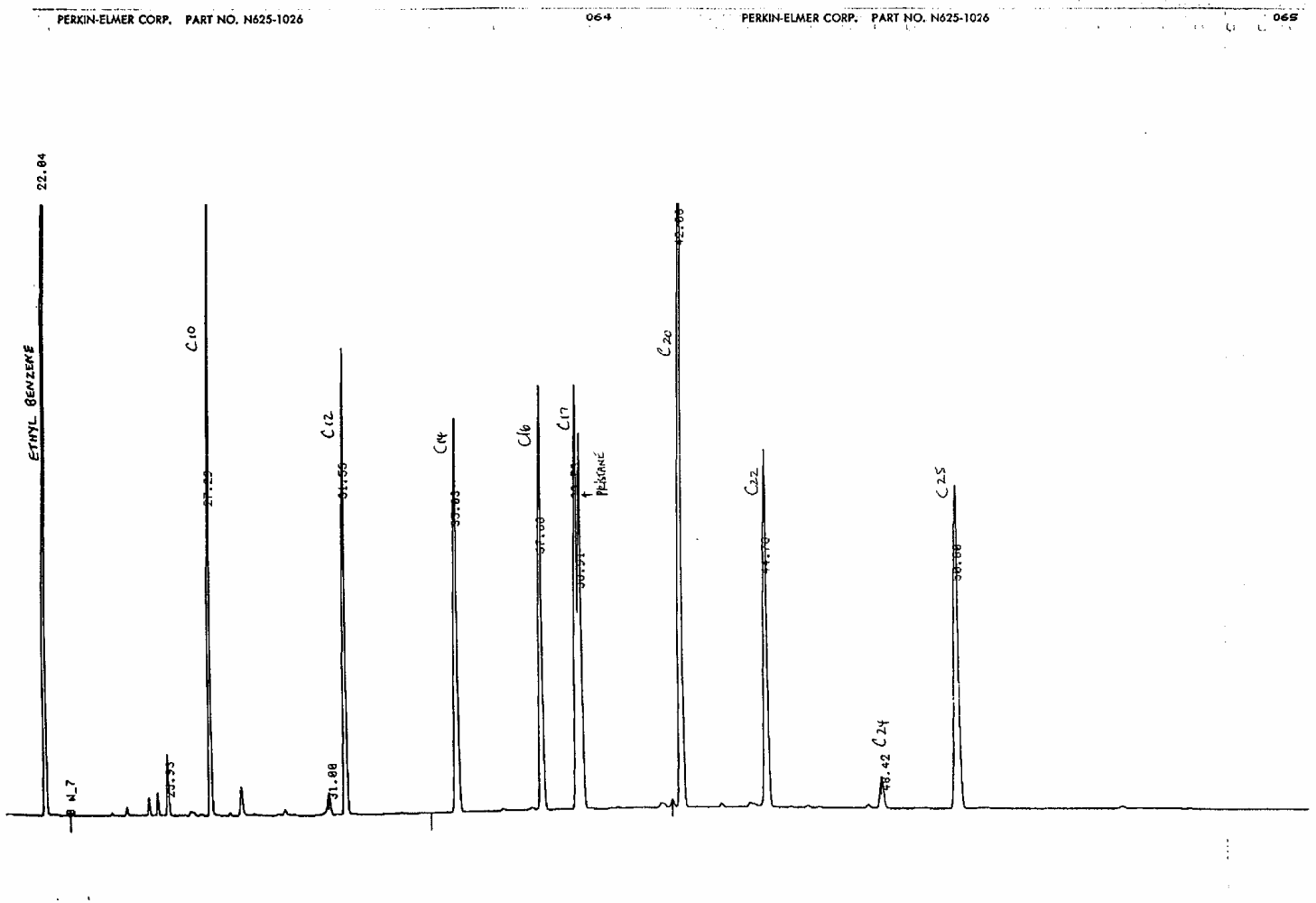


Figure 6. Liquid injection of standard mixture in dichloromethane analysed by gas chromatography to verify retention times of component hydrocarbons.



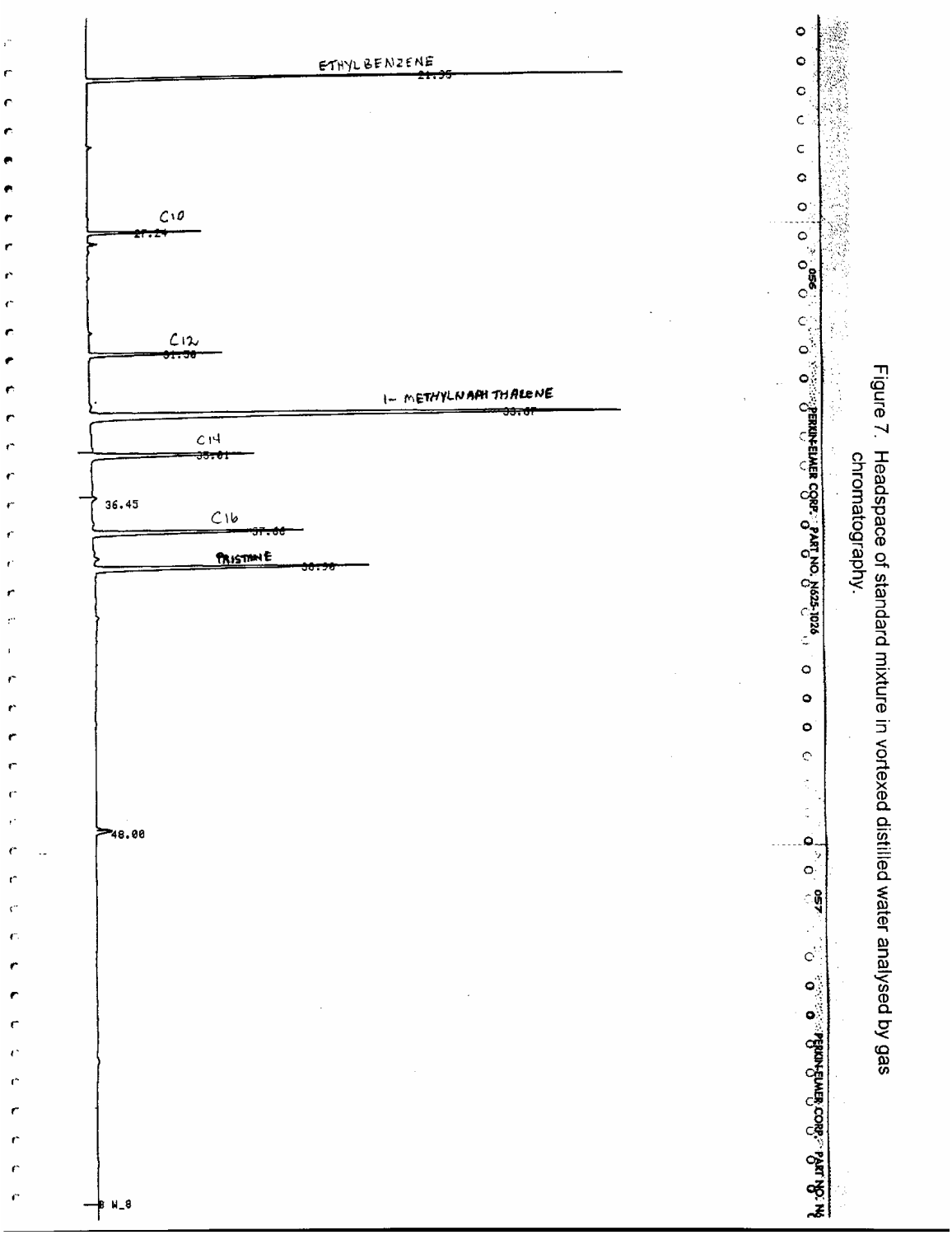
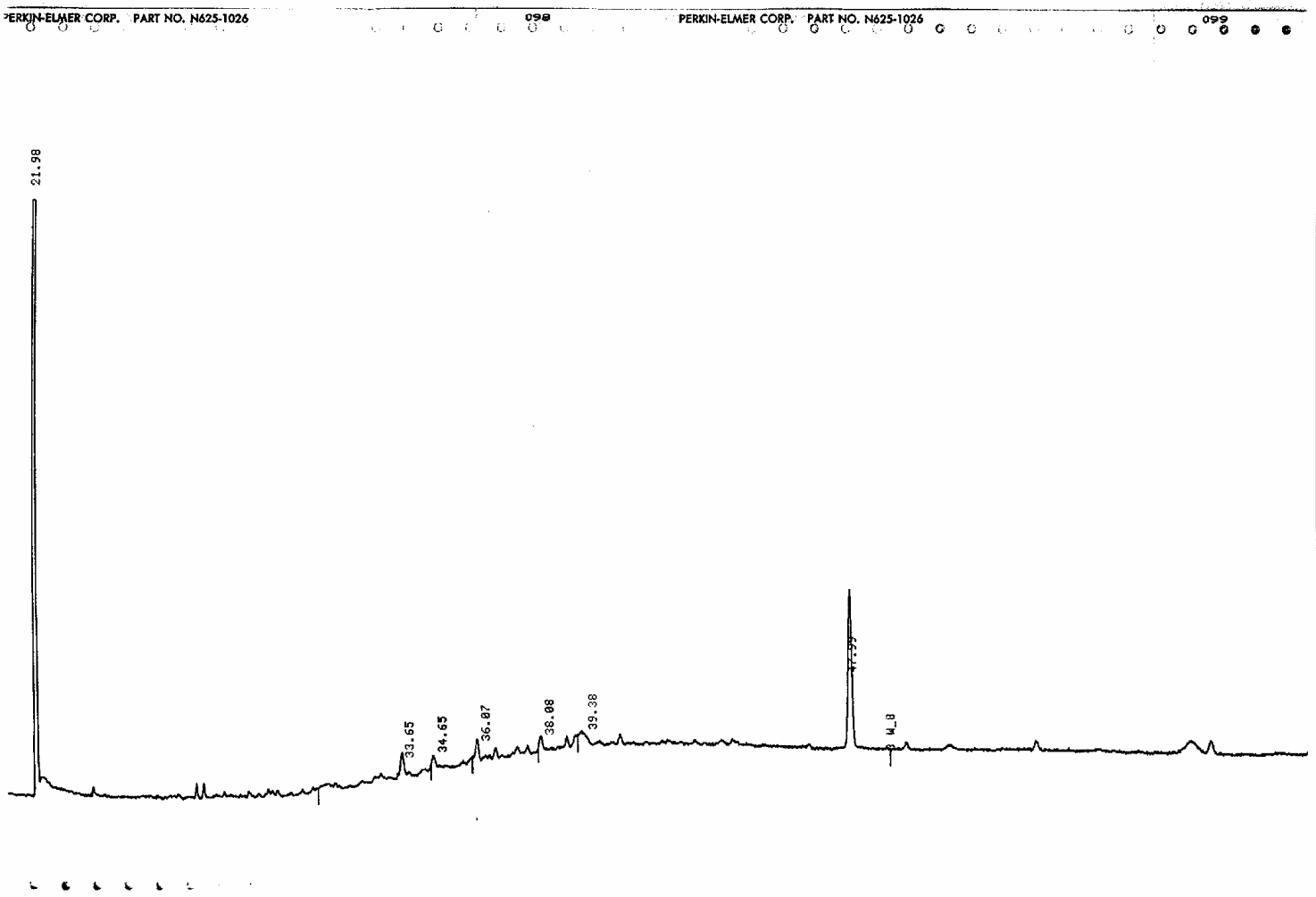


Figure 7. Headspace of standard mixture in vortexed distilled water analysed by gas chromatography.

Figure 8. Headspace of rock crab flesh in vortexed distilled water analysed by gas chromatography.



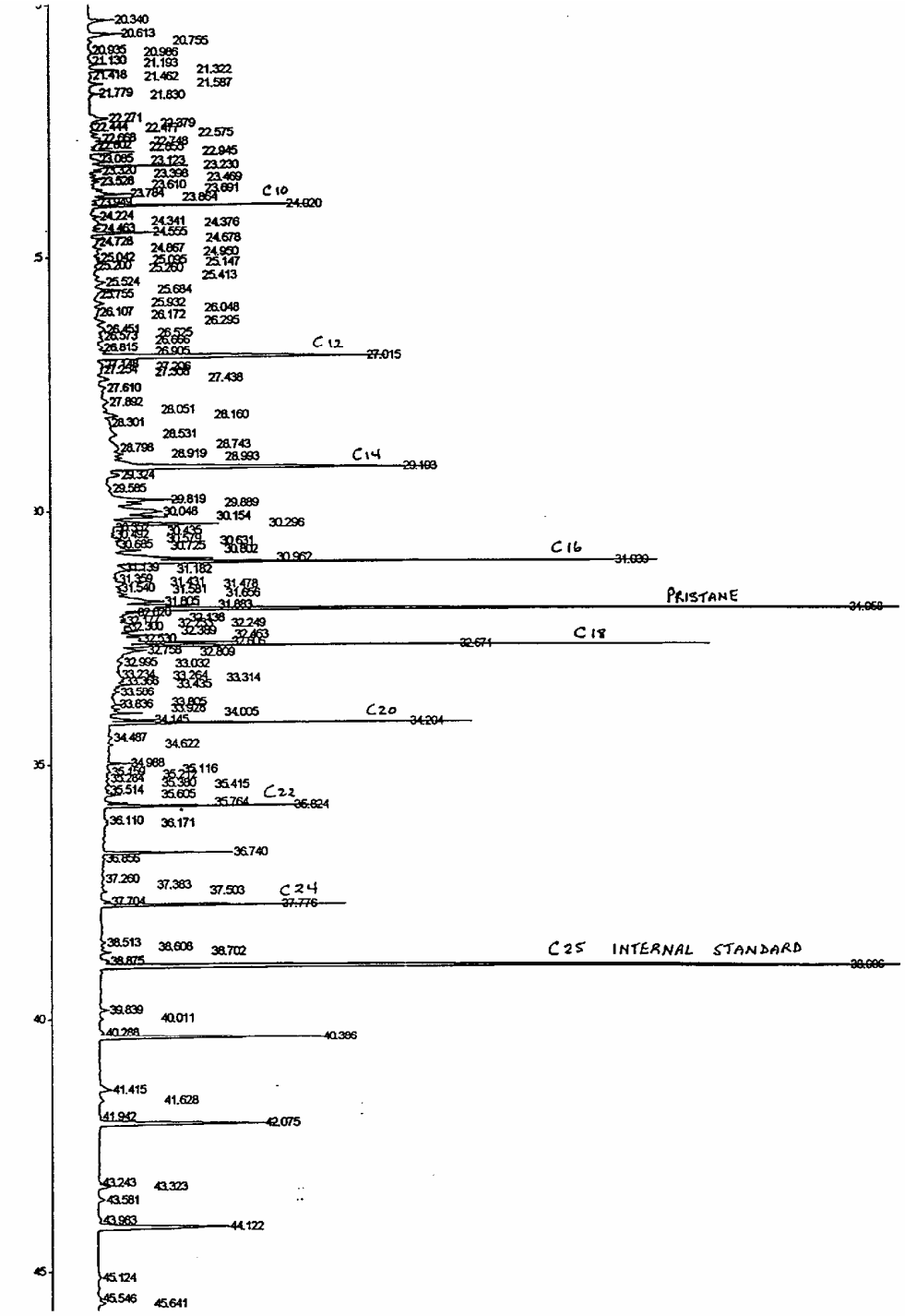


Figure 9. Non-saponifiables of rock crab flesh in hexane analysed by liquid injection on a gas chromatograph.

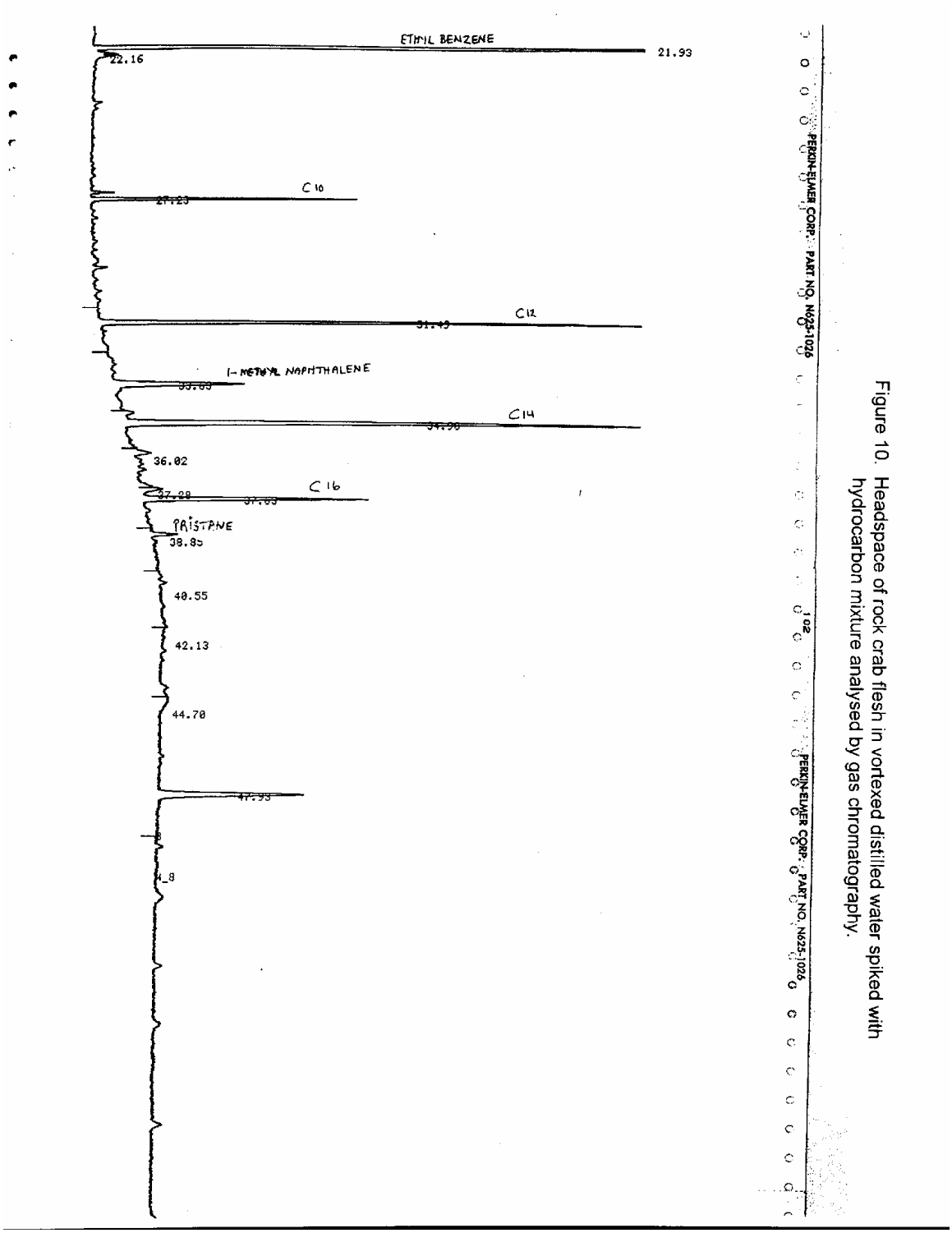


Figure 10. Headspace of rock crab flesh in vortexed distilled water spiked with hydrocarbon mixture analysed by gas chromatography.

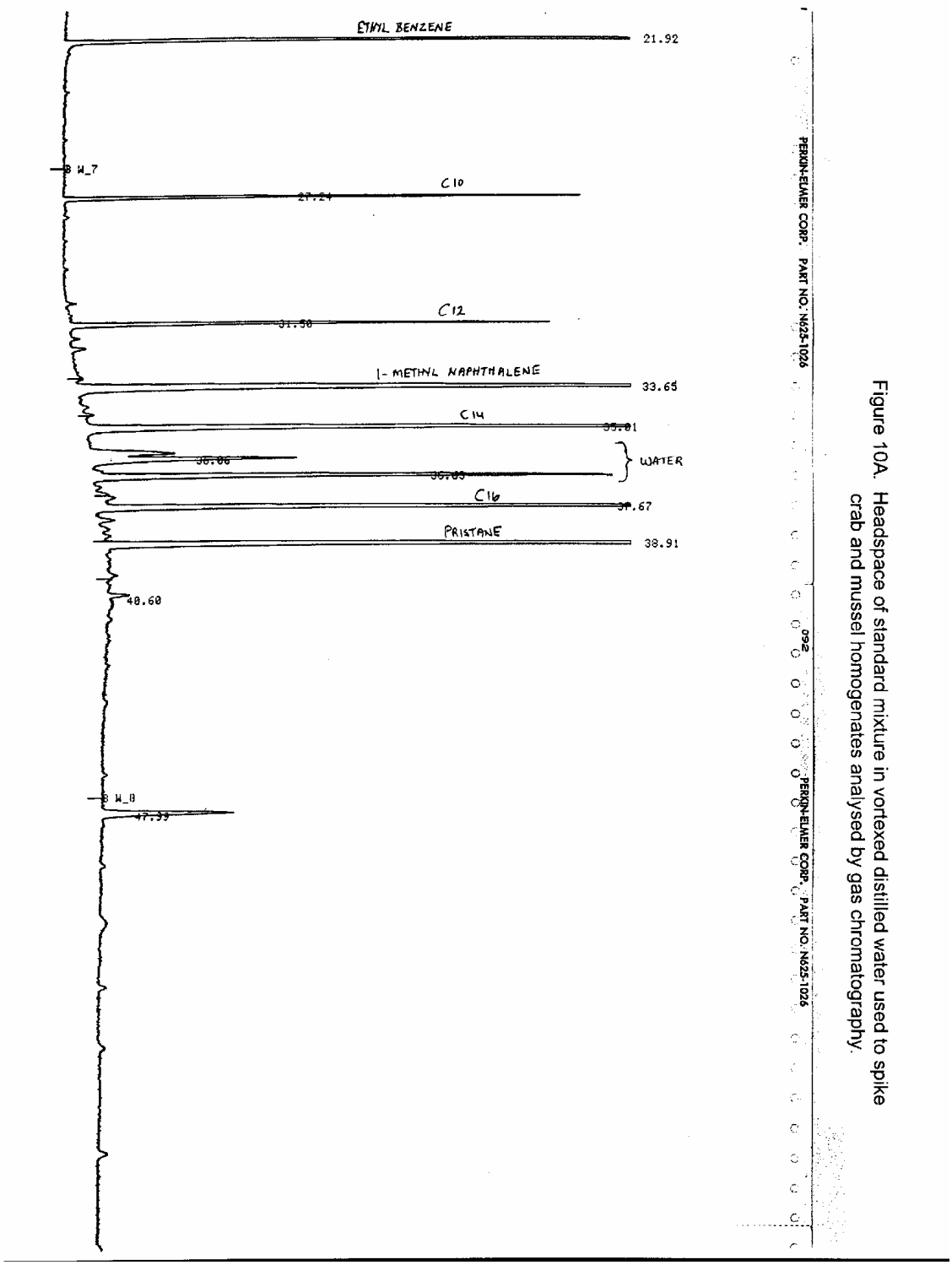


Figure 10A. Headspace of standard mixture in vortexed distilled water used to spike crab and mussel homogenates analysed by gas chromatography.

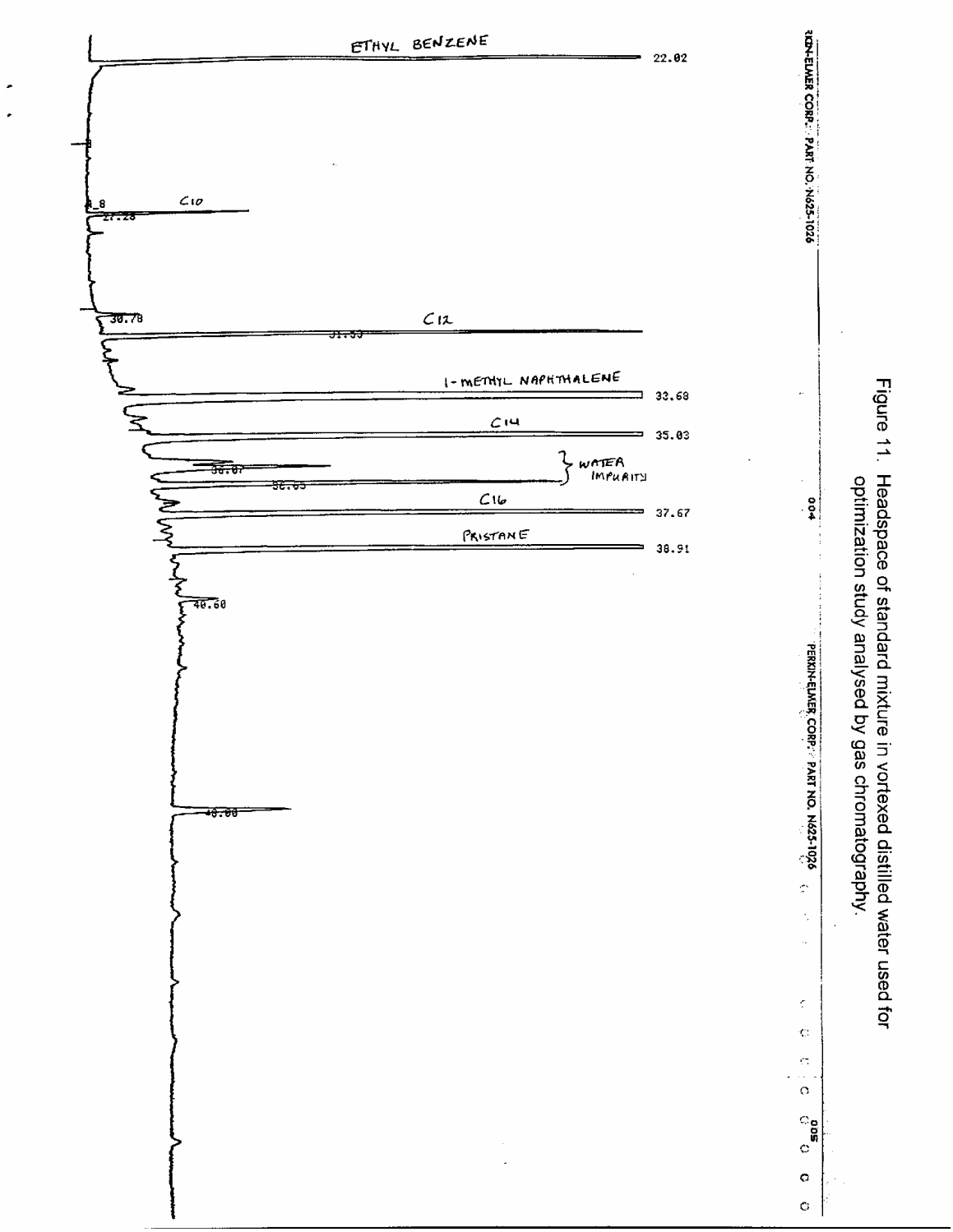


Figure 11. Headspace of standard mixture in vortexed distilled water used for optimization study analysed by gas chromatography.

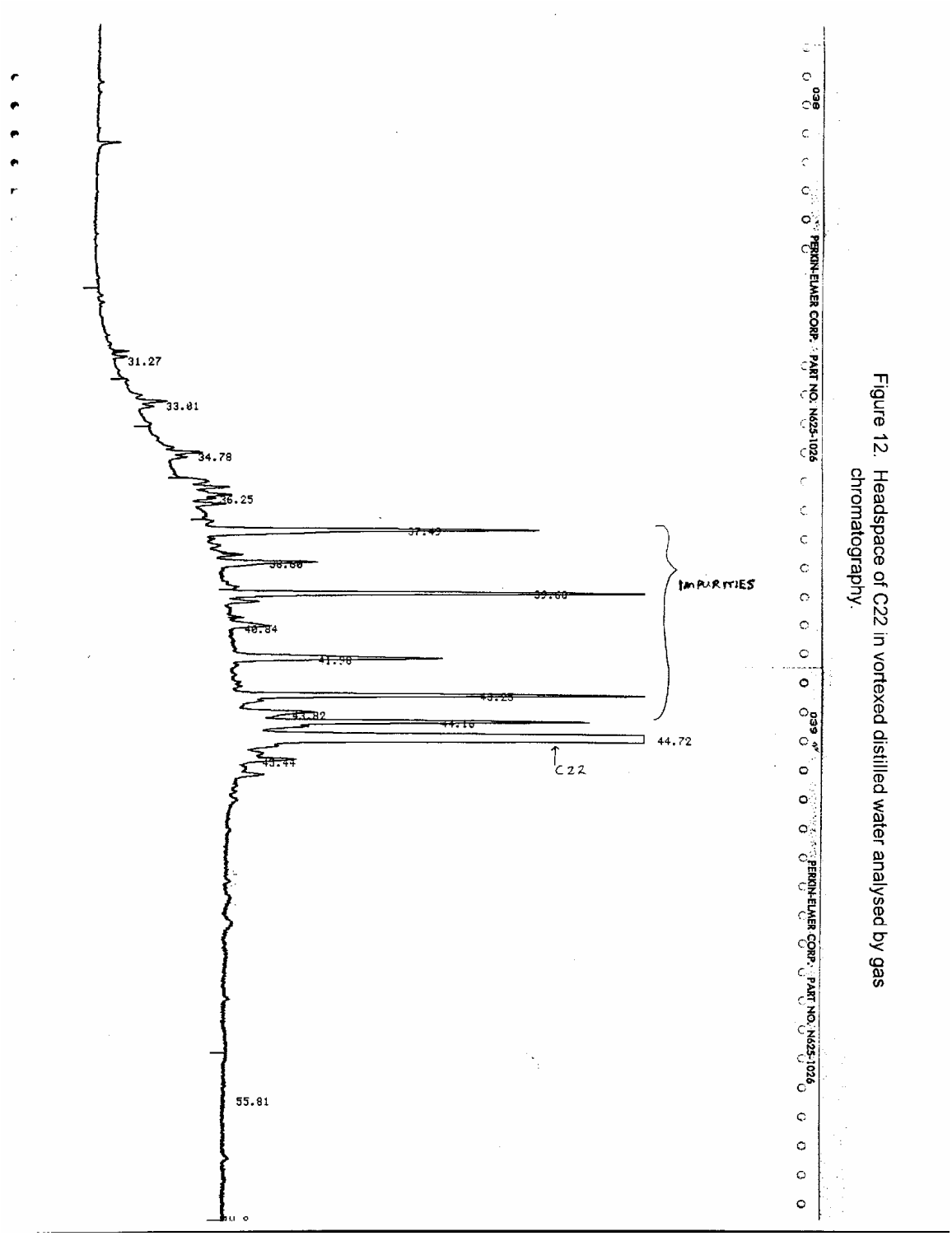
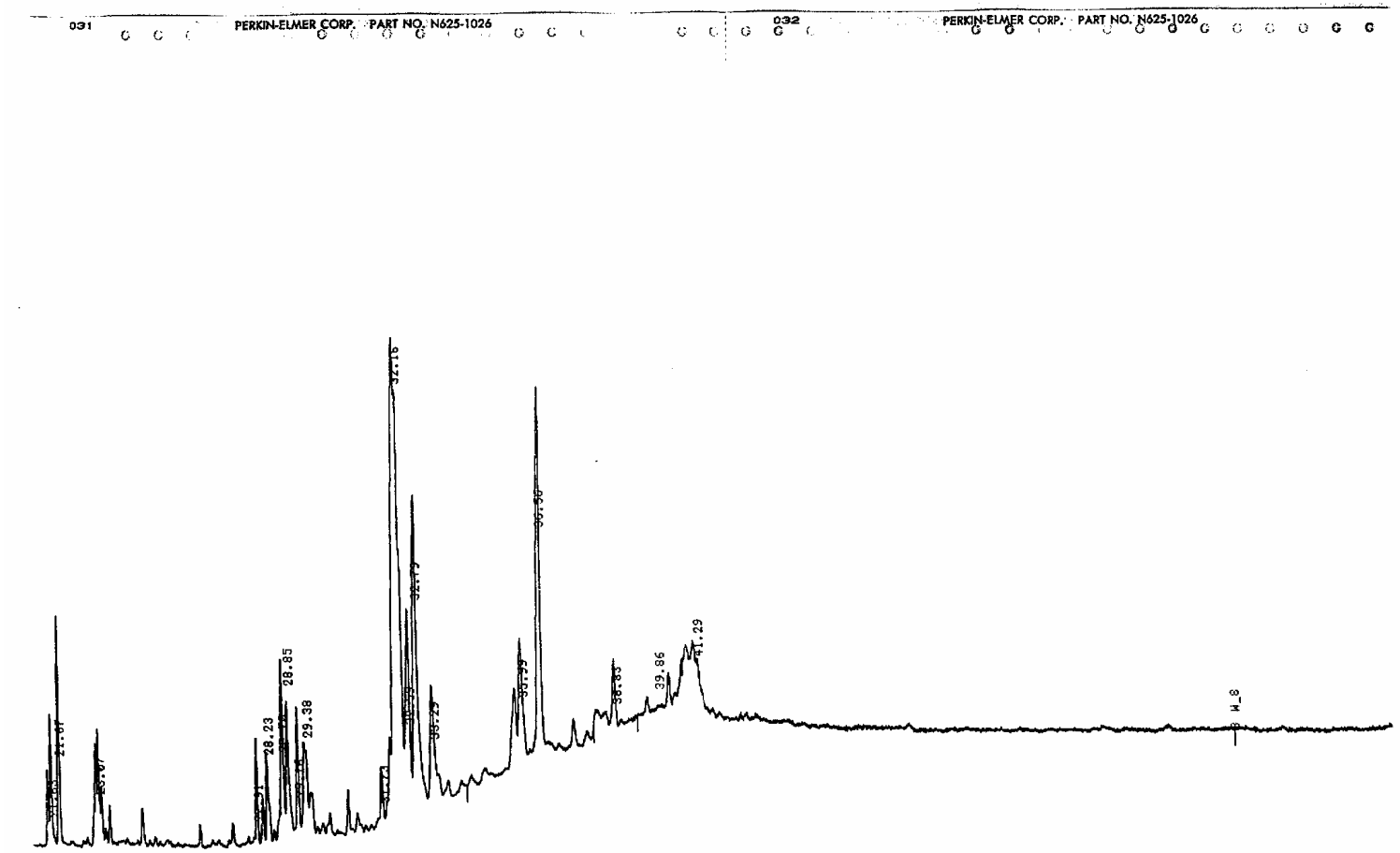


Figure 12. Headspace of C22 in vortexed distilled water analysed by gas chromatography.

Figure 13. Headspace of methyl ethyl ketone in vortexed distilled water analysed by gas chromatography.



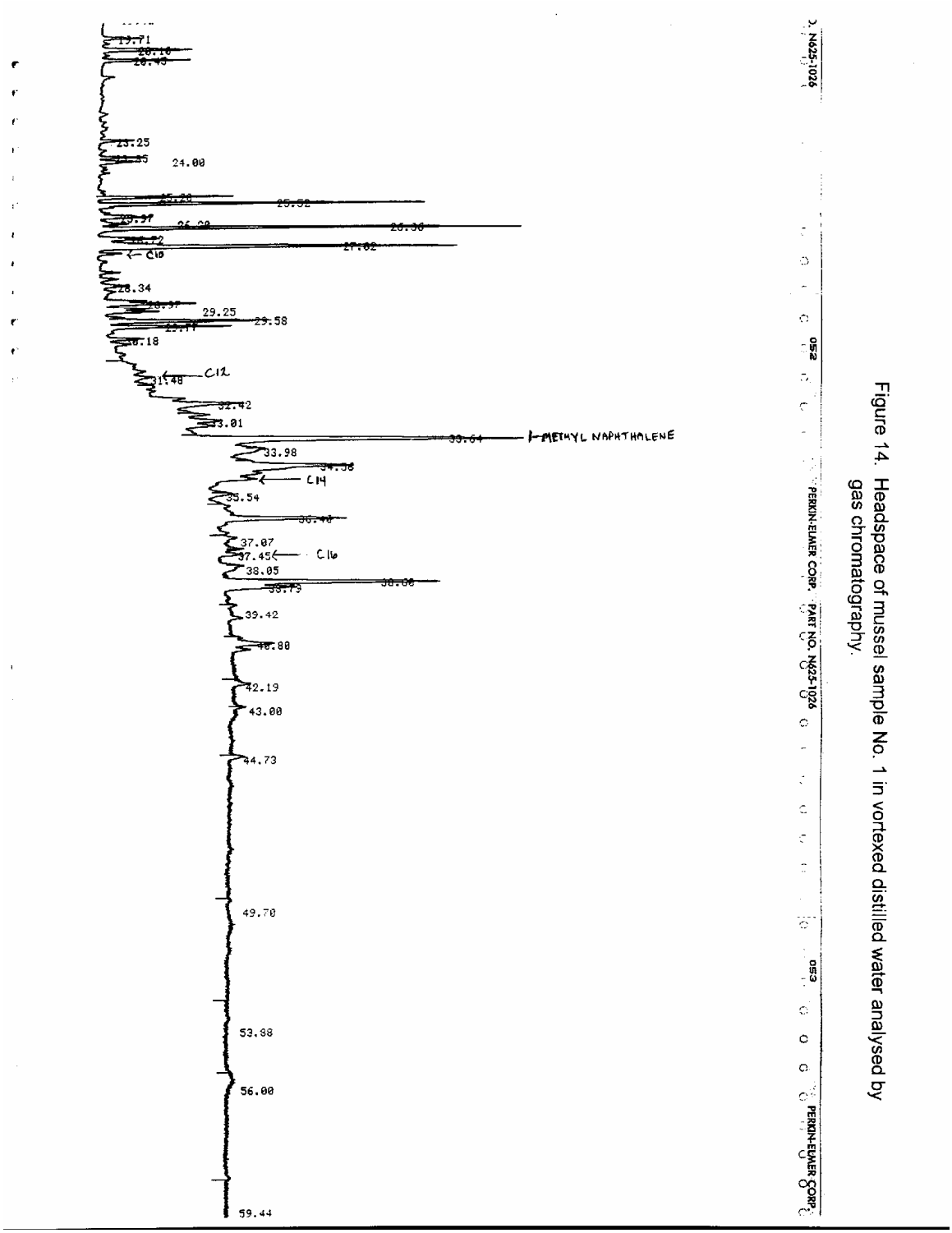


Figure 14. Headspace of mussel sample No. 1 in vortexed distilled water analysed by gas chromatography.

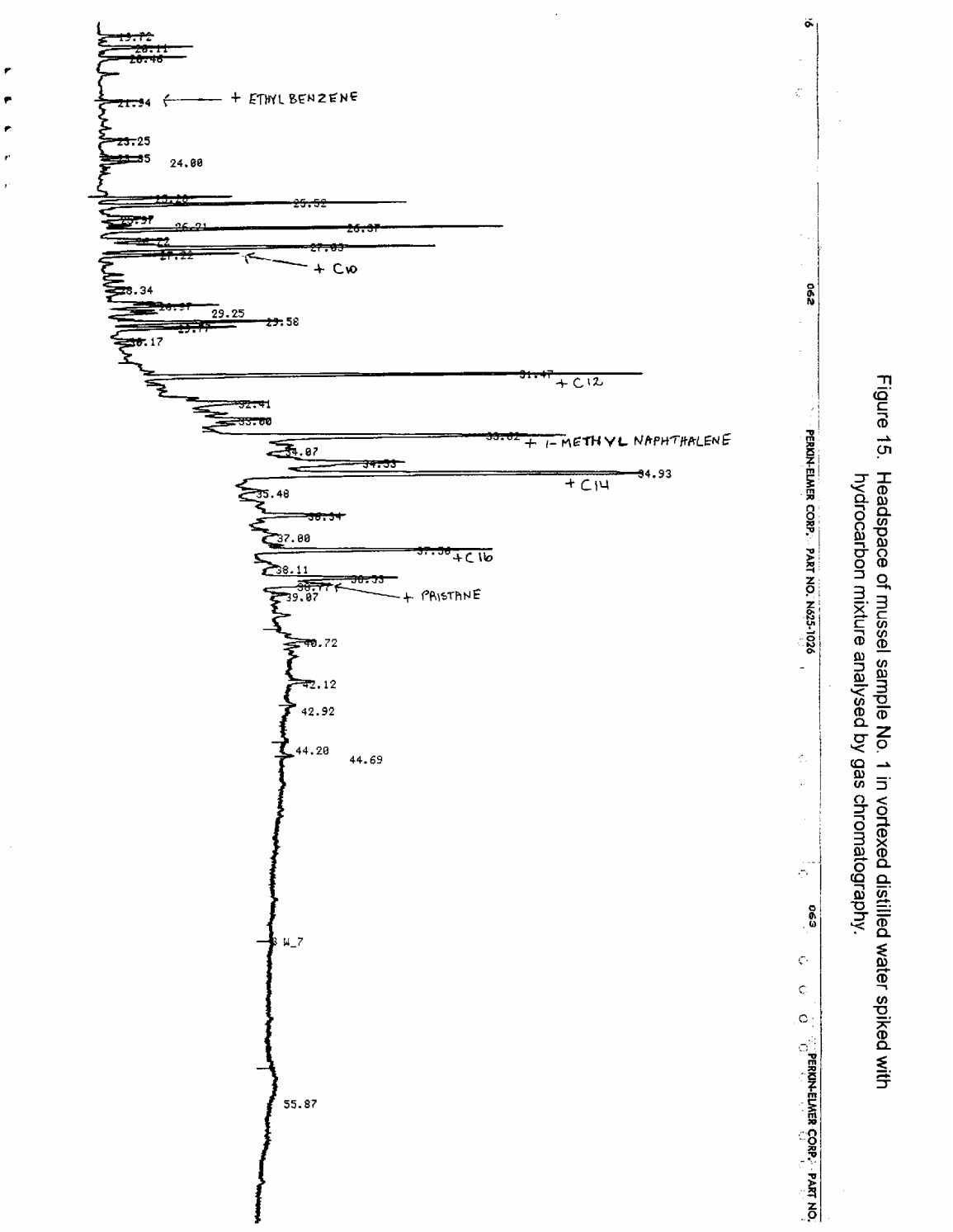


Figure 15. Headspace of mussel sample No. 1 in vortexed distilled water spiked with hydrocarbon mixture analysed by gas chromatography.

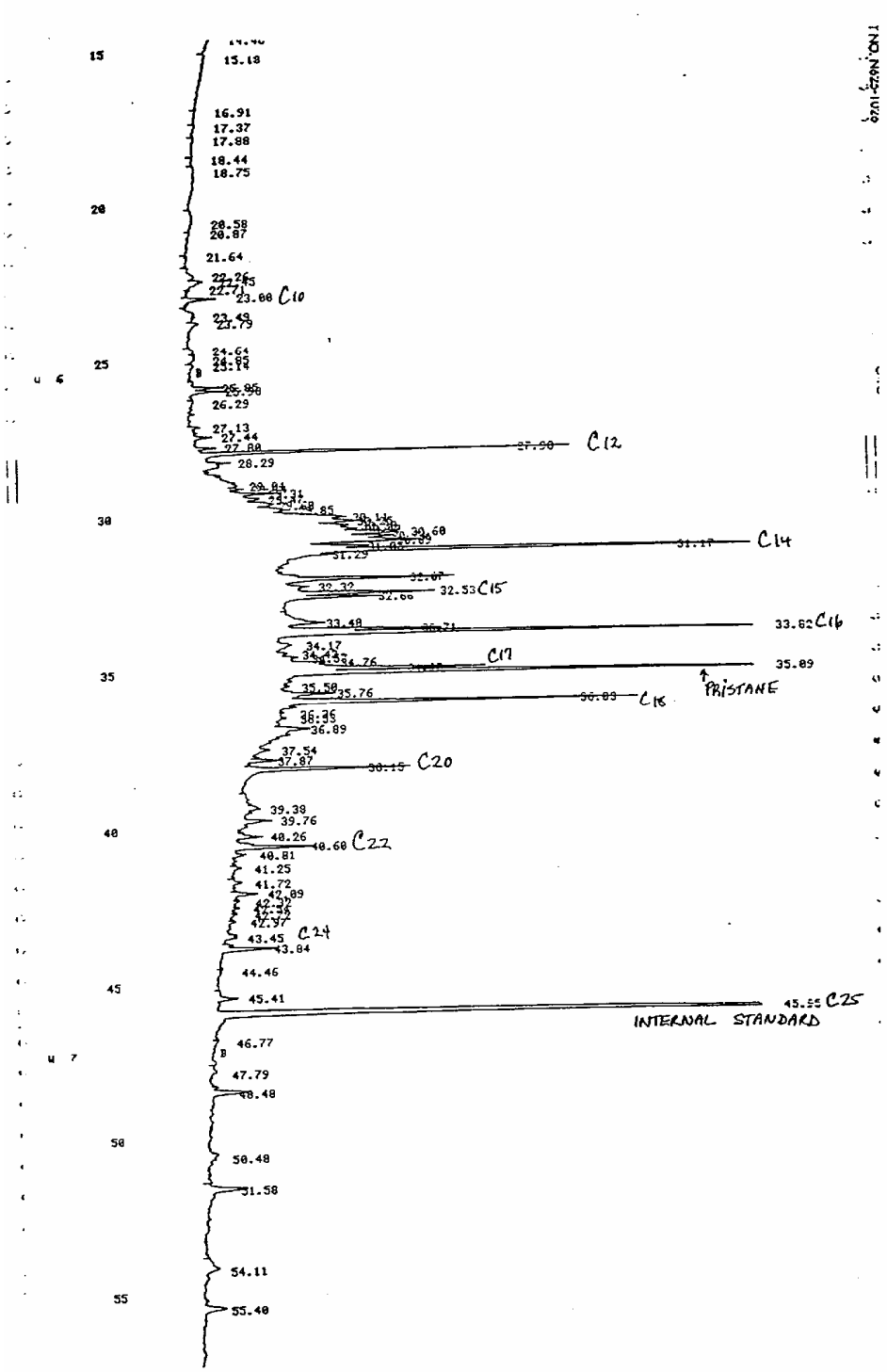


Figure 16. Non-saponifiables of mussel sample No. 1 in hexane analysed by liquid injection on a gas chromatograph.

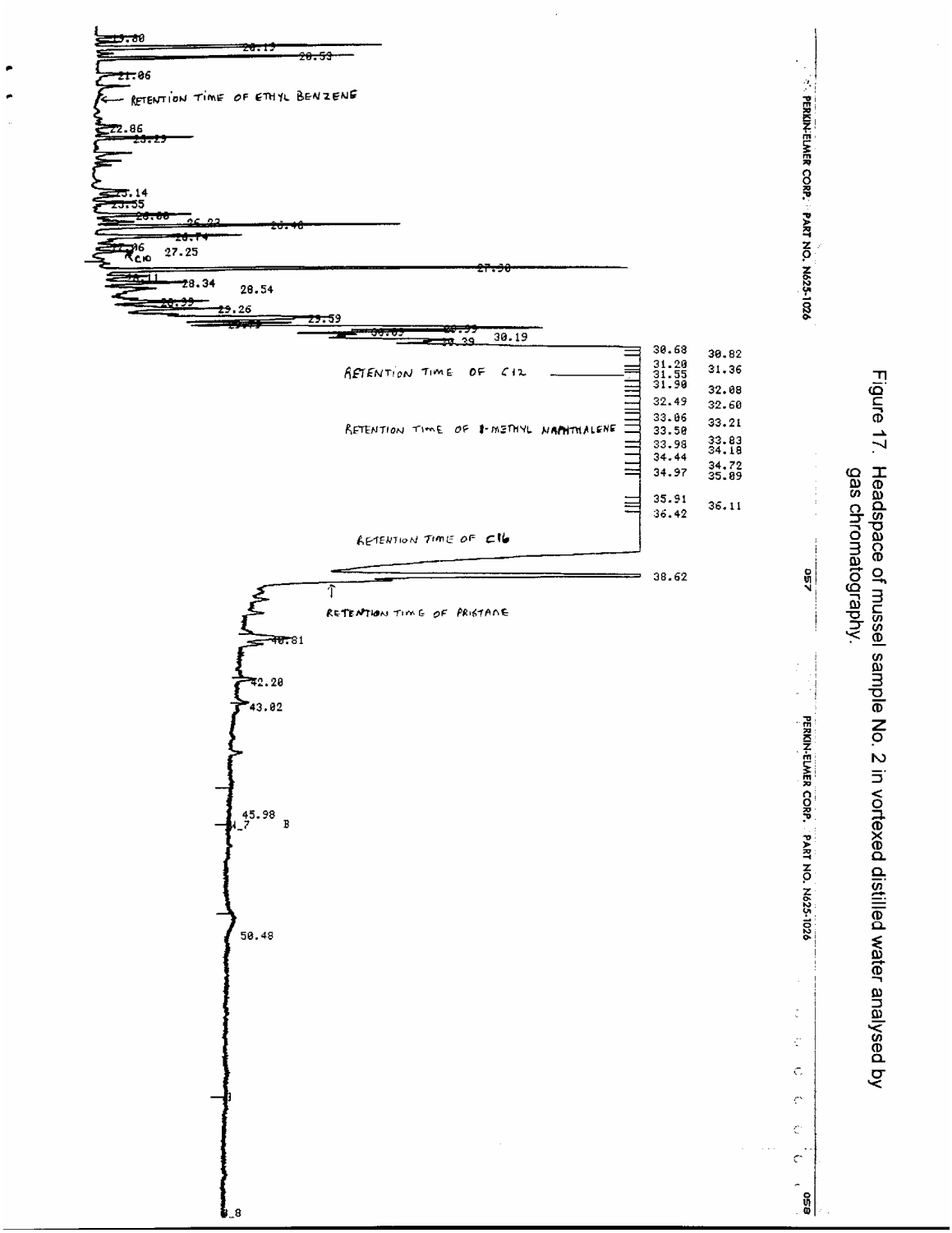


Figure 17. Headspace of mussel sample No. 2 in vortexed distilled water analysed by gas chromatography.

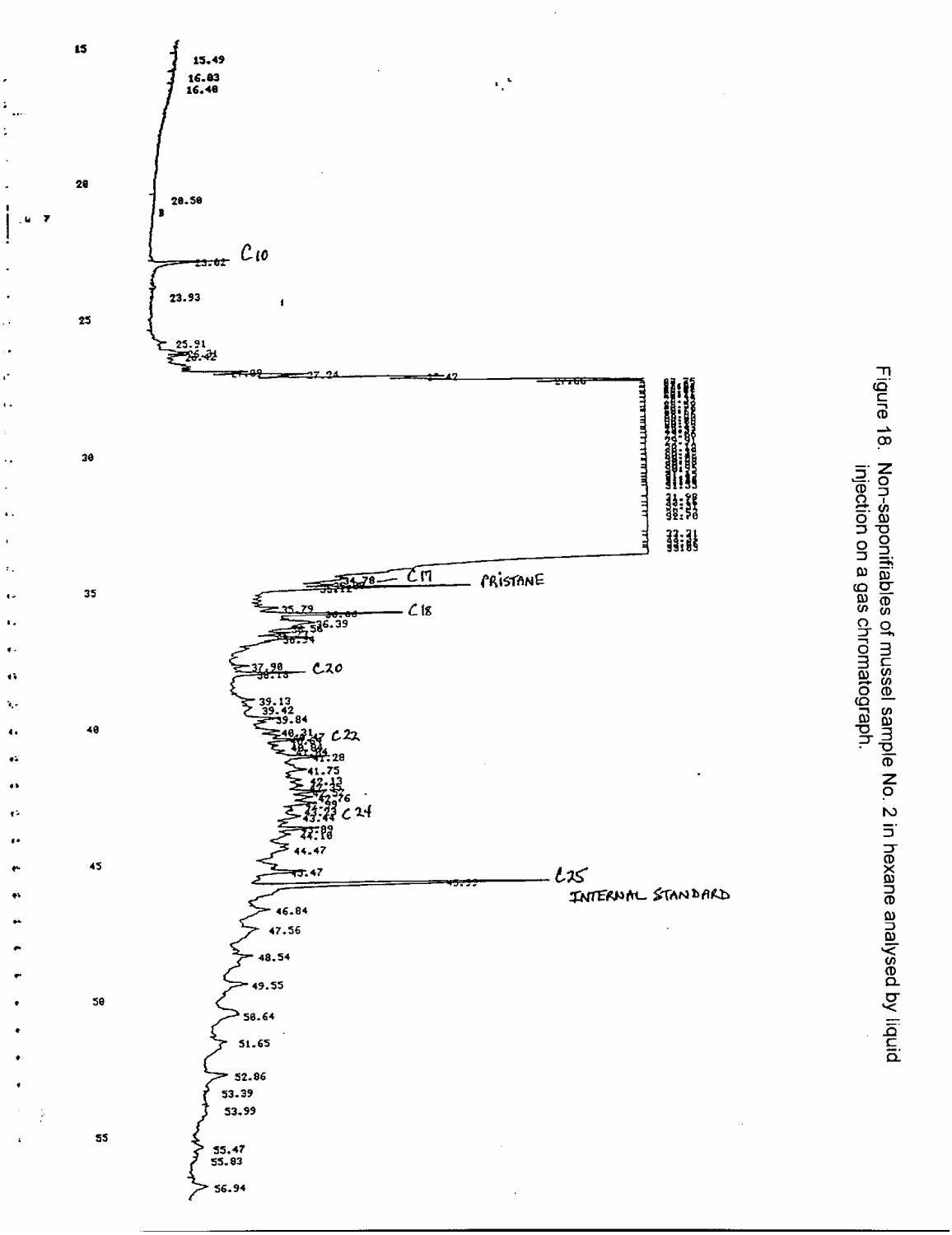


Figure 18. Non-saponifiables of mussel sample No. 2 in hexane analysed by liquid injection on a gas chromatograph.

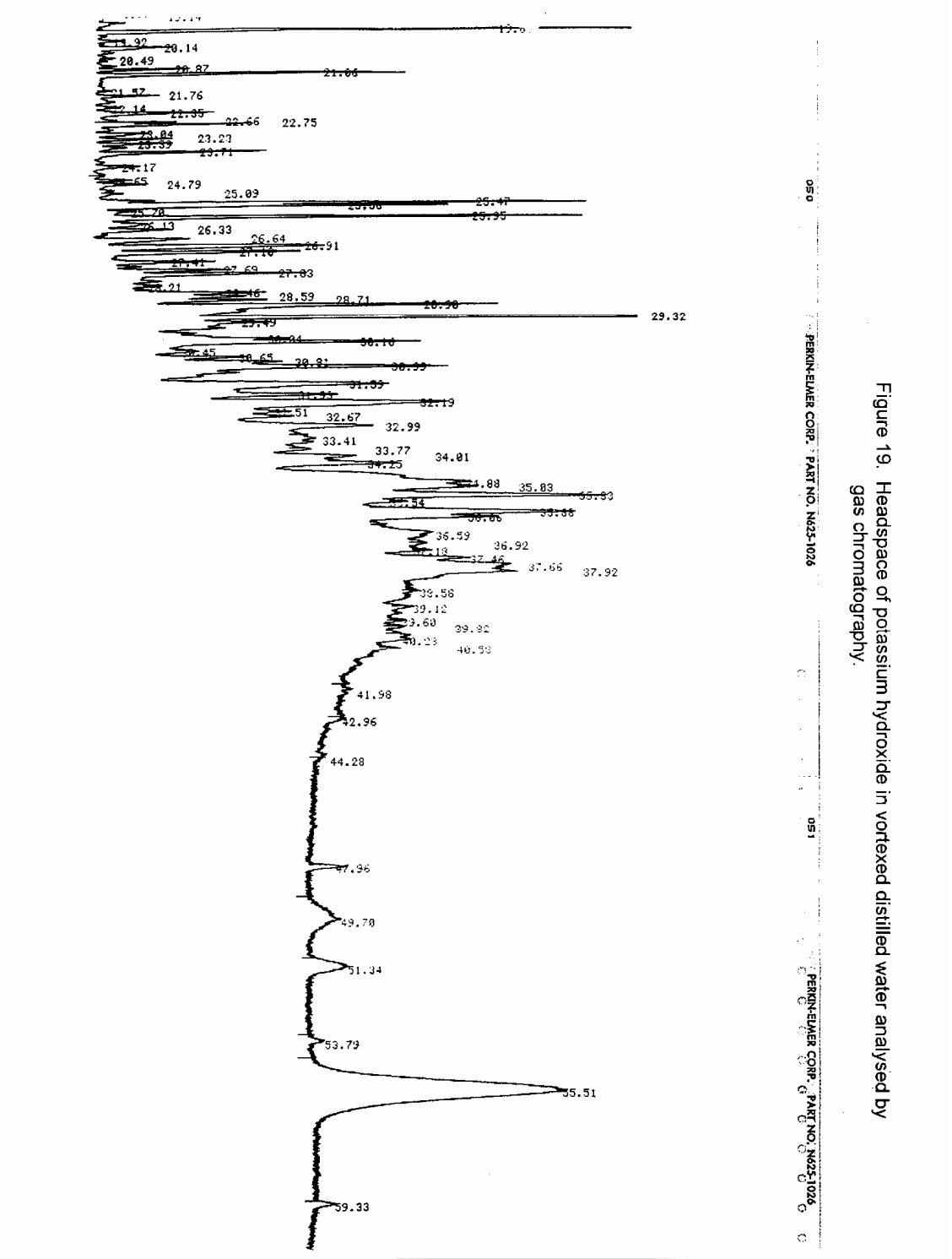


Figure 19. Headspace of potassium hydroxide in vortexed distilled water analysed by gas chromatography.

Appendix B

Zhou, S., R.G. Ackman and, J. Parsons, Very long-chain aliphatic hydrocarbons in lipids of mussels (*Mytilus edulus*) suspended in the water column near petroleum operations off Sable Island, Nova Scotia, Canada, Marine Biology, 126: 499-507, 1996.

Appendix B

Marine Biology (1996) 126: 499–507

S. Zhou · R. G. Ackman · J. Parsons

Very long-chain aliphatic hydrocarbons in lipids of mussels (*Mytilus edulis*) suspended in the water column near petroleum operations off Sable Island, Nova Scotia, Canada

Received: 6 April 1996 / Accepted: 8 May 1996

Abstract Mussels (*Mytilus edulis*) suspended in the water column in 1994 and 1995 for the monitoring of oil drilling operations off Sable Island, Nova Scotia were examined for hydrocarbon profiles, particularly aliphatic hydrocarbons. A spring bloom of phytoplankton occurred during the 90-d suspension period in 1995. Hydrocarbons isolated from the 1995 suspended mussels showed very high concentrations of both biogenic hydrocarbons and very long-chain *n*-alkanes from C_{20} to C_{32}, initially thought to be petrogenic. Both types of hydrocarbons were either not detected or were only present in trace amounts in the mussels suspended in 1994 at similar sites. The biogenic hydrocarbons in the 1995 mussels were apparently of planktonic origin, from the spring bloom, and were dominated by heneicosahexaene (21:6), followed by pristane, heptadecane, and various monounsaturated and polyunsaturated phytenes, heptadecenes, nonadecenes and heneicosenes. They could be readily hydrogenated to yield the basic alkanes. The 1995 mussels suspended within 1 km from the oil well platform were probably slightly tainted by petrogenic hydrocarbons, as evidenced by the detection of phytane and high concentrations of total aliphatic hydrocarbons, whereas the mussels suspended 10 km from the platform showed only high concentrations of biogenic hydrocarbons and the novel long-chain *n*-alkanes. The occurrence of an unusual phytoplankton bloom during the suspension period severely interfered with the petroleum monitoring role of mussels by altering the mussel hydrocarbon profiles through the accumulation into and probably selective depuration of xenobiotic hydrocarbons from the mussel tissues.

Communicated by R. J. Thompson, St. John's

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Introduction

The mussel *Mytilus edulis*, because of its wide distribution, has been considered and used extensively as a most suitable and reliable organism for the monitoring of organic contaminants in estuarine and coastal environments (Philips 1978; Risebrough et al. 1980; Murray et al. 1991; Granby and Spliid 1995). Typically marine organisms, including bivalves, accumulate organic pollutants in their tissues, leading to concentration levels far above those found in the water column (Widdows et al. 1982; Pruell et al. 1986; Ernst et al. 1989; Heras et al. 1992; Zhou and Ackman 1994).

As part of a recently developed project for the monitoring of petrogenic hydrocarbons in the lipids of blue mussel (*Mytilus edulis*) suspended in the water column near Sable Island, Nova Scotia, we observed a novel range (C_{20} to C_{32}) of long-chain aliphatic hydrocarbons in addition to the expected background of aliphatic, methyl-branched and other hydrocarbons (normal range from C_{10} to C_{21}). This novel feature is probably related to an unusual phytoplankton bloom reported on the Scotian Shelf in 1995.

An earlier paper on the lipids of the amphipod *Corophium volutator* from Minas Basin, an arm of the Bay of Fundy lying between Nova Scotia and New Brunswick, Canada, reported 0.2% of aliphatic hydrocarbons in a total of 1.74% extractable lipids (Napolitano and Ackman 1989). These hydrocarbons were similar straight-chain alkanes and ranged from C_{18} to C_{25}. A weak odd-carbon preference was noted, as well as minor amounts of the accompanying isoprenoid hydrocarbons pristane (2,6,10,14-tetramethylpentadecane) and phytane (2,6,10,14-tetramethylhexadecane). The last two hydrocarbons suggested a possible

The gas-liquid chromatography–mass spectrometry (GLC-MS) system consisted of a Perkin-Elmer 990 gas chromatograph and a Finnigan MAT Model 700 Ion Trap Detector (ITD). The GLC was equipped with a fused-silica capillary column, similar to that in the GLC-FID system, passed through a transfer line directly into the ITD inlet. The GLC was operated using helium carrier gas (ultra-high purity grade) at a pressure of 138 kPa. The injector temperature was held at 320 °C, and the GLC-MS transfer line was maintained at 280 °C. Conditions and temperature programs were the same as in the GLC-FID system. The mass spectrometer scanned from m/z 50 to 400. The identity of alkanes was confirmed by their mass spectra and by the injection of authentic hydrocarbons [pristane, phytane (Analabs, Inc., North Haven, Conn.), and a series of *n*-alkanes (C_{10} to C_{35}) (Polyscience Corp., Niles, Ill.)]. Alkenes were tentatively identified by the interpretation of mass spectra and through the hydrogenation of the extracted hydrocarbons.

The calculations of aliphatic hydrocarbon concentrations in mussel tissues prior to 1995 were based on spiking studies on mussels collected in 1993 and 1994 with the internal standard of *n*-pentacosane (Zhou et al. 1996). Due to the unexpected detection of very long-chain *n*-alkanes in the 1995 mussels, the amounts of aliphatic hydrocarbons in the 1995 mussel tissues were obtained through the external standard method by using the average recovery of *n*-pentacosane spiked to the 1995 inshore mussel tissues.

Hydrocarbons isolated from the mussels suspended 10 km (upper and lower) and 1000 m (upper) from the oil well platform were pooled for examination after hydrogenation. Hydrogenation was carried out in dichloromethane in a glass flask under a hydrogen flow of 0.5 liter min^{-1} with PtO_2 catalyst for 2 h. The hydrogen gas was passed over the solvent and thorough mixing achieved with the aid of a magnetic stirring bar. After hydrogenation, the solvent was removed by rotary evaporator, and the hydrocarbons were transferred to a 10 ml centrifuge tube with hexane and dichloromethane. The solvent was then washed with distilled water and centrifuged to avoid any transfer of catalyst. The supernatant hexane/dichloromethane layer was pipetted to a graduated glass centrifuge tube and concentrated under a nitrogen flow.

Results

The mussels (*Mytilus edulis*) collected in 1995 and 1994 exhibited totally different hydrocarbon profiles (compare Fig. 1 with Fig. 2B). Hydrocarbons isolated from the 1994 mussels showed a slightly tainted hydrocarbon profile characterized by the presence of low levels of numerous branched aliphatic petroleum hydrocarbons between C_{10} and C_{14} (Fig. 2B). These petrogenic hydrocarbons were detected in all of the 1994 and 1995 mussels suspended within 1 km of the drilling platform (Figs. 1A, 2B). The origins of those branched alkanes could be traced to the oil base mud used for drilling operations, which displayed a C_{10} to C_{18} hydrocarbon profile identical to the GLC chromatograms recorded during our previous studies on the base mud oil (Zhou et al. 1996). Those

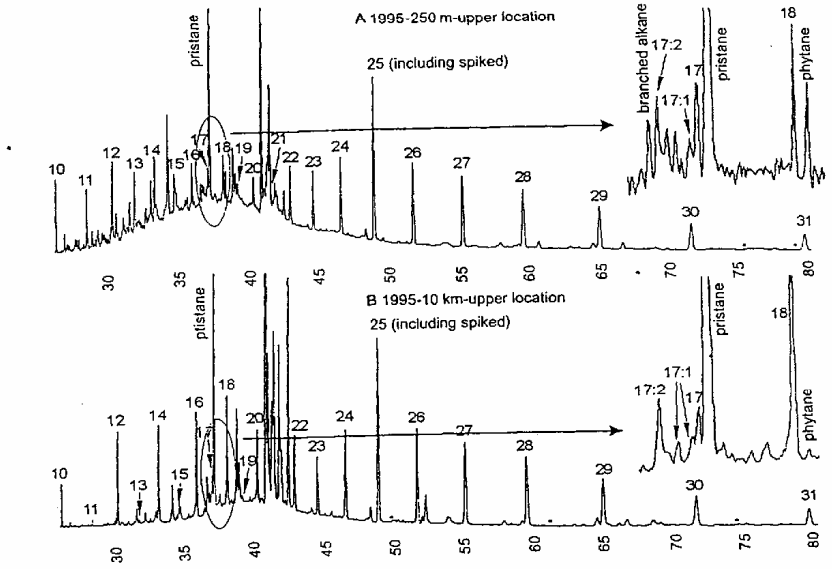

Fig. 1 *Mytilus edulis*. Partial GLC-FID chromatograms of hydrocarbons isolated from mussels suspended in the upper position of the water column in 1995: A 250 m (Site 1, upper) from the oil well drilling platform and B 10 km (Site 4, upper) from the oil well drilling platform. Partial chromatograms in upper right corners are GLC-MS reconstructed ion chromatograms emphasizing the pristane/phytane regions. Numbers above peaks represent carbon numbers of the straight-chain *n*-alkanes. Alkenes were tentatively identified, and identities denoted by the straight-chain carbon number followed by number of double bonds

heneicosahexaene were present at slightly higher concentrations than the two common alkanes. The 1995 inshore mussels were used as a reference sample in this study since there was no sign of the presence of tainting aliphatic hydrocarbons of petroleum origin and the mussels originated from the same mussel farm as the 1994 and 1995 exposure mussels.

A regional phytoplankton bloom occurred in the experimental areas during the 90-d suspension period of the 1995 mussels. This spring bloom led to the temporary closure of shellfish fisheries in southwestern Nova Scotia; fisheries were reopened on 22 May 1995. The hydrocarbon profiles typical of the suspended mussel samples (Fig. 1) collected in 1995 were rather complicated compared with those of the two mussel samples mentioned above and showed the following contrasting characteristics: (1) presence of substantially higher concentrations of total aliphatic hydrocarbons (Table 1); (2) high concentrations of straight-chain alkanes with both odd and even numbers of carbon from C_{10} to C_{32}, those from C_{20} to C_{32} being particularly unexpected; (3) the detection of high concentrations of pristane and phytane; (4) the presence of a wide GLC baseline envelope of hydrocarbons, corresponding to straight-chain alkanes from C_{10} to C_{24}; (5) the presence of high concentrations of straight-chain alkenes with 17, 19 and 21 carbons, and particularly of heneicosahexaene. These were not detected or were present only in very low concentrations in the 1994 suspended mussels and the 1995 inshore reference mussel samples (Fig. 2). The wide GLC baseline envelope of the isolated hydrocarbons was found to be present in all 1995 mussel samples and was centered around heptadecane in the GLC chromatograms (Fig. 1). This wide baseline envelope accounted for a large proportion of the total aliphatic hydrocarbons determined in the 1995 suspended mussels.

The total amounts of aliphatic hydrocarbons and the net tainting amounts calculated as the difference between the inshore mussels and the suspended mussels are shown in Table 1. The 1995 suspended mussels collected from the lower positions of Sites 2 and 4 were among the most heavily (40.57 ppm net; Site 2) and the least heavily (2.25 ppm net; Site 4) hydrocarbon-loaded samples. The amounts of hydrocarbons in the mussel tissues increased from Site 4 to Site 2 and from the upper positions to the lower positions, except for Site 4 where more hydrocarbons were found in the upper mussel sample than in the lower mussel sample. It was observed that the total hydrocarbon levels in the mussel samples from the site nearest to the drilling well platform (Site 1) were slightly lower than those in the mussels collected from Site 2 of the upper and lower positions, respectively. From an overall point of view, the 1995 mussels suspended within 1 km of the drilling well platform were tainted by petrogenic hydrocarbons, whereas mussels 10 km away from the platform contained only trace amounts of petrogenic hydrocarbons. This conclusion is supported by the following observations: (1) the detection of phytane in all mussels suspended within 1 km of the platform; (2) the presence of both odd- and even-carbon-chain *n*-alkanes between C_{10} and C_{20} in mussels within 1 km of the platform, while in the 10 km mussels those odd-chain *n*-alkanes were present in much lower concentrations than the adjacent even-chain *n*-alkanes (Fig. 1); (3) detection of oil base mud hydrocarbons (numerous branched alkanes between C_{10} and C_{14}) in mussels within 1 km of the platform; (4) differences in total amounts of hydrocarbons in mussels collected from different sites (Table 1).

Large proportions of the total hydrocarbons in the mussel samples suspended in 1995 were composed of straight-chain alkanes, ranging from 2.02 to 15.66 ppm (Table 1). Changes in the amounts of straight-chain alkanes among different sites and positions were similar to those of the total accumulated aliphatic hydrocarbons. Calculation of the carbon preference index (CPI) suggested no obvious odd-carbon-chain predominance among *n*-alkanes from C_{23} to C_{32} in any of the eight mussel samples suspended in 1995. Figure 3 shows the profiles of straight-chain alkane distribution in three typical mussel samples. Both the 1995 inshore mussels and the 1994 suspended mussels (Site 2, lower) contained low levels of *n*-alkanes, and *n*-alkanes above octadecane were virtually absent in the 1995 inshore mussels. The profiles of straight-chain alkanes for the 1995 suspended mussels from Sites 1, 2 and 3 are clearly bimodal (Fig. 3), since individual alkane concentrations reached maximum levels at heptadecane and tetracosane, respectively. The *n*-alkane distribution from C_{10} to C_{19} of the first mode is asymmetrical and could probably be further divided into two submodes: one from C_{10} to C_{15} due to the accumulation of oil base mud and the other from

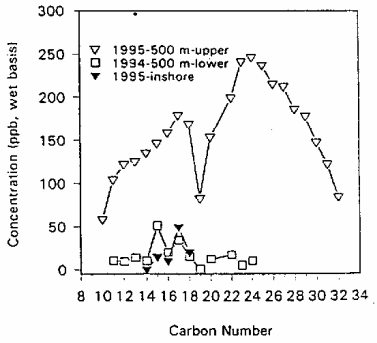

Fig. 3 *Mytilus edulis*. *n*-Alkane distribution in mussels suspended in the water column in 1994 (Site 2, lower) and 1995 (Site 2, upper), and in mussels obtained from an inshore farm in 1995

californianus, are closely related to human population density. Studies have also shown that an increase in environmental contamination leads to declining scope for growth and increasing stress of mussels (Widdows et al. 1995). The complicated hydrocarbon profiles of the 1995 suspended mussels were apparently caused by the spring bloom of phytoplankton during the suspension period. The pristane can be a derivative of the phytol in chlorophyll, but is also one of the major hydrocarbon components present in some phytoplankton and various other algal species (Whittle et al. 1974; Nevenzel 1989). Either way, the high concentration of pristane in the 1995 suspended mussels is a clear indication of intensive feeding on phytoplankton by the mussels.

Straight-chain alkanes are commonly present in almost all marine organisms (Snedaker et al. 1995). The most predominant *n*-alkanes reported in marine organisms are odd-number carbon chains such as heptadecane and pentadecane (Nevenzel 1989), which mainly came from the biosynthesis processes of ingested algal and/or microbial precursors from the marine food web, and probably originated from the decarboxylation of even carbon numbered fatty acids. Most biogenic *n*-alkanes of other chain lengths (both odd-and even-number chains) are usually found only in specific marine organisms. It is interesting to note that very long-, even-chain aliphatic *n*-alkanes from C_{18} to C_{36}, peaking at C_{24} to C_{28}, were found to be the major aliphatic hydrocarbons in both farmed and wild mussels from Galicia Spain (Hermida Ameijeiras et al. 1994). The detection of long-chain *n*-alkanes (C_{20} to C_{32}) in the 1995 suspended mussels and their absence from the 1994 suspended mussels and 1995 inshore mussels probably reflects the very intensive ingestion of phytoplankton from the regional bloom reported in 1995. An identical range of long-chain *n*-alkanes was observed by Napolitano and Ackman (1989) in lipids of the amphipod *Corophium volutator* from Minas Basin, Nova Scotia, and also occurred in the muscle of dabs (*Limanda limanda*) caught in the vicinity of the North Sea Beatrice oil platform (McGill et al. 1987). Those particular long-chain *n*-alkanes have also been found in the water column (Siron et al. 1987), and in a few marine organisms and tissues, including phytoplankton (Whittle et al. 1974; Nagashima et al. 1986). As early as 1979, a significant hydrocarbon fraction was found in lipids of *C. volutator* from Minas Basin, an arm of the Bay of Fundy (Ackman et al. 1979); the hydrocarbon fraction in proportion to total lipids was 0.2 to 0.3% and included pristane, phytane, and C_{18} to C_{35} alkanes (Napolitano and Ackman 1989). Later an examination of foam lipids collected at the upper end of the Bay of Fundy showed a spectrum of long-chain *n*-alkanes ($C_{20}$to C_{39}) similar to our current observations (Napolitano et al. 1992). Both regions were investigated because of high concentrations of *C. volutator* and their role as prey for migratory shorebirds. Diatoms are peculiar to these intertidal mudflats made up of fine particles, and they and their detritus (Jensen and Kristensen 1990) are probably the origin of the hydrocarbons observed in several of the Bay of Fundy samples. A similar planktonic algae is probably the source of the long-chain *n*-alkanes bioconcentrated off Sable Island in 1995. Although we were unable to definitively identify the bloom that was actually observed from the oil well drilling platform (C. Ross private communication), diatoms such as *Pseudonitzshia seriata* were observed in water samples collected in May on the Scotian Shelf (C. Carver private communication). It is interesting that lipids of *Nitzschia pungens* f. *multiseries* grown in batch culture contained 11% hydrocarbons when nutrients were replete (Parrish et al. 1991).

The observation of *n*-octadecene, *n*-heptadecene and *n*-heneicosahexaene in the 1995 inshore reference mussels is probably related to the particular algal species (possibly including macrophytes) which were present only around the mussel farm. These alkenes, if originally present, were probably depurated during the 90-d suspension period in 1994 and not reinforced due to the absence of input of those particular algal hydrocarbons in the water column of the suspension areas during this particular suspension period. The hydrogenation confirmed the presence of heneicosahexaenes, and of monounsaturated and polyunsaturated heptadecenes, nonadecenes, phytenes and heneicosenes in particularly large amounts in the 1995 suspended mussels (Fig. 4B). The C_{21} group of hydrocarbons could not possibly have been petrogenic, but is a characteristic of algae (Blumer et al. 1970, 1971). High concentrations of very long-chain biogenic *n*-alkenes, C_{31} and C_{33}, with 2, 3, and 4 double bonds, have been found in *Mytilus edulis* from the North Sea and were suggested to be from a dietary source of marine alga (Rowland and Volkman 1982).

Multiple sources of hydrocarbons might have contributed to the presence of high concentrations of abnormal aliphatic hydrocarbons in the 1995 suspended mussel samples. The detection of phytane in marine organisms is normally accepted as an indicator of the presence of petrogenic hydrocarbons, since this component is commonly found in petroleum products and is recalcitrant to biodegradation (Schaeffer et al. 1979). The reported presence of large quantities of phytoplankton near the oil drilling platform played an important role in our conclusions on the origin of various abnormal hydrocarbons and strengthened those on the relatively minimal bioaccumulation of petrogenic hydrocarbons related to oil-drilling in the indicator mussels. Phytoplankton may play a role in hydrocarbon bioconcentration by physically absorbing any suspended hydrocarbons in the water column before their hydrocarbon load is finally deposited in the mussel tissues. This proposed pathway of bioconcentration by phytoplankton via surface absorption rather than ingestion is discussed briefly for polyaromatic

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Appendix C

A partial listing of publications relating experience with SPME to show the variety of applications

1. Gago-Martinez, Ana, Maria J. Nogueiras, Sandra Rellán, and Juan Prado, Optimization of Solid-Phase Microextraction for the Gas Chromatography/Mass Spectrometry Analysis of Persistant Organic Pollutants, Journal of AOAC International Vol. 87. No.4: 1021-1027, 2004.

2. Marsili, Ray, ed., Flavor, Fragrance, and Odor Analysis, (Food Science and Technology Series/115), Marcel Dekker, Inc., New York, NY, 2004.

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5. Rocha, Silvia, Vitor Ramalheira, António Barros , Ivonne Delgadillo, and Manuel A. Coimbra, Headspace Solid Phase Microextraction (SPME) Analysis of Flavor Compounds in Wines. Effect of the Matrix Volatile Composition in Relative Response Factors in a Wine Model, J. Agric. Food Chem., 49: 5142-5151, 2001.

6. Palma-Harris, Cheryl, Roger F. McFeeters, and Henry P. Fleming, Solid-Phase Microextraction (SPME) Technique for Measurement of Generation of Fresh Cucumber Flavor Compounds, J. Agric. Food Chem., 49: 4203-4207, 2001.

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10. Elmore J. Stephen, Mehmet Ali Erbahadir, and Donald S. Mottram, Comparison of Dynamic Headspace Concentration on Tenax with Solid Phase Microextraction for the Analysis of Aroma Volatiles, J. Agric. Food Chem., 45: 2638-2641, 1997.

11. Haahr, A.-M., et al., Flavour Release of Aldehydes and Diacetyl in Oil/Water Systems, Food Chemistry, 71: 355-362, 2000.

12. Cam, D., S. Gagni, N. Lombardi, and M.O. Punin, Solid-Phase Microextraction and Gas Chromatography- Mass Spectrometry for the Determination of Polycyclic Aromatic Hydrocarbons in Environmental Solid Matrices, J. Chromatogr. Sci., Vol. 42: 329-335, 2004.

Appendix D

Marsili, R., Monitoring Light-Induced, Off-Flavor Chemicals in Milk by SPME, Sigma-Aldrich Co., T498259, 1998.

SPME A Faster, Simpler Sample Prep Technology

Winter 1998

SPME for Food and Beverage Applications

Analysts in the food and beverage industry continue to develop new SPME applications for extraction of flavors and odors. The article on this page describes the extraction of off-flavors in milk, the result of exposure to light. The applications on page 2 describe the performance of SPME in extracting pyrazines from peanut butter, as well as natural and artificial flavors from hard candy.

Monitoring Light-Induced, Off-Flavor Chemicals in Milk by SPME

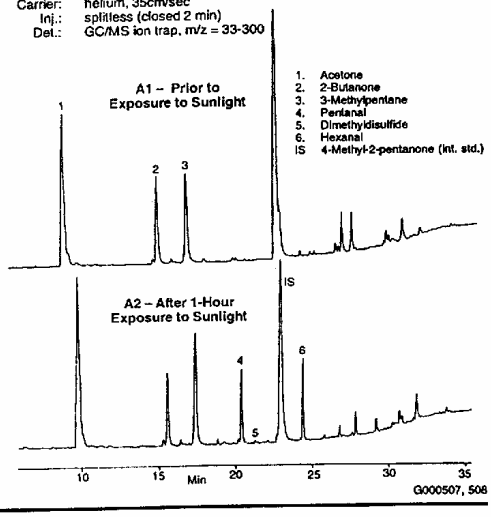
R. Marsili, Dean Foods Technical Center, Rockford, IL

Off-flavors can occur in milk by a variety of mechanisms. One of the most common causes of these off-flavors is exposure to light. It is estimated that exposure of milk samples to fluorescent lights in the supermarket dairy case is responsible for the development of off-flavors in some 80% of samples sold.

Although it provides the high sensitivity needed to monitor light-induced off-flavor reactions, dynamic headspace gas chromatography/mass spectrometry (DH-GC/MS) is time consuming and lacks precision.

Figure A. Milk Sample Off-Flavors by SPME-GC/MS

Sample:	3g of 2% milk + 10µL internal standard solution (20µg/mL 4-methyl-2-pentanone) (9mL GC vial)
SPME Fiber:	PDMS/Carboxen, 75µm film
Cat. No.:	57318
Extraction:	headspace, 15 min with constant stirring at 45°C
Desorption:	5 min, 250°C
Column:	Supel-Q™ PLOT, 30m x 0.32mm ID
Cat. No.:	24242
Oven:	70°C (2 min) to 140°C at 6°C/min (2 min hold) then to 220°C at 6°C/min (5 min hold)
Carrier:	helium, 35cm/sec
Inj.:	splitless (closed 2 min)
Det.:	GC/MS ion trap, m/z = 33-300



The author used both DH-GC/MS and SPME-GC/MS to monitor milk samples in glass bottles exposed to sunlight for approximately one hour. An SPME fiber coated with 75µm polydimethylsiloxane (PDMS)/Carboxen™ was capable of extracting low ppb levels of pentanal, hexanal, and dimethyldisulfide – off-flavor byproducts. Figure A1 shows a chromatogram of 2% milk prior to exposure to sunlight; Figure A2 shows the same milk sample after exposure to sunlight for 1 hour.

SPME-GC/MS proved to be superior to the dynamic headspace method. SPME eliminates background, artifact, and carryover peaks which are observed in DH-GC chromatograms. SPME also is more accurate, faster, easier, and less expensive than DH. Furthermore, SPME-GC/MS provides sensitivities equivalent to DH-GC/MS.

Acknowledgment
All information and illustrations in this article were submitted by Ray Marsili, Dean Foods Technical Center, Rockford, IL 61108.

ACS Presentations

In addition to providing the information shown on this page, the author made the following detailed presentation at the American Chemical Society 216th National Meeting in Boston, MA, August 23-27, 1998. If you would like a copy of the paper, please use the postcard on page 4 of this newsletter.

The Application of Dynamic Headspace and Solid-Phase Microextraction Gas Chromatographic Techniques for Studying Off-Flavors in Milk

R.T. Marsili and N. Miller, Dean Foods Co., PO Box 7005, Rockford, IL 61108

The following SPME presentations also were presented at the ACS meeting:

SPME Method Development for Headspace Analysis of Volatile Flavor Compounds

D.D. Roberts and P. Pollien, Nestlé Research Center, Vers-Chez-les-Blanc, 1000 Lausanne 26, Switzerland

Analysis of Orange Soda Flavor Volatiles Using Headspace SPME and GC-MS

V. Barrett, Sunkist Growers Inc., 760 E. Sunkist St., Ontario, CA 91761

Solid Phase Microextraction of Volatiles from Foods Using a Porous Carbon Fiber

B. Page, G. Lacroix, and D. Weber, Food Research, Health Protection Branch, Health Canada, Ottawa, Canada K1A OL2.

SUPELCO T498259

Natural and Artificial Flavors in Hard Candy

SPME can be applied to the comparison of artificial flavoring and natural flavoring. Using a 1cm StableFlex™ SPME fiber coated with divinylbenzene/Carboxen™/polydimethylsiloxane (DVB/Carboxen/PDMS), we extracted the components of two hard candies with cherry flavoring. One sample was labeled as having artificial flavoring (Figure A1), and the other was labeled as having both natural and artificial flavorings (Figure A2).

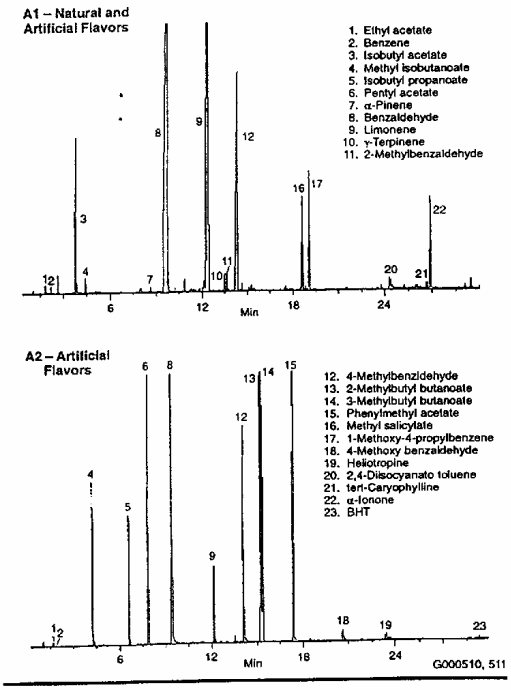
The two candies contained some common ingredients, primarily benzaldehydes. The candy with natural flavoring included a variety of terpinene-type compounds commonly found in citrus fruits. The artificially-flavored candy did not contain these compounds, except for a small amount of limonene. Identification of the components was not confirmed with standards, but was based on mass spectral determination.

The Carboxen portion of the fiber, layered under a DVB coating, extracts small analytes such as ethyl acetate, benzene, and other small esters. The DVB extracts larger analytes such as caryophyllene and BHT. The distinct characteristics of the multiple components of the fiber coating allowed extraction of a wide molecular weight range of compounds with one fiber.

Figure A. Cherry Flavoring in Hard Candy

Sample: 0.5g candy in 5mL water in a 15mL vial
SPME Fiber: DVB-Carboxen-PDMS StableFlex
Cat. No.: 57328-U
Extraction: headspace, 30 min at 40°C
Desorption: 270°C for 5 min

Column: Meridian MDN-5, 30m x 0.25mm x 0.25µm film
Cat. No.: 24096
Oven: 45°C (1.5 min) to 260°C at 4°C/min
Inj.: split or splitless with 0.75mm liner, 270°C
Det.: ion trap mass spectrometer, m/z = 33-400 at 0.6 sec/scan
Selected ions used for quantitation.



Pyrazines in Peanut Butter

The analysis of semivolatile flavor compounds in fat matrices is difficult. The analytes tend to reside in the fat and are not removed easily. To drive enough of the analytes of interest into the headspace for traditional headspace analysis, the sample must be heated to more than 100°C. This can cause the formation of degradation byproducts and reduce analytical accuracy. SPME allows the use of lower temperatures to drive the analytes into the headspace because the SPME fiber concentrates the analytes.

Pyrazines, the primary flavor components in peanut butter, form when peanuts are roasted. We used a 1cm DVB/Carboxen/PDMS StableFlex SPME fiber to extract pyrazines from a peanut butter sample. We detected 21 pyrazines ranging in concentration from <1ppb to >500ppb (Figure B, peaks 6-26). The SPME fiber also extracted volatile components such as hexanal, dimethyldisulfide, and 1-methyl pyrrole (peaks 1-5). With a SUPELCOWAX™ 10 column, only two of the pyrazines co-eluted.

(contd. on next page)

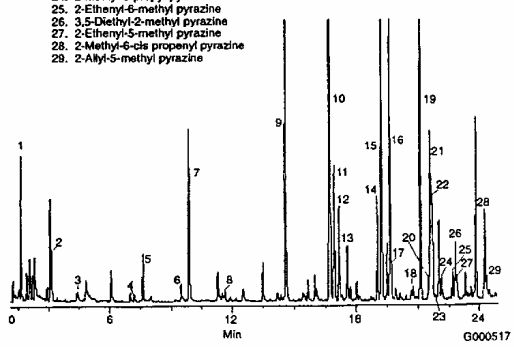
Figure B. Peanut Butter

Sample: 5g peanut butter in 40mL vial
SPME Fiber: DVB-Carboxen-PDMS StableFlex
Cat. No.: 57328-U
Extraction: headspace, 30 min at 65°C in heating block
Desorption: 270°C for 5 min

Column: SUPELCOWAX 10, 30m x 0.25mm x 0.25µm film
Cat. No.: 24079
Oven: 40°C (5 min) to 230°C at 4°C/min
Inj.: splitless/split, closed 0.5 min, 270°C, with 0.75mm liner
Det.: ion trap mass spectrometer, m/z = 30-350 at 0.6 sec/scan
Selected ions used for quantitation.

Some Volatile Components in Peanut Butter
1. Carbon disulfide
2. 3-Methylbutanal
3. Pentanal
4. Dimethyl disulfide
5. Hexanal
6. 1-Methyl pyrrole
7. 4-Methyl-pentene-2-one
8. Heptanal

Pyrazines in Peanut Butter
9. 2-Methyl pyrazine
10. 2,5-Dimethyl pyrazine
11. 2,3-Dimethyl pyrazine
12. 2-Ethyl pyrazine
13. 2,6-Dimethyl pyrazine
14. 2-Ethyl-6-methyl pyrazine
15. 2-Ethyl-5-methyl pyrazine
16. Trimethyl pyrazine
17. 2-Ethyl-3-methyl pyrazine
18. 2,6-Diethyl pyrazine
19. 2-Ethyl-3,5-dimethyl pyrazine
20. 2,3-Diethyl pyrazine
21. 2-Methyl-5-isopropyl pyrazine
22. 3-Ethyl-2,5-dimethyl pyrazine
23. 5-Methyl-2-propyl pyrazine
24. 2-Methyl-5-propyl pyrazine
25. 2-Ethenyl-6-methyl pyrazine
26. 3,5-Diethyl-2-methyl pyrazine
27. 2-Ethenyl-5-methyl pyrazine
28. 2-Methyl-6-cis propenyl pyrazine
29. 2-Allyl-5-methyl pyrazine



Pyrazines in Peanut Butter (contd.)

Some of the peak identifications were confirmed with standards, but most were based on mass spectral interpretation. Therefore, identification of all of the isomers may not be absolute.

Quantifying analytes in a food matrix can be difficult. We achieved our quantitation of methyl pyrazine and the dimethyl pyrazines (Table 1) by determining the area counts of a selected ion for each pyrazine in a known weight of peanut butter. We spiked a second vial containing a known weight of peanut butter with a known amount of each pyrazine standard (in this case 188ppb with respect to the weight of peanut butter). Using the following formula, we determined the amount of pyrazines in the peanut butter:

$$\text{Area counts (spiked p. butter)} - \text{Area counts (unspiked p. butter)} = \text{Area counts (spiked pyrazine)}$$

Area counts (spiked pyrazine) = 188ng/g for each pyrazine

$$\text{Pyrazines in ppb} = \frac{188 \times \text{area counts (unspiked peanut butter)}}{\text{area counts (spiked pyrazine)}}$$

Table 1. Quantitation of Pyrazines (ppb)

2-Methyl pyrazine	158
2,5-Dimethyl pyrazine	526
2,3-Diemthyl pyrazine	47
2,6-Dimethyl pyrazine	16

The values listed in Table 1 are similar to those reported in the journal article listed below.

Reference
Ku, K.L., Lee, R.S., Young, C.T. and Chiou, R.Y.Y. *Roasted Peanut Flavor and Related Compositional Characteristics of Peanut Kernels of Spring and Fall Crops Grown in Taiwan.* J. Agric. Food Chem. (1998) 46: 3220-3224.

Seminars and Exhibits

Water Quality Conference

San Diego, CA, Nov. 1-3

We will highlight a new odor screening method for geosmin and methyl isoborenol at 5ppt concentration.

Northeastern Association of Forensic Sciences

Newport, RI, Nov. 5-6

We will discuss the use of SPME for direct derivatization of amphetamines from urine in street drug screening.

Eastern Analytical Symposium

Somerset, NJ, Nov. 14-16

We will provide an in-depth comparison of artificial and natural flavors in foods.

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