DETERMINATION OF COMPONENTS OF THE HONEY BEE QUEEN (*Apis mellifera* L.) MANDIBULAR GLAND PHEROMONE AS FLUORESCENT PYRENYL ESTER DERIVATIVES.

by

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Determination of components of the Honey Bee Queen (Apis mellifera L.) Mandibular Gland Pheromone as Fluorescent Pyrenyl Ester Derivatives

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Abstract

A method has been investigated for determining the two most abundant components of the queen honey bee (*Apis mellifera* L.) mandibular pheromone, (E)-9-keto-2-decenoic acid (ODA) and (R,E)- and (S,E)-9-hydroxy-2-decenoic acid (HDA) at levels expected in functioning honey bee colonies. ODA and HDA were derivatized with 1-pyrenyldiazomethane to form highly fluorescent esters. Separation of the pyrenyl esters from other compounds present in a biological sample was achieved by high performance liquid chromatography (HPLC) on commercially available reverse phase columns. This study reports the investigation of various derivatization, HPLC, and detection conditions in order to establish a sensitive technique to monitor the pheromone components. This method has been applied to the detection of queen mandibular pheromone components on treated honey bee workers as preliminary studies for the investigation of the pheromone flux in a honey bee colony.

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List of abbreviations

BrMMC	4-bromomethyl-7-methoxycoumarin
DIP-CIMS	direct insertion probe chemical ionization mass spectrometry
DIP-EIMS	direct insertion probe electron impact mass spectrometry
GC	gas chromatography
HDA	(R,E)- and (S,E)-9-hydroxy-2-decenoic acid
HOB	methyl p-hydroxybenzoate
HPLC	high performance liquid chromatography
HVA	4-hydroxy-3-methoxyphenylethanol
NMR	nuclear magnetic resonance spectroscopy
ODA	(E)-9-keto-2-decenoic acid
PDAM	1-pyrenyldiazomethane
PMT	photomultiplier tube
ppm	parts per million
Qeq	queen equivalent
QMP	queen mandibular pheromone
TLC	thin layer chromatography
UV	ultraviolet

1. Introduction

1.1 The honey bee, *Apis mellifera* L.

The honey bee is a very advanced social insect of great economic importance. Honey bees not only produce honey but they also pollinate important crops. A honey bee colony consists of one queen, many thousands of workers, and a variable number of drones, each with their own duties within the colony. The queen is the only fertile female in the colony and her most important function is egg laying. The queen is continuously groomed and fed by workers. The workers are also female but generally sterile, and are responsible for tasks in the nest, including activities such as cell cleaning, brood and queen tending, receiving nectar, packing pollen, building comb and foraging.[1] The drones are males whose only apparent significant function is mating.

1.2 The queen mandibular pheromone (QMP)

All animals must communicate with other members of their species. Even insects with very limited social behavior can, for example, locate food, communicate alarm and establish territories. Honey bees are highly social insects and have developed very intricate dance and chemical communication systems. Chemicals used for communication among members of the same species are called pheromones. Primer pheromones are those which elicit physiological effects while releaser pheromones are those which elicit behavioral effects.

The honey bee queen produces several compounds, those produced in the mandibular gland are the most studied since they are involved in a multitude of functions. The queen mandibular pheromone blend consists of (E)-9-keto-2-decenoic acid (ODA; 200 μg), (R,E)- and (S,E)-9-hydroxy-2-decenoic acid (HDA; 80 μg), methyl *p*-hydroxybenzoate (HOB; 20 μg), and 4-hydroxy-3-methoxyphenylethanol (HVA; 2 μg) (the quantities are the average amount found in the mandibular glands of a laying queen and are defined as 1 queen equivalent (Qeq)[2] Figure 1.2). Queen mandibular pheromone is the first primer pheromone to be identified[3,4,5] that is involved in the control over the reproductive functioning of the colony.

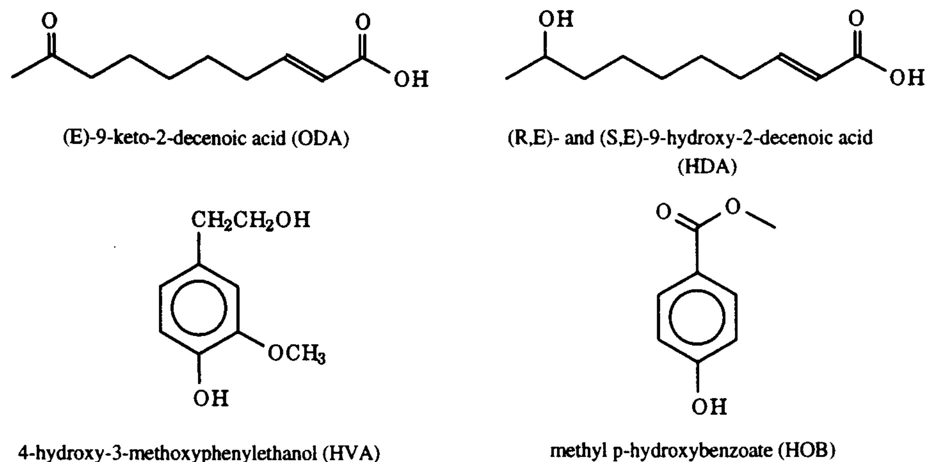

Figure 1.2 The honey bee queen mandibular pheromone blend.

Due to her attractive pheromones,[6] there is usually a circle of six to ten worker bees formed around the queen which is referred to as the retinue. Worker bees in the retinue, referred to as messenger bees, lick and antennate the queen, obtaining pheromone from her. Those messengers licking the queen obtain the greatest amount of pheromone. The pheromone is further transferred by these messenger bees in subsequent contacts with other bees.

Although queen mandibular pheromone was isolated and identified based on a worker retinue response, it has been shown to elicit several other responses such as inhibition of queen rearing,[4,6,7] drone attraction,[8] attraction of swarming workers to their queen[4,9] and the stimulation of foraging behavior under some colony conditions.[10]

1.3 Previous quantitative queen mandibular pheromone studies

Slessor *et al.*[11] analyzed ODA by conversion into its trimethylsilylated derivative followed by gas chromatographic (GC) detection. They described the chemical composition of the five components of the honey bee queen pheromone by analyzing mandibular gland extracts during the life of queens of different ages, colony exposure and backgrounds. Detection limits of about 10^{-9} g were obtained.

Naumann *et al.*[12] have studied the production, secretion and transmission of ODA. Pheromone production and exudation by the queen was also analyzed by gas chromatography of the trimethylsilylated ODA. Further experiments were performed with radioactively labeled ODA[13] to follow the transmission of the pheromone between bees. For example, tritiated ODA incorporated into the mandibular gland pheromone was applied to a lure, a worker was allowed to contact the lure, the worker was then dissected and washed and the amount of tritiated pheromone in different organs was determined by liquid scintillation counting.

They found that the queen produces about 200 μg of ODA per day in her mandibular glands. An equilibrium amount of about 423 ng of ODA can be washed from her body and is therefore available for removal by workers. Workers that made prolonged contact (licking) were able to remove about 121 μg from the queen per day and antennating messengers removed only 14 μg from the queen per day. The queen deposits 3 μg per day on the wax which acts as both a sink and a transmission substrate. Licking

messengers can transfer 10 μg per day of ODA per day in subsequent contacts with licking workers, where as only 1 μg per day is transferred to an antennating worker.

A concern in Naumann *et al*'s study was that the [^{3}H]-ODA was possibly catabolized by the queen or workers after application but prior to analysis. Tritiated, catabolized products would also be detected by scintillation counting, therefore it is possible that not only ODA was followed.

1.4 Objective

The objective of this project was to investigate an alternative method which is more sensitive than gas chromatography and allowed the pheromone to be followed specifically.

The study of pheromone transmission has implications for the proper design and use of pheromones in bee management. For example, Winston *et al.*[9] studied the role of queen mandibular pheromone and colony congestion in the inhibition of swarming. They found that supplemental pheromone applied on slides delayed swarming in the uncongested but not congested colonies and that pheromone application by aerosol spray was more effective than pheromone on slides in the congested colonies. Their results provided evidence that pheromone transmission is slowed as colonies grow and become congested , resulting in diminished pheromone-based inhibition by the queen of workers behaviors, leading to the rearing of new queens and subsequent swarming. These findings are of economic importance because good honey production is not obtained from colonies that have swarmed, since their worker population is reduced below the foraging strength necessary to produce surplus honey.

Naumann *et al.*[12] found by following radioactively labeled ODA that direct contacts between a retinue worker and other bees resulting in 27.2 ± 10.2 pg of ODA being transferred in 5 min. The chosen analytical method must therefore be able to detect these small amounts of pheromone. We have used fluorescence detection since it is not

uncommon to achieve picogram detection limits with this technique, three orders of magnitude more sensitive than GC studies.

The major components of the queen mandibular pheromone, the carboxylic acids, were reacted with a fluorescent tag. There are several precolumn fluorescent derivatization reagents commercially available for the HPLC determination of carboxylic acids. The tagged acids were then separated by high performance liquid chromatography (HPLC) followed by fluorescence detection. Since the HPLC retention time is relatively constant under given conditions, the queen mandibular components free of their possible catabolic products were followed, HPLC studies were therefore more selective than radioactive studies. Sample pretreatment was not required and the reaction mixture was directly injected onto the HPLC.

2. Instrumentation

NMR spectra were recorded with a Bruker 400 MHz instrument and chemical shifts were reported in parts per million (ppm). Mass spectra (MS) were recorded on a Hewlett Packard 5895B GC-MS system by electron impact (EI) ionization or by chemical ionization (CI) using isobutane as the ionizing gas. Melting points were determined on a Fisher-Johns apparatus. Ultraviolet (UV) spectra were recorded on a Philips PU8720 UV/VIS scanning spectrophotometer.

2.1 Separations

All separations were performed with a Waters LC625 HPLC with Waters Nova Pak™ reversed phase C18 analytical columns (300 mm x 3.9 mm i.d. or 250 mm X 4.6 mm i.d.). The acetonitrile used was HPLC grade. All solvents were filtered with 0.45 μm sieve filters under vacuum to remove particles and operating solvent reservoirs were purged with helium. The solvent systems used for separations varied and are discussed in the text and figure captions.

2.2 Fluorescence detection

Fluorescence detection allows increased sensitivity and selectivity. The molecule of interest is excited by absorbing energy (a photon) supplied by an excitation source. The excited molecule then lowers its energy state by releasing energy in the form of heat or light. A molecule in the first singlet excited state (S_1) can drop to the singlet ground state (S_0) giving off energy in the form of light. This generally happens within 10^{-9} s and is called fluorescence. It almost always occurs from the S_1 to S_0 transition. Energy is lost when the excited molecule relaxes to the lowest vibrational level of S_1, therefore the emission wavelength is always longer than that of absorption. Both the optimal excitation and emission wavelengths characteristic of the fluorescent molecule of interest are determined, decreasing the chance of detecting fluorescent side reaction products or reagent decomposition products.

The detection system used in these experiments consisted of a Hewlett Packard 1046A programmable fluorescence detector followed by a Hewlett Packard 3396 series 2 integrator.

The excitation source was a Xenon flash lamp that could be operated at flash frequencies of either 55, 110, or 220 Hz. The radiation emitted by the lamp was collected and focused onto the entrance slit of the excitation monochomator. The excitation monochromator was set to pass light at the absorption maximum of the analyte molecule of interest and this radiation was focused into the flow cell. The holographic concave

grating was the main part of the monochromator, its surface contained 1200 grooves per millimeter. The entrance slit to the excitation monochromator was a 1.4 mm circular slit and the exit slit of the emission monochromator was a 4 mm x 4 mm square slit. The flow cell was a 5μl cylindrical quartz chamber. The anode to cathode voltage of the photomultiplier could be varied to change the gain of the detector.

3. Fluorescent tags for carboxylic acids

Relatively few organic compounds exhibit intense fluorescence. Molecules that exhibit fluorescence normally consist of rigid structures containing a planar aromatic ring. The chosen reagent and its degradation products should be either non-fluorescent or be easily separable from the desired derivative.

Since the study of biologically important fatty acids is of great interest, many precolumn fluorescent derivatization reagents have been developed for the determination of carboxylic acids by HPLC. All these derivatizations are esterification reactions in which a label affording high sensitivity by optical detection is introduced.

The fluorescent reagents are divided into three groups. First, there are activated halogenomethyl derivatives such as 4-bromomethyl-7-methoxycoumarin.[14] These fluorescent reagents react only at elevated temperatures in the presence of base catalysts. Esterification occurs by nucleophilic substitution at the reagent by the carboxylate anion.

Second, there are diazomethane derivatives such as 9-anthryldiazomethane.[15] Aryl diazoalkanes have been developed because they react readily with carboxylic acids at room temperature to form intensely fluorescent esters. Unfortunately, several of these diazo compounds possess limited stability.

Third, are the aliphatic amine derivatives such as 1-(5-dimethylamino-1-naphthylsulphonyl)piperazine.[16] Aliphatic amine derivatives require an activation agent such as dicyclohexyl carbodiimide. The agent converts the carboxylic acid group into the COCN group in high yield. This reactive intermediate is now susceptible to attack by the reagent.

For our studies, the reaction of reagent and substrate must be simple and reproducible since it must be performed on the very small amounts of pheromone present in a biological sample. The recommended methods have often been rigorously tested on gram quantities but are inappropriate in cases where only microgram or less quantities are available. In this work, two fluorescent derivatization reagents which are suitable for separation by reverse phase HPLC were investigated.

4. 4-Bromomethyl-7-methoxycoumarin as a fluorescent tag

The first fluorescent reagent we chose to investigate was 4-bromomethyl-7-methoxycoumarin (BrMMC) as all components of QMP could be derivatized and followed. BrMMC reacts at elevated temperatures in the presence of a base catalyst. Esterification occurs by the nucleophilic substitution at the reagent by the carboxylate anion (Figure 4).

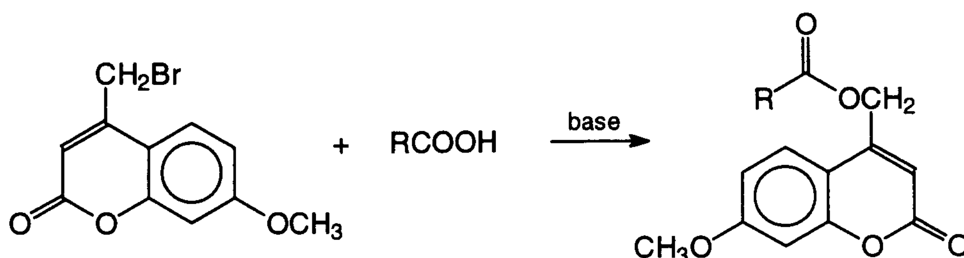

Figure 4 Synthesis of fluorescent coumarin esters.

BrMMC has been widely used to derivatize biologically important substances such as biotin,[17] pesticides,[18] herbicides,[19] barbital[20] and food additives.[21] Farinotti *et al.*[22] studied various short chain fatty acids (C_2-C_5) which are intermediates in the metabolism of amino acids such as leucine, isoleucine and valine. These fatty acids also affect the flavor and aroma of cheese, wine and fruits. The acids were derivatized with BrMMC and separated by reverse phase HPLC. To determine the detection limit, a sample containing the various short chain fatty acid derivatives was diluted successively and aliquots were injected on to the HPLC column. The detection limit was about 0.5 picomole (signal/noise=3).

4.1 Materials

BrMMC and triethylamine were purchased from Aldrich Chemical Company (Milwaukee, WI) and were used without further purification. ODA, HDA and QMP blend was obtained from Phero Tech Inc. (Delta, B.C.). The Phero Tech Inc. QMP blend consisted of ODA (59.2%), HDA (35.5%, enantiomeric excess=67.7%-), HOB (4.74%), and HVA (0.47%).

When flash column purification was required, the appropriate solvent system was determined by thin layer chromatography (TLC). TLC plates were prepared from silica gel 60G. For the detection of compounds, the plates were sprayed with 10% aqueous sulfuric acid and heated.

4.2 Preparation and purification of coumarin esters

Several of the base catalysts reported[14,23-25] in the literature were investigated and we have found that triethylamine proved to be the easiest and most efficient for our small scale reactions. To ODA (1 mg, 5.4×10^{-6} mol) was added BrMMC (4 mg, 1.7×10^{-5} mol) and triethylamine (2 mg, 2.0×10^{-5} mol) in 7 ml acetone. The mixture was heated at 80°C for four hours. The reaction products were precipitated by adding water. The precipitate was separated and washed several times with water. The coumarin ester of ODA (Figure 4.1a), previously unreported, was purified by recrystallization from ether as white crystals (m.p.=80-83°C) that exhibited the following spectral characteristics:

DIP-CIMS m/z (relative intensity) 373(M+1, 5); 185(100); 167(85).

^{1}H NMR ($CDCl_3$) ppm 1.25-1.65(6H, H5'6'7', m); 2.13(3H, H10', s); 2.27(2H, H4', m); 2.45(2H, H8', t, J=8Hz); 3.90(3H, H12, s); 5.32(2H, H11, s); 5.92(1H, H2', d, J=16Hz); 6.45(1H, H3, s); 6.84(1H, H8, s); 6.88(1H, H6, d, J=8 Hz); 7.10(1H, H3', dt, $J_{3'2'}$=16 Hz, $J_{3'4'}$=7 Hz); 7.48(1H, H5, d, J=8 Hz).

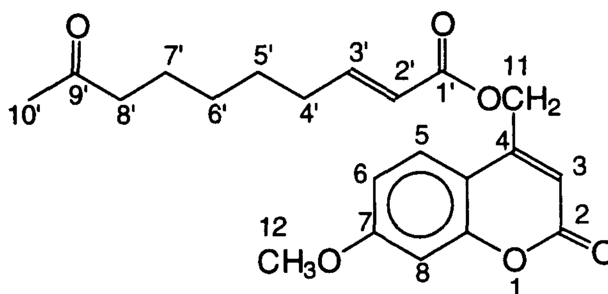

Figure 4.2 Structure of the fluorescent coumarin ester of ODA.

To HDA (1 mg, 5.1×10^{-6} mol) was added BrMMC (4 mg, 1.7×10^{-5} mol) and triethylamine (2 mg, 2.0×10^{-5} mol) in 7 ml acetone. The mixture was heated at 80°C for four hours. The reaction products were precipitated by adding water. The precipitate was separated and washed several times with water. The coumarin ester of HDA, previously unreported, was recrystallized from ether as white crystals (m.p.=59-61°C) that exhibited the following spectral characteristics:

DIP-CIMS m/z (relative intensity) 375(M+1, 7); 191(100); 187(29); 169(38).

^1H NMR ($CDCl_3$) ppm 1.20-1.60(12H, H5'6'7'8'10', m); 2.25(2H, H4', m); 3.48(1H, H9', m); 3.90(3H, H12, s); 5.32(2H, H11, s); 5.92(1H, H2',d, J=16 Hz); 6.45(1H, H3, s); 6.84(1H, H8, s); 6.88(1H, H6, d, J=8 Hz); 7.10(1H, H3', dt, $J_{2'3'}$=16 Hz, $J_{3'4'}$=7 Hz) 7.48(1H, H5, J=8 Hz).

The BrMMC derivative of palmitic acid (m.p.=81-85°C) was identically synthesized and exhibited the following spectral characteristics:

DIP-CIMS m/z (relative intensity) 445(M+1, 70); 257(70); 191(100).

^1H NMR ($CDCl_3$) ppm 0.87(3H, CH_3, t, J=8 Hz); 1.20-1.40(28H, CH_2, m) 2.45(2H, H2', t, J=8Hz); 3.89(3H, H12, s); 5.27(2H, H11, s); 6.34(1H, H3, s); 6.85(1H, H8, s); 6.92(1H, H6, d, J=8 Hz); 7.40(1H, H5, d, J=8 Hz).

The derivative of 9-ketodecanoic acid (m.p.=78-82°C), previously unreported, exhibited the following spectral characteristics:

DIP-CIMS m/z (relative intensity) 375(M+1, 40); 249(100); 235(39); 207(35); 191(22); 174(50).

^{1}H NMR ($CDCl_3$) ppm 1.25-1.65(10H, H3'4'5'6'7', m); 2.11(3H, H10', s); 2.45(4H, H2'8', m); 3.89(3H, H12, s); 5.26(2H, H11, s); 6.35(1H, H3, s); 6.85(1H, H8, s); 6.88(1H, H6, d, J=8 Hz); 7.43(1H, H5, d, J=8 Hz).

All samples, both solid or in solution, were wrapped in foil for protection from light and kept in the freezer (-10°C) until required.

4.3 High performance liquid chromatography

In order to avoid long column equilibration times, isocratic elution of the coumarin derivatives was preferred. Furthermore, if gradient elution is used the column equilibration time between each run must be identical in order to obtain reproducible peak areas.

The ideal HPLC solvent system was found by injecting samples of reagent, purified ODA coumarin ester and HDA coumarin ester. The amount of water in acetonitrile was increased by 5% increments until the coumarin esters were baseline resolved. Optimal separation of coumarin esters occurred when eluted with water-acetonitrile (1:1, operating pressure=3040 psi) and a flow rate of 0.75 ml/min (Table 4.1). The palmitic acid derivative remained on the column until eluted with 100% acetonitrile.

Table 4.3 Retention times of BrMMC esters. Isocratic elution (1:1 water-acetonitrile), Nova Pak™ C18 column (300 mm x 3.9 mm i.d., flow rate=0.75 ml/min), fluorometrically detected at 400 nm with excitation at 229 nm.

Carboxylic acid derivatized	Retention time (min)
HDA	12.3
ODA	15.9
9-ketodecanoic acid	17.9
BrMMC reagent	3.7, 4.7, 10.8

High water percentages in the HPLC solvent cause very high operating pressures due to their high viscosity and are unsuitable for routine operation. With water-acetonitrile (35:65) the pressure decreases (2460 psi) and only the ODA derivative (retention time 6.3 min) separates from the unreacted fluorescent reagent (retention time 5.6 min).

4.4 Excitation and emission spectra

The optimal excitation and emission wavelengths of the ODA derivative were determined by trapping the sample in the flow cell by stopping the solvent flow just as the retention time of the ODA ester was reached. The contents of the flow cell were then scanned and the wavelength at which the compound emitted the most fluorescence was chosen. There were two wavelengths where fluorescence intensity was high, 229 nm and 311 nm (Figure 4.4a). The emission spectra at these two excitation wavelengths were very similar with that of 229 reaching a fluorescence intensity maximum double that reached with excitation at 311 nm. The optimal emission wavelength was 400 nm (Figure 4.4b).

The excitation and emission maxima for the HDA derivative were identical. Changes in the composition of the water-acetonitrile elution solvent had no effect on the excitation and emission spectra of the purified coumarin esters. A 389 nm cut-off filter was chosen to remove stray light longer than the excitation wavelength but shorter than the emission wavelength. The absorption spectrum of the cut-off filter was obtained, the absorbance at 400 nm was 0.1 (80% transmittance).

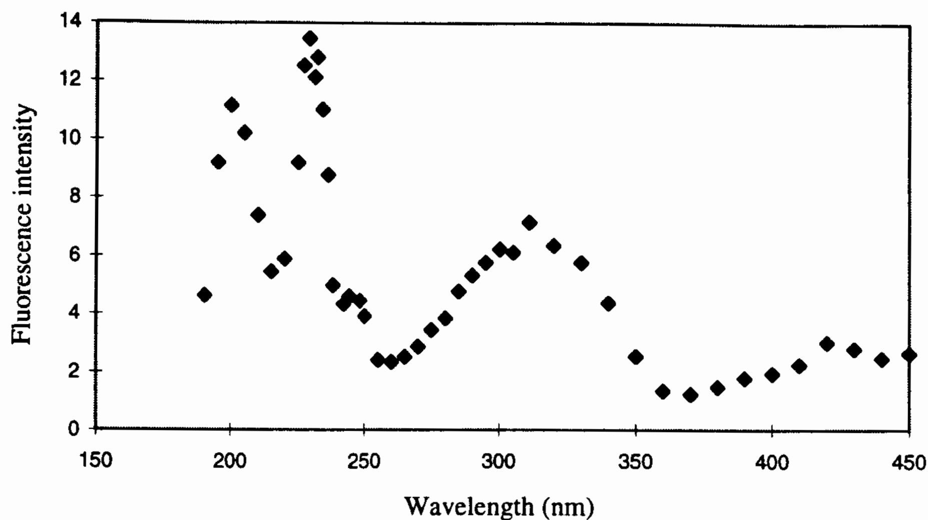

Figure 4.4a Excitation spectrum of the ODA coumarin derivative. Emitted light intensity is measured at all wavelengths. Separation by isocratic elution (35:65 water-acetonitrile) on a Nova Pak™ C18 column (300mm x 3.9mm i.d., flow rate=1ml/min).

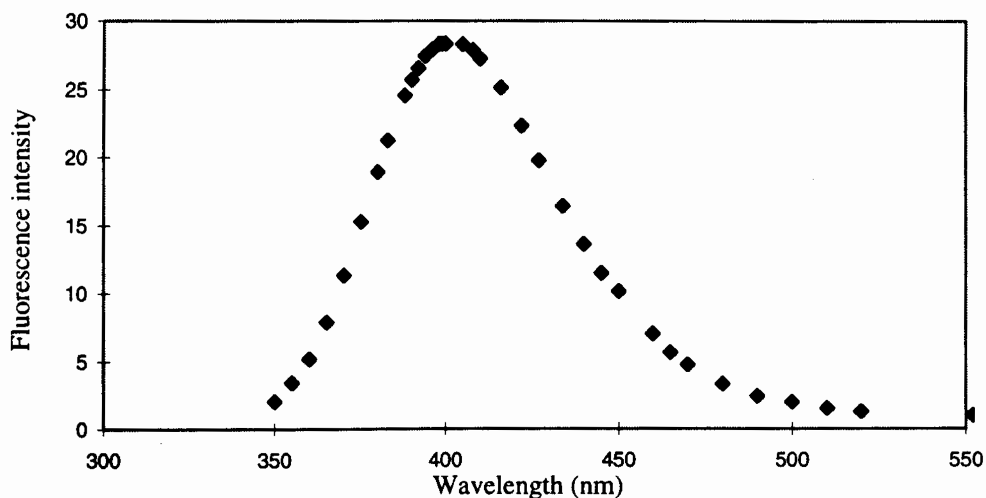

Figure 4.4b Emission spectrum of the ODA coumarin derivative. Excitation wavelength=229 nm. Separation by isocratic elution (35:65 water-acetonitrile) on a Nova Pak™ C18 column (300mm x 3.9mm i.d., flow rate=1ml/min).

Another emission spectrum was obtained after the time required to determine the optimal excitation and emission wavelengths (10 minutes) and the fluorescence intensity had decreased to 90% of its original intensity indicating either photodegradation, as found by Lloyd,[26] or diffusion of the fluorophore out of the detector flow cell had occurred. If photodegradation occurred, it should not significantly affect the results since very little time was spent in the flow cell of the detector because the HPLC flow was continuous for routine analyses.

4.5 Calibration

Standard solutions of increasing concentrations of the ODA coumarin ester in acetonitrile were prepared, three aliquots of each standard were injected and the fluorescence intensities recorded. Figure 4.5 shows the calibration curve and Table 4.4 lists the mean fluorescence intensities (n=3) and the standard errors of these means.

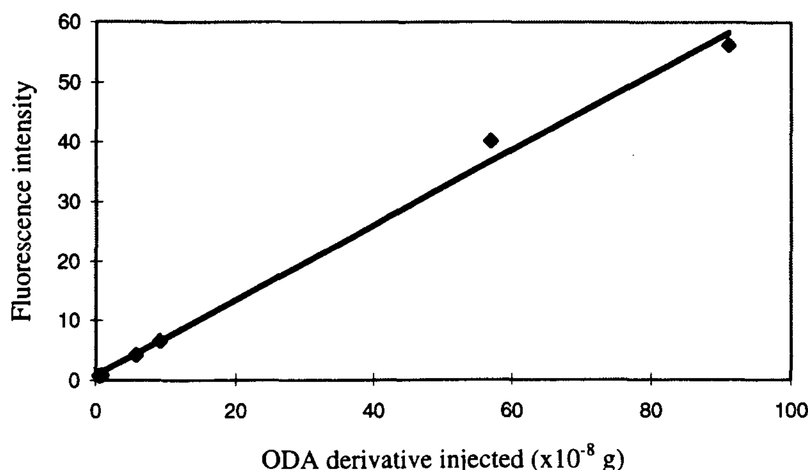

Figure 4.5 Calibration of the ODA coumarin ester by successive dilution of a synthetic standard (r^2=0.9946, y=0.63x+0.82). Separation by isocratic elution (35:65 water-acetonitrile) on a Nova Pak™ C18 column (300mm x 3.9mm i.d., flow rate=1ml/min), fluorometric detection at 400 nm with excitation at 229 nm.

Table 4.5 Mean fluorescence intensities and the standard error of the mean (n=3) at several ODA coumarin ester concentrations by serial dilution. Separation by isocratic elution (35:65 water-acetonitrile) on a Nova Pak™ C18 column (300mm x 3.9mm i.d., flow rate=1ml/min), fluorometric detection at 400 nm with excitation at 229 nm.

ODA ester injected ($x10^{-8}$ g)	Mean fluorescence intensity (n=3)	Standard error
0.57	0.72	0.02
0.91	0.81	0.02
5.7	4.17	0.02
9.1	6.60	0.04
57	40.11	0.24
91	56.26	0.76

4.6 Queen extract

Many compounds were expected to be present in a biological sample and interfering peaks were a potential problem. A queen head containing all the mandibular pheromone components was crushed in acetonitrile. BrMMC (0.0030 g) and triethylamine (0.0058 g) were added to the acetonitrile extract and heated overnight at 65°C. The reaction mixture was passed through a silica column (acetonitrile) to remove excess base or acid. The mixture was then diluted to 10 ml and a 25 μl aliquot was injected onto the HPLC. There were no peaks that interfered with either the ODA derivative peak (retention time=6.3 min). The column was then flushed with acetonitrile to remove any non polar compounds. A typical chromatogram of a queen extract is given in Figure 4.6.

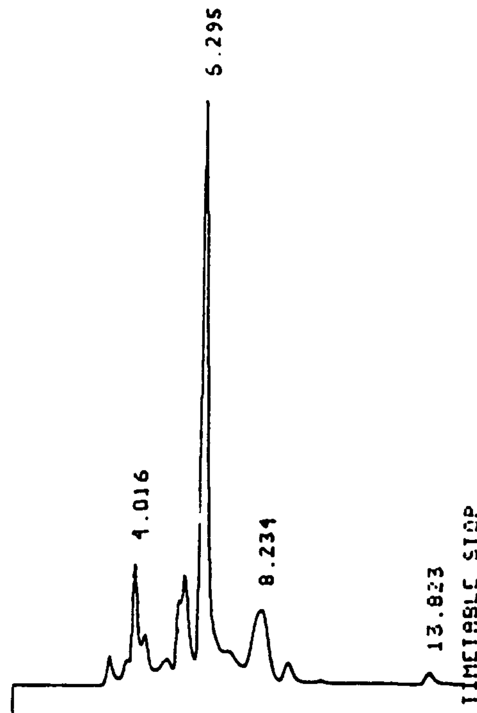

Figure 4.6 Typical chromatogram of a BrMMC derivatized queen head extract. Isocratic elution (35:65 water-acetonitrile) Nova-Pak™ C18 column (300 mm x 3.9 mm i.d.), fluorometric detection at 400 nm with excitation at 229 nm, PMT=10.

4.7 Discussion

By the dilution of a synthetic standard, about $1x10^{-9}$ g of ODA was the minimum detectable amount (signal/noise=4), determined by comparing the peak height of the ODA ester signal to that of the background noise. This is approximately the amount detectable by gas chromatographic analysis of silylated samples and does not represent an improvement in sensitivity.[11]

Lloyd[26] found that the use of esters of 4-hydroxymethyl-7-methoxycoumarin in determinations by gradient elution HPLC was limited by poor fluorescence yields in non-polar solvents and the possible formation of molecular aggregates such as micelles at high water concentrations. They have shown the fluorescence quantum yields to be as low as 0.02 in non-hydrogen bonding solvents such as acetonitrile. Our results are similar in that the fluorescence intensity of the ODA ester increased with the amount of water in the solvent system (Figure 4.6). At each new water concentration, the optimal excitation and emission wavelengths were determined and found to be unchanged. At 35% water in acetonitrile the maximum yield is obtained, although concentrations of greater than 40% water in acetonitrile could not be investigated due to pressure limitations, the HPLC system shuts down at 3500 psi. There seems to be no explanation for why the fluorescence intensity increased while the Stokes shift remained unchanged.

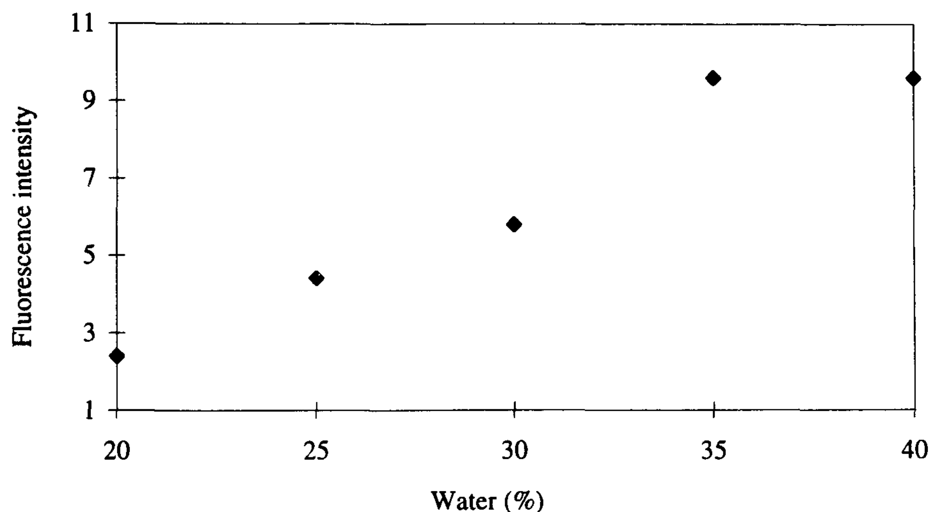

Figure 4.7 Variation of fluorescence intensity of the ODA coumarin ester with the concentration of water in acetonitrile in the HPLC solvent system. Isocratic elution (35:65 water-acetonitrile) on a Nova-Pak™ C18 column (300 mm x 3.9 mm i.d.), fluorometric detection at 400 nm with excitation at 229 nm.

As high operating pressures result with the high water concentrations necessary for high fluorescence quantum yields and optimum separation, the best analysis conditions were unobtainable. BrMMC seemed to be an inappropriate reagent for the study of QMP at levels expected in the colony, therefore a new fluorescent reagent was investigated.

5. 1-Pyrenyldiazomethane as a fluorescent tag

A reasonably stable aryl diazoalkane, 1-pyrenyldiazomethane[27] (PDAM) was investigated. PDAM was chosen as a fluorescent tag since the reaction occurs under mild conditions, requires no catalyst, and produces highly fluorescent esters. The carboxylic acid components of QMP are derivatized with PDAM at room temperature in acetonitrile through the attack of the carboxylic acid to replace the diazo functionality (Figure 5). PDAM was purchased from Molecular Probes Inc. (Eugene, Oregon) and was used without further purification.

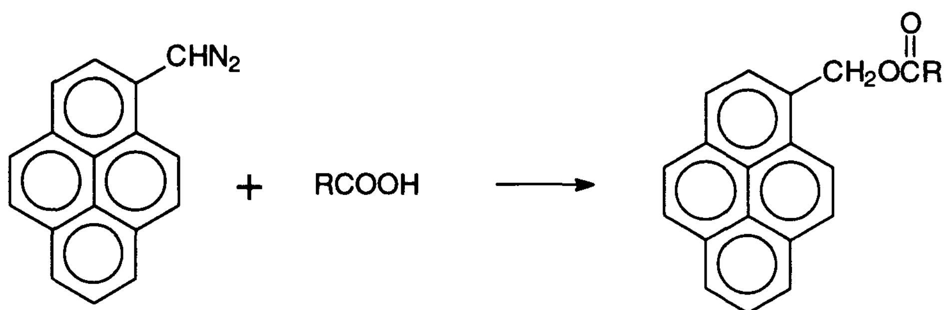

Figure 5 Synthesis of fluorescent PDAM esters.

PDAM has been widely used to derivatize biologically important substances such as methylmalonic acid,[28] cortolic and cortolonic acids[29] and the prostaglandins.[27] Yoshida *et al.*[30] studied biotin, which is a coenzyme essential in amino acid or carbohydrate metabolism. Biotin was reacted with PDAM and the ester derivative was separated on a reverse phase column. Pharmaceutical preparations containing biotin and other vitamins and a control serum spiked with biotin were derivatized with PDAM. Samples of 500 µg

of biotin were derivatized and aliquots were injected onto the HPLC for separation. Successive dilution of a standard was performed to find the detection limit of 100 fmol (signal/noise=3) with high reproducibility.

There were no experiments performed where small amounts (1 ng) of biotin were derivatized and the entire reaction mixture injected onto the column.

5.1 Preparation and purification of esters

To ODA (5.5 mg, 3×10^{-5} mol) was added PDAM (6.1 mg, 2.5×10^{-5} mol) in 7 ml ethyl acetate-acetonitrile (1:1)(Figure 5.1). After 1 hour at room temperature the disappearance of the orange color of the reagent was complete and the solution had turned yellow. The ODA ester (Figure 5.1) was purified by flash column chromatography (2:1 hexane-ethyl acetate), the separation was monitored by TLC developed in the same solvent. A yellow powder that exhibited the following spectral characteristics was obtained:

DIP-EIMS m/z (relative intensity); 398(M, 45); 215(100); 201(7).

^{1}H NMR ($CDCl_3$) ppm 1.30-1.65(6H, H5'6'7', m); 2.10(3H, H10', s); 2.15(2H, H4', m); 2.40(2H, H8', t, J=7 Hz); 5.35(2H, H11, s); 5.85(1H, H2', d, J=16 Hz); 7.00(1H, H3', dt, $J_{2'3'}$=16 Hz, $J_{3'4'}$=6.5 Hz); 8.00-8.35(9H, H2-10, m).

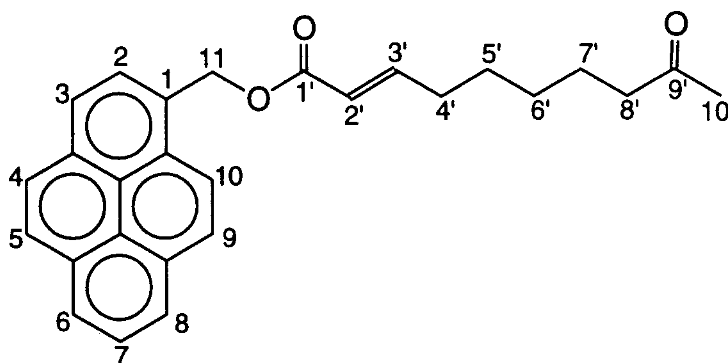

Figure 5.1 Structure of the pyrenyl ester of ODA.

To HDA (6.0 mg, 3.2×10^{-5} mol) was added PDAM (7.2 mg, 3.0×10^{-5} mol) in 7 ml ethyl acetate-acetonitrile (1:1). The reaction was complete after 1 hour. The PDAM ester of HDA was purified by flash column chromatography (11:7 hexane-ethyl acetate) resulting in a yellow powder that exhibited the following spectral characteristics:

DIP-EIMS: m/z (relative intensity); 400($M^{+\cdot}$, 13); 232(94); 203(96); 215(100).

^{1}H NMR ($CDCl_3$) ppm 1.15(3H, H10', d. J=7 Hz); 1.25-1.60(8H, H5'6'7'8', m); 2.20(2H, H4', m); 3.75(1H, H9', m); 5.42(2H, H11, s); 5.80(1H, H2', d, J=16 Hz); 7.00(1H, H3', dt $J_{3'4'}$=6.5 Hz, $J_{2'3'}$=16.0 Hz); 8.00-8.40(9H, H2-10, m).

All samples, both solid or in solution, were wrapped in foil for protection from light and kept in the freezer (-10°C) until required.

5.2 Decomposition of the reagent

The decomposition rate of PDAM in ethyl acetate has been reported[27]. It was found that 15% of the PDAM decomposed over 20 days at -20°C and 80% decomposed over 20 days at room temperature. Since the entire reaction mixture was injected onto the HPLC where the solvent system is acetonitrile-water mixtures, we wished to dissolve and store the PDAM in acetonitrile. PDAM dissolved in acetonitrile was kept in the freezer (-10°C) and examined by HPLC over a period of 27 days (Figure 5.2). There are several peaks present in the chromatogram, even immediately after purchasing the PDAM. The relative area (reported by the integrator) of the major peak (retention time=2.5 min., 100% acetonitrile) was 92% on the first day monitored and decreased to approximately 65% of its initial intensity over 27 days at -10°C.

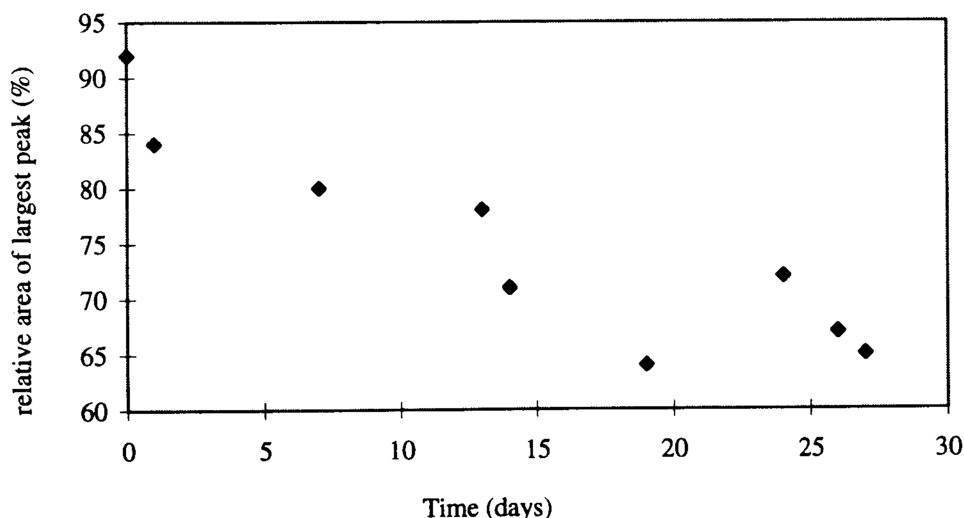

Figure 5.2 Apparent decomposition of PDAM stored in acetonitrile at -10°C. Isocratic elution (acetonitrile) Nova-Pak™ C18 column (300 mm x 3.9 mm i.d.), flow rate = 1ml/min. Fluorometric detection at 388 nm with excitation at 232 nm.

When concentrated solutions of PDAM were required, it was dissolved in acetonitrile-ethyl acetate (1:1) since the solubility of PDAM in acetonitrile is limited. For routine analyses, fresh solutions of PDAM were made every 3 or 4 days, wrapped in foil and stored in the freezer.

5.3 High performance liquid chromatography

The ideal HPLC solvent system was found by injecting samples of reagent, purified ODA pyrenyl ester and HDA pyrenyl ester. The amount of water in acetonitrile was increased by 5% increments until the pyrenyl esters were baseline resolved. Optimal separation of pyrenyl esters was obtained with 20% water in acetonitrile (operating pressure=2900 psi). The retention times of several derivatized acids were recorded (Table 5.3).

Table 5.3 Retention times of PDAM esters. Isocratic elution (20:80 water-acetonitrile), flow rate 1ml/min, Nova Pak™ C18 column (300 mm x 3.8 mm i.d.). Fluorometric detection at 388 nm with excitation at 232 nm.

Carboxylic acid derivatized	Retention time(min)
ODA	9.3
HDA	8.6
9-hydroxydecanoic acid	9.1
(E)-10-hydroxy 2-decenoic acid	9.0
10-hydroxydecanoic acid	9.6
lactic acid	3.9
formic acid	5.1
acetic acid	5.7
propionic acid	7.1
pentanoic acid	11.5
hexanoic acid	15.1
PDAM and decomposition product	2.5, 3.5

The shorter (250 mm. x 4.6 mm. i.d.) Nova Pak™ C18 column required a greater amount of water (25%, flow rate=1ml/min, operating pressure=1780 psi) in order to achieve good separation of the ODA (16.0 min) and HDA (14.3 min) pyrenyl esters.

5.4 Excitation and emission spectra

For the ODA ester, the excitation spectrum was found to exhibit two absorptions where fluorescence intensity is high, 232 nm and 242 nm (Figure 5.4a). The emission scans were very similar for these two excitation wavelengths with that of 232 nm reaching a slightly higher (5%) maximum fluorescence intensity. The spectra were therefore obtained with the excitation wavelength set to 232 nm. The optimal emission wavelength was 388 nm (Figure 5.4b).

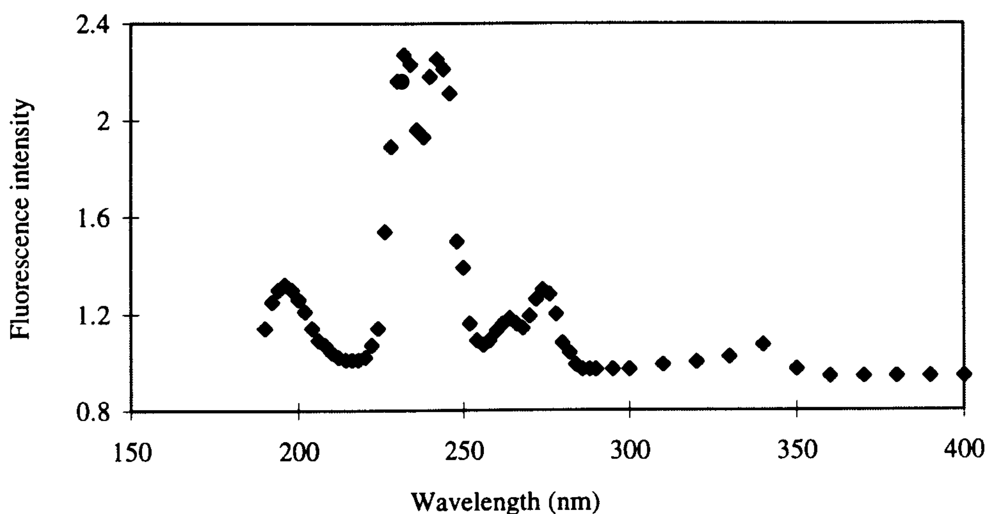

Figure 5.4a Excitation spectrum of the ODA pyrenyl ester. Emitted light intensity is measured at all wavelengths. Isocratic elution (25:75 water-acetonitrile), flow rate=1 ml/min on a reverse phase Nova Pak™ C18 column (250 mm x 4.6 mm i.d.).

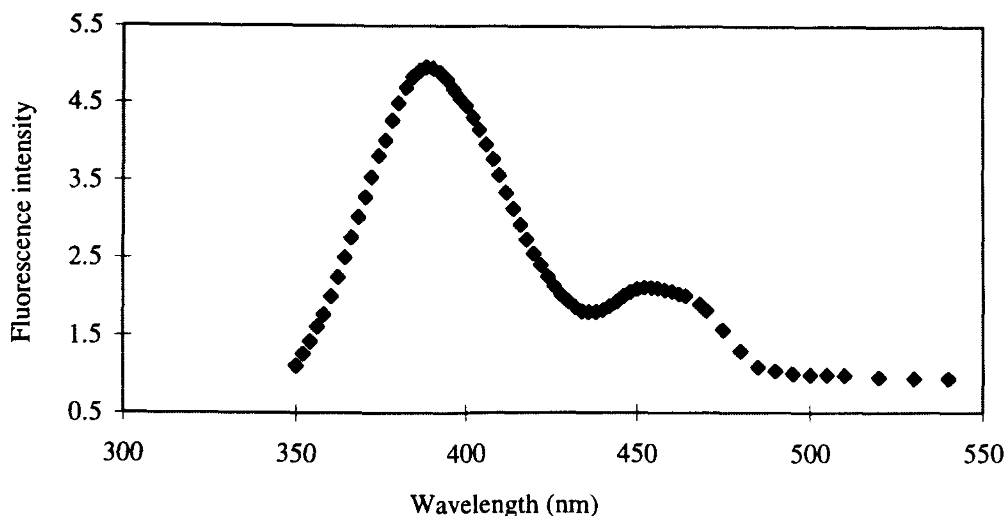

Figure 5.4b Emission spectrum of the ODA pyrenyl ester. Excitation wavelength =232 nm. Isocratic elution (25:75 water-acetonitrile), flow rate=1 ml/min on a reverse phase Nova Pak™ C18 column (250 mm x 4.6 mm i.d.).

Emission around 460 nm was also observed in the solvent system alone when excited at 232 nm. It is therefore likely due to a solvent contamination. The excitation and emission maxima for the HDA derivative were identical. Changes in the composition of the water-acetonitrile elution solvent had no effect on the excitation and emission spectra of the purified esters. Another emission spectrum was obtained after the time required to determine the optimum excitation and emission wavelengths (~15 minutes) and the fluorescence intensity had not decreased. The amount of time spent in the flow cell of the fluorescence detector did not ever exceed this time since the HPLC flow for routine analyses was continuous. A 370 nm cut-off filter was chosen to remove stray light longer than the excitation wavelength but shorter that of emission. The absorption spectrum of the cut-off filter was obtained, the absorbance at 388 nm was 0.1 (80% transmittance).

5.5 Effect of the photomultiplier tube (PMT) setting

The voltage across the dynodes inside the photomultiplier defines the PMT gain. If a concentrated sample is injected when the PMT gain is too high, the peaks go off scale and are not properly integrated. It is therefore important to know the effect of the PMT gain on the integrated area since it was necessary to compare areas detected at different PMT settings. At each PMT setting, identical amounts were injected and the fluorescence intensities of the ODA and HDA esters were monitored (Figure 5.5). It was found that the intensity doubles with each step up to a PMT gain of 14. Above this the fluorescence intensity was less than expected, increasing only by a factor of 1.2. All subsequent measurements were done at PMT settings of 14 or less.

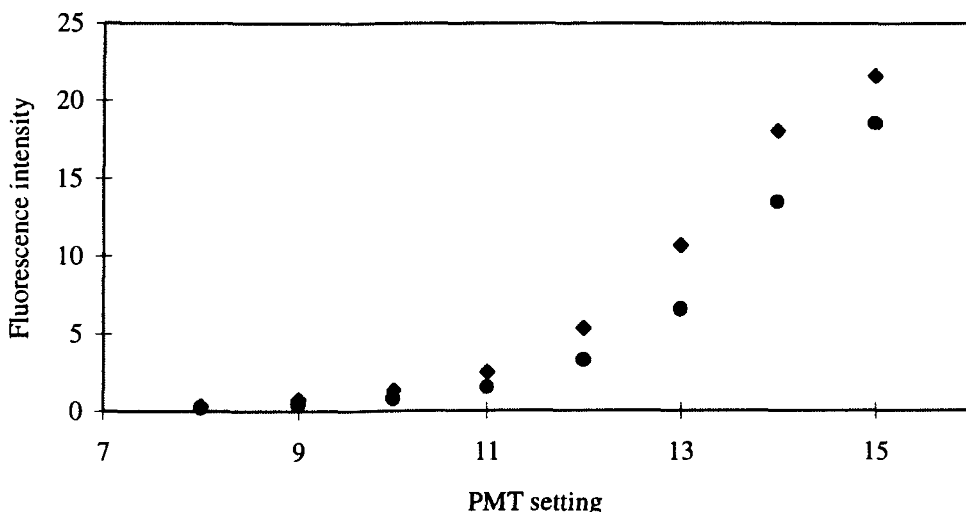

Figure 5.5 Effect of the PMT gain on the fluorescence intensity of the ODA ♦ (3.12×10^{-8} g) and HDA ● (1.87×10^{-8} g) fluorescent esters. Separation by isocratic elution (25:75 water-acetonitrile), flow rate=1 ml/min on a reverse phase Nova Pak™ C18 column (250 mm x 4.6 mm i.d.). Fluorometric detection at 388 nm with excitation at 232nm.

5.6 Calibration by dilution of synthetic standards

A sample of 1.1 mg ODA and 0.64 mg HDA synthetic QMP was derivatized and serial diluted. Aliquots of 10 μl of each sample were injected and the mean (n=3) fluorescence intensities recorded. Figure 5.6 shows the calibration and table 5.6 lists the mean fluorescence intensities and the standard errors of these means.

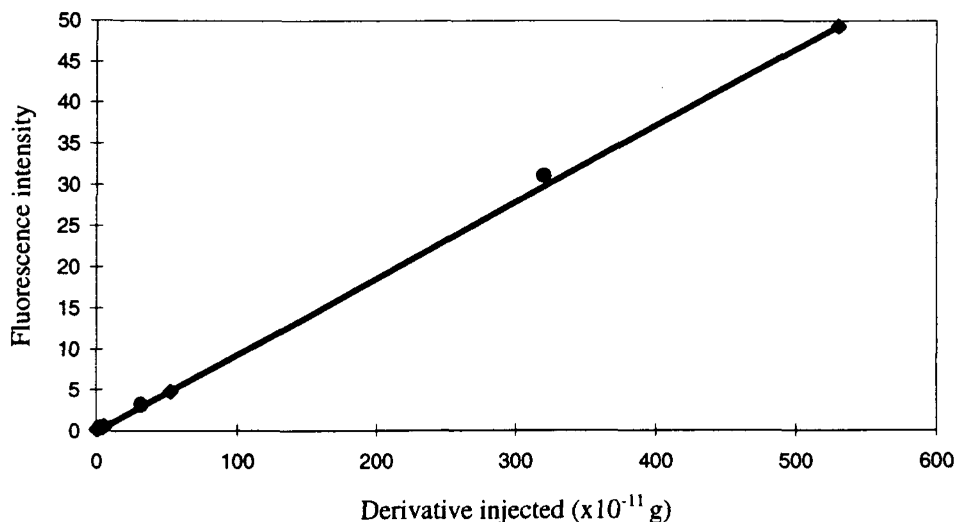

Figure 5.6 Calibration of the ODA ♦ and HDA • esters by serial dilution of synthetic standards. Linear regression performed on ODA data: $r^2=1$, $y=0.093x-0.013$ Separation by isocratic elution (25:75 water-acetonitrile), flow rate=1 ml/min on a reverse phase Nova Pak™ C18 column (250 mm x 4.6 mm i.d.). Fluorometric detection at 388 nm with excitation at 232nm.

Table 5.6 Mean fluorescence intensities (n=3) and the standard error of the mean at several ODA and HDA pyrenyl ester concentrations by successive dilution of a synthetic standard. Separation by isocratic elution (25:75 water-acetonitrile), flow rate=1 ml/min on a reverse phase Nova Pak™ C18 column (250 mm x 4.6 mm i.d.). Fluorometric detection at 388 nm with excitation at 232nm.

Ester injected ($x10^{-11}$ g)		Mean fluorescence intensity		Standard error	
ODA	HDA	ODA	HDA	ODA	HDA
0.53	0.32	0.12	---	0.02	---
5.3	3.2	0.52	0.32	0.04	0.01
53	32	4.78	3.15	0.03	0.04
530	320	49.30	31.04	0.40	0.09

5.7 Optimization of reaction conditions at low substrate concentrations

Derivatization of known small amounts of pheromone, rather than serial dilution of a stock solution closer simulates the conditions encountered when derivatizing biological samples. Reaction rates are slower at the very low concentrations of substrate encountered. To obtain efficient, reproducible labeling, optimal reaction conditions such as the reaction vessel, time, and reagent concentration must be established.

5.7a Reaction vessel

Since the amount of substrate is very small, the solvent volume used was kept to a minimum. For routine analyses, only 3-5 μl of solvent was used. Glass capillaries cut to 3 cm and sealed at one end were found to be of an appropriate size. The reagent and sample were added and the capillaries were flame sealed. The entire reaction mixture was directly injected onto the HPLC without any pretreatment.

To determine if the soft glass surface was responsible for the unacceptable variations in fluorescence intensities of samples containing identical amounts of ODA initially encountered, silylated reaction vials were prepared. The capillaries were filled with a 5% solution of $Cl_2Si(CH_3)_2$ in toluene and heated at 94°C for two hours. The capillaries were rinsed first with toluene and then methanol and dried (94°C). Samples of $1x10^{-9}$ g of ODA were derivatized in both silylated and unsilylated glass capillaries and the fluorescence intensities compared. The results indicated that the fluorescence intensity yield was 23% higher in silylated capillaries. All subsequent reactions were carried out in silylated capillaries.

5.7b Reaction temperature and time

Uniform heating of the sealed capillaries was accomplished by complete submersion in a heated oil bath. In most reported studies[27-30] derivatization was carried out at either room temperature or 40°C. Due to the low concentrations, all subsequent analyses were done at 40°C.

The optimal reaction time was investigated by following the formation of the ODA and HDA esters. In a small reaction vial made from a Pasteur pipette, PDAM (3.4×10^{-5} g) in acetonitrile-ethyl acetate (1:1, 90 µl) and ODA and HDA (5×10^{-8} and 3×10^{-8} g respectively, 10 µl acetonitrile) were allowed to react. The silylated vial was stoppered with a glass plug covered with Parafilm. Aliquots of 5 µl were removed several times during the reaction, separated by HPLC and the fluorescence intensity of both the ODA and HDA derivative peaks were monitored (Figure 5.7a). The fluorescence intensity reached its approximate maximum after about 7 hours.

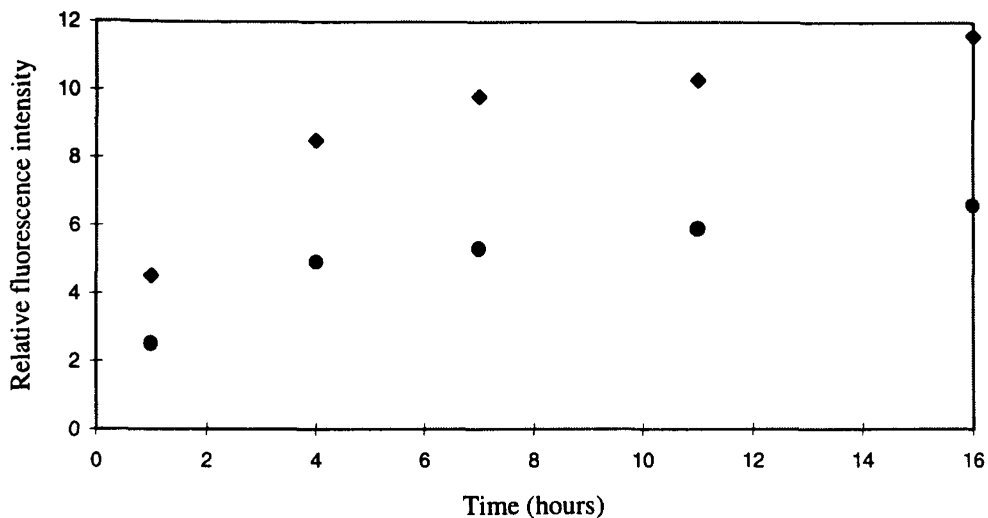

Figure 5.7a Formation of the ODA ♦ and HDA ● derivatives as a function of time at 40°C in silylated glassware. Separation by isocratic elution (25:75 water-acetonitrile), flow rate=1 ml/min on a reverse phase Nova Pak™ C18 column (250 mm x 4.6 mm i.d.). Fluorometric detection at 388 nm with excitation at 232nm.

For comparison a similar study was conducted using unsilylated glassware. The fluorescence intensity reached its maximum after about 8 hours but then began to decrease (Figure 5.7b). Presumably, the ester formed is undergoing a reaction at the basic or acidic sites on the soft glass surface of the capillary tube.

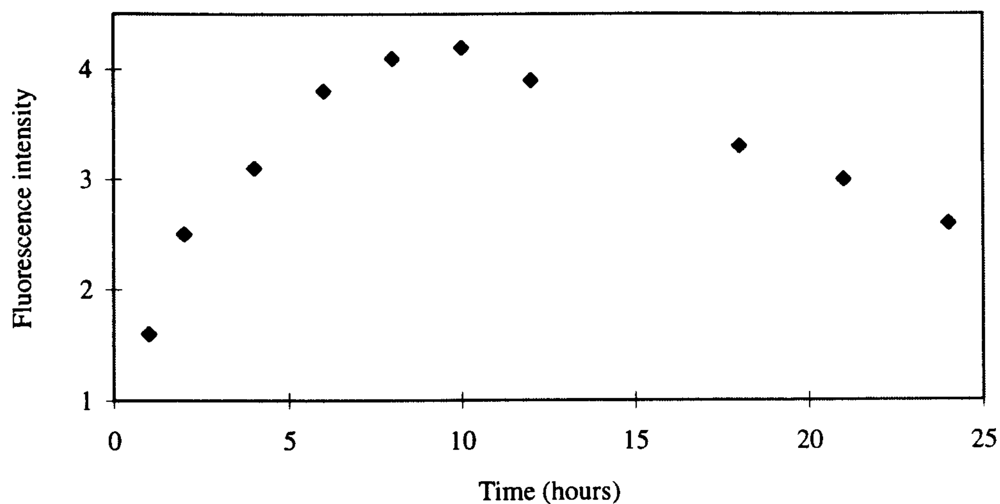

Figure 5.7b Formation of the ODA derivative as a function of time at 40°C in unsilylated glassware. Separation by isocratic elution (25:75 water-acetonitrile), flow rate=1 ml/min on a reverse phase Nova Pak™ C18 column (250 mm x 4.6 mm i.d.). Fluorometric detection at 388 nm with excitation at 232nm.

5.7c Reagent concentration

The effect of the PDAM concentration on the reaction yield was investigated and the results are shown in Figure 5.7c and the mean fluorescence intensities and standard errors of the means are listed in Table 5.7c. A stock solution of PDAM (4 mg) in ethyl acetate-acetonitrile (1:1, 10 ml) was prepared. Samples of 1×10^{-9} g of ODA were derivatized in silylated capillaries by adding either 0.5 μl (2.7×10^{-7} g), 2 μl (10.8×10^{-7} g) or 5 μl (27×10^{-7} g) of the stock PDAM solution. Three replicates at each amount of reagent were prepared. The capillaries were flame sealed and heated to 40°C overnight. The samples were then transferred by rinsing (acetonitrile, 25 μl) into larger vials made from Pasteur pipettes. The entire sample was injected onto the HPLC.

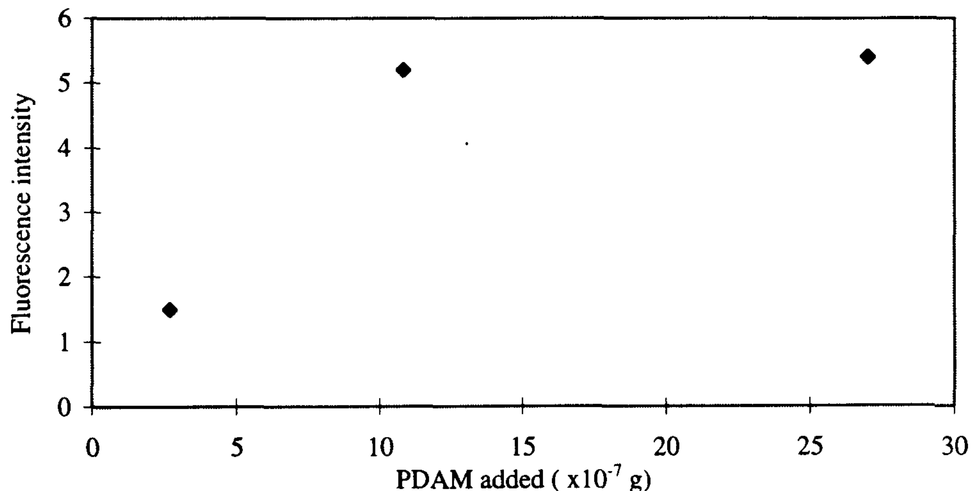

Figure 5.7c Formation of the PDAM ester of ODA upon addition of different amounts of PDAM. Separation by isocratic elution (25:75 water-acetonitrile), flow rate=1 ml/min on a reverse phase Nova Pak™ C18 column (250 mm x 4.6 mm i.d.). Fluorometric detection at 388 nm with excitation at 232nm.

Table 5.7c Mean fluorescence intensities and the standard error of the mean (n=3) upon addition of different amounts of PDAM. Separation by isocratic elution (25:75 water-acetonitrile), flow rate=1 ml/min on a reverse phase Nova Pak™ C18 column (250 mm x 4.6 mm i.d.). Fluorometric detection at 388 nm with excitation at 232nm.

PDAM added ($x10^{-7}$ g)	Mean fluorescence intensity of the ODA ester	Standard error
2.7	1.5	1.2
10.8	5.2	0.2
27.0	5.4	0.2

The fluorescence intensities of the samples containing 0.5 μl of stock solution were substantially lower than those containing 2 and 5 μl. All subsequent small scale derivatizations were performed using 2-3 μl of PDAM stock solutions.

5.7d Stability of PDAM esters

The stability of the PDAM esters under formation conditions was monitored over a period of 25 hours. Purified ODA (5.3×10^{-8} g) and HDA (3.2×10^{-8} g) fluorescent esters were heated to 40°C with reagent (3.4×10^{-5} g) in a silylated Pasteur pipette vial and the fluorescence intensity monitored. The results indicated that no discernible decomposition of the fluorescent esters had occurred after 20 hours at 40°C (Figure 5.7d).

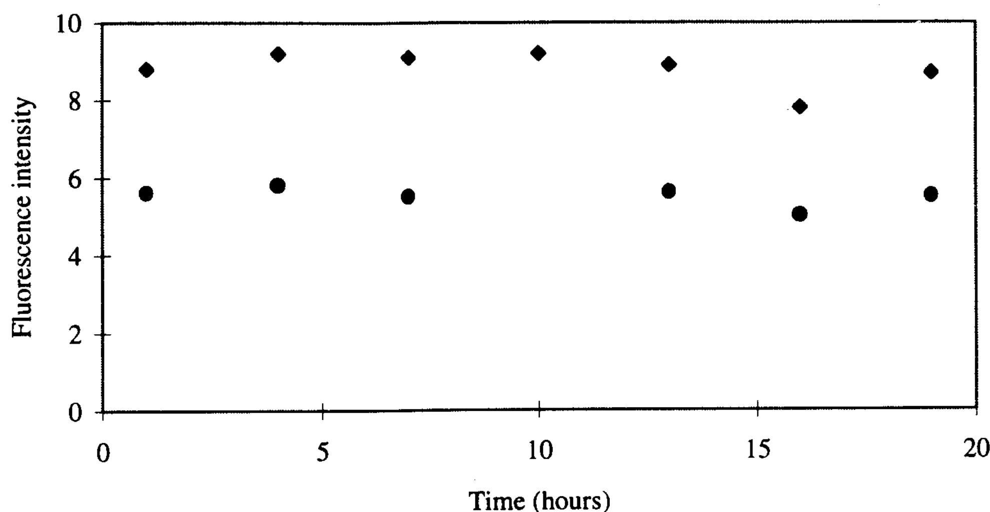

Figure 5.7d Stability of the ODA♦ and HDA• esters (measured as fluorescence intensity) as a function of time at 40°C. Separation by isocratic elution (25:75 water-acetonitrile), flow rate=1 ml/min on a reverse phase Nova Pak™ C18 column (250 mm x 4.6 mm i.d.). Fluorometric detection at 388 nm with excitation at 232nm.

5.8 Calibration at low substrate concentrations

Samples of known quantities of synthetic QMP were derivatized in silylated capillaries. Three replicates were done at each concentration and the mean fluorescence intensities (n=3) measured. The PMT setting must be increased at lower concentrations of ODA, therefore two calibration curves were prepared. Figure 5.8a and 5.8b show the calibrations and Tables 5.8a and 5.8b show the mean fluorescence intensities and the standard errors in these means.

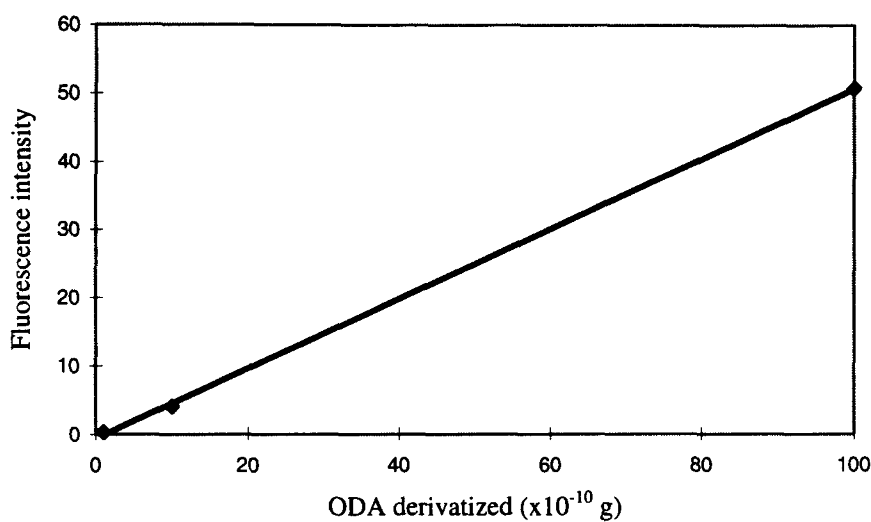

Figure 5.8a Calibration of the ODA fluorescent esters from 10^{-10} to 10^{-8} g (r^2=0.9998, y=0.5128x-0.6167). Separation by isocratic elution (25:75 water-acetonitrile), flow rate=1 ml/min on a reverse phase Nova Pak™ C18 column (250 mm x 4.6 mm i.d.). Fluorometric detection at 388 nm with excitation at 232nm. PMT=11.

Table 5.8a Mean fluorescence intensities and the standard error of the mean (n=3) at different ODA ester amounts (10^{-10} to 10^{-8} g). Separation by isocratic elution (25:75 water-acetonitrile), flow rate=1 ml/min on a reverse phase Nova Pak™ C18 column (250 mm x 4.6 mm i.d.). Fluorometric detection at 388 nm with excitation at 232nm. PMT=11

ODA ester injected (x10^{-10} g)	Mean fluorescence intensity	Standard error
1	0.27	0.02
10	4.1	0.27
100	50.7	2.94

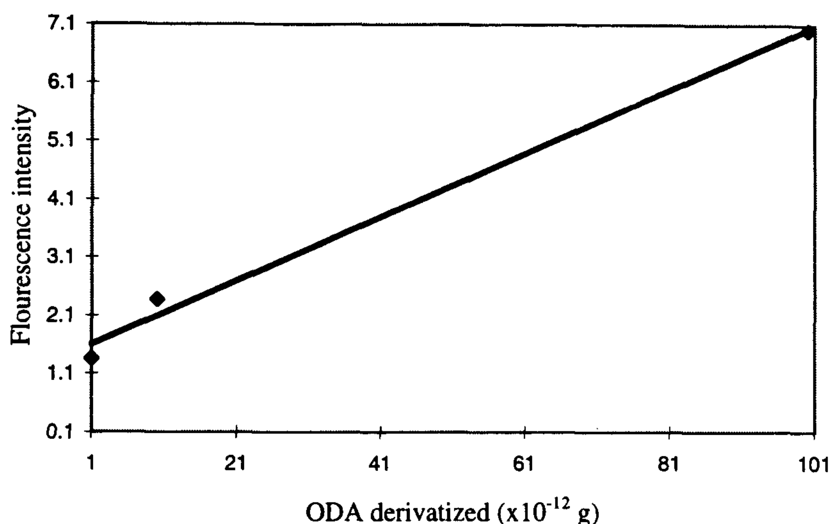

Figure 5.8b Calibration of the ODA fluorescent ester from 10^{-10} to 10^{-12} g (r^2=0.9926, y=0.0548x+1.5511). . Separation by isocratic elution (25:75 water-acetonitrile), flow rate=1 ml/min on a reverse phase Nova Pak™ C18 column (250 mm x 4.6 mm i.d.). Fluorometric detection at 388 nm with excitation at 232nm. PMT=14.

Table 5.8b Mean fluorescence intensities and the standard error in the mean (n=3) at different ODA ester amounts (10^{-12} to 10^{-10} g). Separation by isocratic elution (25:75 water-acetonitrile), flow rate=1 ml/min on a reverse phase Nova Pak™ C18 column (250 mm x 4.6 mm i.d.). Fluorometric detection at 388 nm with excitation at 232nm. PMT=14

ODA ester injected ($x10^{-12}$ g)	Mean fluorescence intensity	Standard error
1	1.36	0.41
10	2.37	0.40
100	7.01	0.89

Pyrene derivatives have been shown to form eximers with increased concentration.[31] Eximers do not seem to be forming since at the higher concentrations studied the calibration curves do not deviate from linearity.

6. Application of method

This method was investigated in order to be applied to the transmission of the queen mandibular pheromone of the honey bee among workers to study and quantify the pheromone flux in a honey bee colony. Preliminary experiments were carried out to test if the developed method is appropriate for studies in the colony.

A queen extract was derivatized in order to be sure that there are no compounds which elute at similar times as the ODA ester. The recovery of pheromone from treated workers was measured.

6.1 Queen extract

Many compounds are present in a biological sample and interfering peaks are a potential problem. A queen was killed by freezing. The head was crushed in acetonitrile and washed three times (3x15 μl) with fresh solvent. The extract was then diluted to 10 ml (acetonitrile), a 20 μl aliquot was removed and the solvent evaporated. PDAM was added and allowed to react overnight. A 3 μl aliquot was injected onto the HPLC, a typical chromatogram is given in Figure 6.1. The retention time of the ODA ester is 16.0 min. The other peaks are presumably unreacted reagent and reagent decomposition peaks.

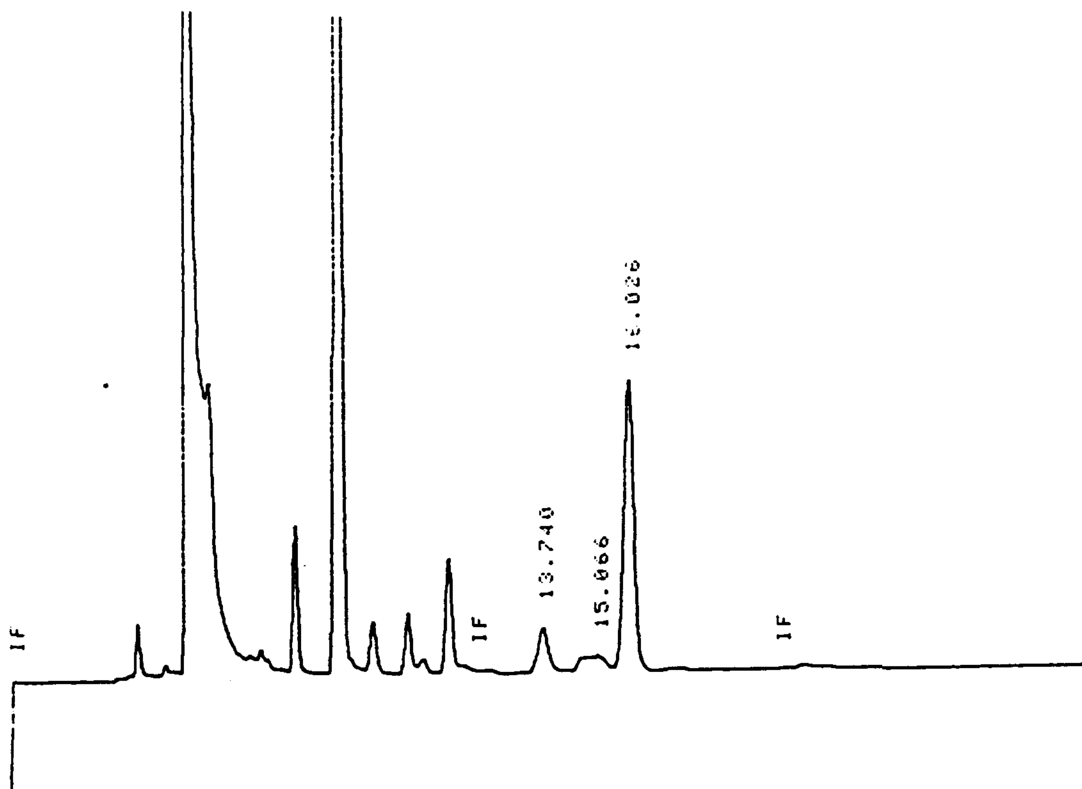

Figure 6.1 Typical chromatogram of a PDAM derivatized queen head extract. Isocratic elution (25:75 water-acetonitrile) Nova-Pak TM C18 column (250 mm x 3.9 mm i.d.) flow rate=1 ml/min. Fluorometric detection at 388 nm with excitation at 232nm. PMT=8.

6.2 Recovery of pheromone from treated workers

Workers of unknown age were collected from a queenright colony in the SFU apiaries. Fifteen workers were killed by freezing, ten of which were treated topically with 1 x 10^{-9}g of ODA on the dorsal surface of the abdomen. The remaining five untreated workers were used as untreated controls. Immediately after evaporation of the solvent the workers were washed. Two methods of washing the workers were used. Four treated and two untreated workers were each suspended by an antenna and 5 x 50 μl of methanol was dribbled over the corpse into a small vial made from a Pasteur pipette. The combined methanol washes were evaporated with a stream of air and transferred by rinsing with methanol (15μl) into silylated capillaries.

Six treated and three untreated workers were placed in a 2 ml fritted disk Buchner funnel wetted with 50 μl of methanol. The worker was washed with 5 x 50 μl then removed and the filter was further rinsed with 2 x 50 μl. The combined methanol washes were again evaporated with a stream of air and the residue was transferred with rinsing (methanol, ~15μl) into silylated capillaries. The solvent was allowed to evaporate overnight. PDAM was added and the capillaries were flame sealed and kept at 40°C overnight.

The washes done by suspension by an antenna and those with the funnel were done on different days, therefore standards of QMP containing similar amounts of solvent as the biological samples were also prepared on both days and the amounts of ODA in the washes determined from these standards.

Table 6.2 Amount of ODA recovered from treated workers. Separation by isocratic elution (25:75 water-acetonitrile), flow rate=1 ml/min on a reverse phase Nova Pak™ C18 column (250 mm x 4.6 mm i.d.). Fluorometric detection at 388 nm with excitation at 232nm. PMT=12.

Treatment and method	Relative fluorescence intensity	Corresponding amount of ODA ($x10^{-10}$ g)
Untreated workers suspended by an antenna	0.6	2.7
	0.5	2.0
Treated workers ($1x10^{-9}$ g ODA) suspended by an antenna	7.6	42
	1.0	5
	3.7	20
	2.6	14
Untreated workers washed in a funnel	0.7	3.2
	2.3	5.7
	47	77
Treated workers ($1x10^{-9}$ g ODA) washed in a funnel	1.1	3.8
	0.25	2.4
	3.0	6.8
	1.2	3.9
	5.7	11
	3.5	7.6

The amounts of ODA recovered from the untreated workers was highly variable. This might be due to endogenous ODA on the workers when they were retrieved from the colony. Naumann *et al.*[12] has shown that the amount of ODA that can be washed from the cuticle decreases with time spent isolated from the queen. They found that after only 60 minutes, over half of the ODA had been internalized. To ensure that endogenous ODA levels are low on untreated workers, a further test could be done where workers would be collected in a small cage and allowed to sit a few hours before treatment and analysis.

6.3 Discussion

BrMMC was an inappropriate reagent for the study of QMP at levels expected in the colony. The reaction requires the formation of the carboxylate anion before esterification occurs, this extra step led to reduced yields at very low substrate concentrations. High water concentrations in the HPLC solvent system required for separation produced high operating pressures and the fluorescence quantum yield appeared to be low in acetonitrile rich solvent. The detection limit was 1×10^{-9} g of ODA, found by successively diluting a purified standard.

The pyrenyldiazomethane reagent offers the advantage of a simple, sensitive and selective method for the chromatographic analysis of QMP components. The carboxylic acid components of the pheromone blend are derivatized under mild conditions in the absence of a catalyst to produce highly fluorescent esters.

Solutions of PDAM in acetonitrile are stable for 4 days stored in the freezer, the PDAM esters are stable for months. Optimal reaction conditions prove to be derivatization in a silylated capillary which is sealed and submersed in oil at 40°C overnight.

Separation of PDAM esters was accomplished on a reverse phase C18 column. The entire reaction mixture was directly injected onto the HPLC without any pretreatment. In order to maintain a reasonable analysis time, the HDA ester was not

separated from the reagent and reagent decomposition peaks, which is present in a large excess, but certainly could be by adjusting the HPLC solvent system to gradient elution. HDA was therefore not determined in the queen extracts and recovery from workers experiments.

The PDAM esters were detected fluorometrically at 388 nm with excitation at 232 nm. Calibration curves at different amplifications were prepared. Samples containing quantities of ODA expected in the colony were derivatized with a large excess of PDAM and analyzed. $1x10^{-12}$ g of the ODA ester was successfully derivatized and detected.

Preliminary studies were carried out to test if the investigated method is appropriate for studies in a honey bee colony. A queen extract was derivatized and there were no other components of the extract that interfered with the separation of ODA. ODA was successfully recovered from treated workers.

HPLC separation followed by fluorometric detection of the ODA pyrenyl ester appears to be a very sensitive technique to follow the transmission of QMP in the honey bee colony.

7 Summary

A method for the quantitative determination of the two most abundant components of the queen honey bee mandibular pheromone, ODA and HDA, has been investigated. ODA and HDA were derivatized with 1-pyrenyldiazomethane in sealed silylated capillaries submersed in oil at 40°C overnight. The entire reaction mixture is injected onto the HPLC without any pretreatment. The separation of esters was achieved with a reverse phase C18 column with a mobile phase of 25% water in acetonitrile and a flow rate of 1 ml/min. The esters were detected fluorometrically at 388 nm with excitation at 232 nm. 1×10^{-12} g of ODA was derivatized and detected. Preliminary experiments which apply the method to the study of pheromone flux in a honey bee colony were completed. A queen extract was derivatized and ODA was successfully recovered from treated workers.

ODA derived from individual bees in a colony should be detectable by derivatizing with PDAM, separation by HPLC, and detection by fluorescent emission. This methodology should provide a sensitive method of estimating the flux of queen pheromone throughout a functioning honey bee colony.

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