PENICILLIUM ROQUEFORTI TOXINS:

DETECTION AND QUANTITATION BY

REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

A Thesis
Submitted to the Faculty
of
Graduate Studies
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by
Karin Siemens

In Partial Fulfillment of the
Requirements for the Degree
of
Master of Science

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This thesis is dedicated to my parents who have continually affirmed the value of education, and who have given me the encouragement, patience and understanding I needed to achieve my goals.

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Sincerest gratitude is extended to my family. I am indebted to my sister, Renita for putting up with me and standing by me, and to my parents, Helene and Gerhard Siemens for their unconditional love and support throughout my studies.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
$CDCl_3$	Deuterated chloroform
^{13}CNMR	Carbon-13 Nuclear Magnetic Resonance
ELISA	Enzyme-Linked Immunosorbent Assay
ε	Extinction coefficient
HPLC	High-performance liquid chromatography
^{1}H NMR	Proton Nuclear Magnetic Resonance
ID_{50}	Dose for 50% inhibition
IR	Infrared
LD_{50}	50% lethal dose
MHz	Megahertz
NMR	Nuclear Magnetic Resonance
NP	Normal phase
PBS	Phosphate buffered saline
PR	*Penicillium roqueforti*
PRI	*Penicillium roqueforti* imine
PR-OH	*Penicillium roqueforti* alcohol
PRT	*Penicillium roqueforti* toxin
R_F	Relative mobility
RIA	Radioimmunoassay
RP	Reversed-phase
TLC	Thin-layer chromatography
TMS	Trimethylsilane
UV	Ultraviolet
YES	Yeast extract with sucrose

ABSTRACT

Methods for the determination and quantitation of *Pencillium roqueforti* toxin (PRT) and its imine form were investigated. As a result of these studies, two novel reversed-phase high-performance liquid chromatography (HPLC) methods have been developed. These techniques were used to detect and quantify both types of toxins in blue-veined cheese and were also used to study the interconversion of these toxins in a variety of media. The first HPLC system, employing a mobile phase comprised of methanol and water (65:35, v/v) at a flow rate of 1.0 ml/min, had a quantitation limit of 3 ng of PRT, and a detection limit of 2 ng of the toxin. Precision analyses based on numerous runs resulted in highly reproducible retention times with an average variation coefficient of 1.6%. The procedure was very accurate with mean recoveries of PRT from spiked culture medium and blue cheese extracts of 96.8 and 100.4%, respectively. Analyzed samples showed no interference of the matrix in the detection of PRT, and this allowed studies on the stability of PRT in cheese to be conducted. After extraction of the spiked cheese sample a mean recovery of only 48.6% of PRT was obtained, which suggested that PRT is unstable in cheese.

The development of the HPLC method arose from attempts to devise a method which could be used to assess and validate a monoclonal antibody-based immunochemical technique for discrimination and detection of both toxins. In order to establish a highly specific immunochemical assay, attempts were made to chemically modify PRT, in order to develop an adequate PRT-protein conjugate for the

subsequent production of a monoclonal antibody specific for either PRT or PR imine. The initial modification step involving the alkaline hydrolysis of PRT, resulted in the PR alcohol form. Numerous procedures were employed to further succinylate the PR alcohol into the hemisuccinate form of the toxin. However, the production of a protein conjugate was not feasible due to the unsuccessful formation of the PR-hemisuccinate.

Alterations of the HPLC procedure permitted a sensitive concomitant determination of PRT and a chemical derivative of PRT, PR imine. Both compounds were adequately resolved with a selectivity factor of 3.18. This method, using a solvent system of acetonitrile and water (60:40, v/v) at a flow rate of 1.0 ml/min, resulted in a quantitation limit of 1.5 ng for PRT and 1.0 ng for PR imine. The retention times (5.86 min and 3.38 min for PRT and PR imine, respectively), and peak heights for both compounds were highly reproducible. The accuracy assessment of the method resulted in mean PRT and PR imine recoveries of 89.2 and 110.6%, respectively. The analysis of PRT and PR imine in cheese sample extracts also showed no interference of the sample matrix.

Consequently, the second reversed-phase HPLC method was used to analyze 60 samples of blue cheese and 6 samples of blue cheese dressing for the presence of PR imine and PRT. PR imine was found in 50 of the cheese samples analyzed in amounts ranging from 19.2 to 41.9 μg/kg of cheese, although no PRT was detected. Analysis of blue cheese dressing showed no evidence of PR imine or PRT. In addition to the analyses of these toxins occurring naturally in cheese, the stability of PR imine and PRT in spiked cheese samples was also investigated using this procedure. PRT when spiked into cheese almost completely disappeared when stored. This was associated with a corresponding increase in the amount of PR imine present.

Furthermore, the conversion of PR imine to PRT in the presence of fetal bovine serum was demonstrated. As a result of this conversion, a reduction in the amount of PR imine spiked into fetal bovine serum was observed. After two days of serum storage, only 6-7% of the original PR imine remained. The reduction of PRT that resulted from PR imine conversion, was also observed, but this is most likely due to the sequestering effect of serum proteins.

FORWORD

This thesis is a compilation of individual manuscripts. Although these manuscripts are interrelated, each is, for the most part, independent from the other, and has been prepared in accordance with the instructions given by the specified journal. Manuscript I, describes the determination of *Penicillium roqueforti* toxin (PRT) by reversed-phase high-performance liquid chromatography (HPLC). This paper was published in the Journal of Chromatography. The next manuscript discusses attempts to conjugate PRT in order to develop a monoclonal antibody to this toxin. Manuscript III expands on the reversed-phase HPLC method for the detection of PR imine, an analogue of PRT. This manuscript was submitted to the Journal of Applied and Environmental Microbiology. Finally, manuscript IV, reports the detection of the PR imine form naturally occurring in blue cheese, as assessed by reversed-phase HPLC. This manuscript, as the results are of unique toxicological significance, was submitted to the Journal of Food Protection.

1. INTRODUCTION

Mycotoxins are toxic chemical substances produced by certain moulds and their toxicity syndromes in animals are referred to as mycotoxicoses. Mycotoxins and mycotoxicoses have existed for decades. Ergotism or "holy fire" is the earliest and best known mould-associated disease, and was common in central Europe from the ninth to the 14th century. Since then frequent outbreaks of different mycotoxicoses have occurred, in animals as well as humans after the ingestion of foods contaminated with various mycotoxins. These mycotoxins have since been identified as being primarily associated with such fungal species as *Penicillium, Aspergillus, Fusarium, and Alternaria* (Davis and Diener, 1978).

Although about 300 mycotoxins have already been identified, the number of known mycotoxins continues to increase (Scott, 1990). In a recent update on mycotoxin contamination, the most concern was expressed about aflatoxins, produced by *Aspergillus flavus* and *Aspergillus parasiticus* (Jelinek *et al.*, 1989). This was closely followed by a concern for ochratoxin A, produced by the *Aspergillus* species *ochraceus, melleus,* and *sulphureus*, as well as *Penicillium* species *viridicatum* and *cyclopium*. These mycotoxins have already undergone a rigorous evaluation with regards to their potential as health hazards, and this has provided a basis for setting up regulations, in order to maintain food safety.

A mycotoxin that has not yet been as fully studied is *Penicillium roqueforti* toxin (PRT). PRT is a toxic secondary metabolite secreted by several strains of *Penicillium roqueforti*. This is of concern as it is deliberately introduced into cheese.

However, PRT converts to its imine form in cheese, the most important reaction involving PRT in cheese. Since the discovery of PRT in 1972 by Still *et al.*, as a contaminant in grains and silage consumed by cattle, this mycotoxin has prompted a series of investigations, regarding the safety of blue-veined cheeses.

Mould cultures have been routinely used without hestitation in cheesemaking. A report suggesting that such cultures might present a hazard to the consumer's health by the formation of toxic metabolites (Engel and Teuber, 1989) now creates some concern.

Although physico-chemical and immunochemical techniques have been developed to quantify and evaluate the biological effects of this toxin, there is concern as to the sensitivity and specificity of these procedures to accurately monitor PRT and its chemical analogues.

This thesis reports an attempt at developing methods used in the analysis of PRT and its imine analogue, thereby improving previous methods both in sensitivity and specificity.

2. REVIEW OF LITERATURE

2.1 Introduction

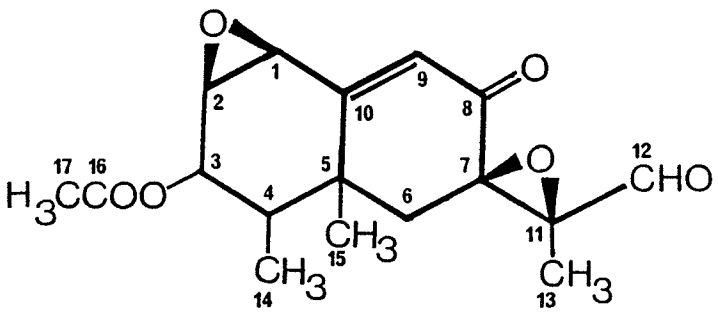
Penicillium roqueforti toxin (PRT) is a mycotoxin of possible health significance, and is a known carcinogen in rats (Polonelli *et al.*, 1982). PRT was first obtained from a strain of *P. roqueforti* isolated from mouldy silage. Death was reported for cattle eating silage contaminated with this mould, while ingestion was associated with abortion and placental retention in dairy cattle (Wei *et al.*, 1973). PRT has also been detected as a metabolite from strains of *P. roqueforti*, used primarily as a source of proteolytic and lipolytic enzymes during the maturation of blue-veined cheeses. These findings are of a concern primarily from a public health point. In addition, research on the production and detection of this mycotoxin is of particular interest due to its importance for the dairy industry.

2.2 Structure of PRT

2.2.1 Chemical and Physical Properties

The chemical structure of PRT (Figure 2.1) has been established (Wei *et al.*, 1975) as a sesquiterpenoid with a molecular weight of about 320. The structure and absolute chemical configuration of PRT has been reported by Baert *et al.* (1980). Purified PRT is a colourless crystalline substance with a melting point between 155 and 157°C, and is soluble in chloroform, methanol, acetone, ethanol, carbon tetrachloride, diethyl ether, and hydrochloric acid and sodium hydroxide aqueous solutions. It is, however, insoluble in hexane and water. The ultraviolet spectrum has

Figure 2.1 The chemical structure of *Penicillium roqueforti* toxin (PRT).

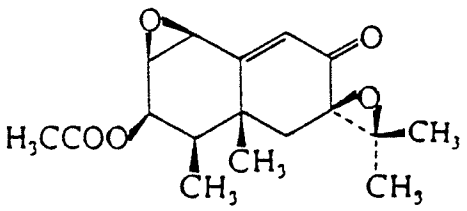

O
1
2
9
10
8
O
17 16
H3CCOO
3
4
5
6
7
O
12
CHO
CH3
15
CH3
14
11
CH3
13

λ_{max}^{EtOH} at 247 nm (ε=9000). In sufficiently purified samples the fluorscence of the toxin can be detected under ultraviolet light as a characteristic dark-blue spot at an R_F of about 0.63 on silica gel thin layer chromatography plates with methanol-chloroform (4:96, v/v) as the solvent, and becomes visable in ordinary light as a yellow spot. The toxin can also be visualized by spraying the plate with 50% sulfuric acid, where it appears immediately as a yellow spot, which after charring at 230°C turns yellowish-brown (Wei *et al.*, 1973).

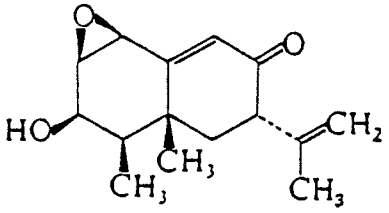
The carbon skeleton of PRT is of the eremophiline type (Betina, 1989a). Several other closely related secondary metabolites with the same carbon skeleton have also been isolated (Moulé *et al.*, 1977, Li and Chang, 1985, and Betina, 1989a) and they include: eremofortins A, B, C, D and E (Figure 2.2, a-e). Alkaloid roquefortine is another metabolite produced by *P. roqueforti* (Ohmono *et al.*, 1975 and Ohmono *et al.*, 1977).

Nuclear magnetic resonance (NMR) can be used to identify and distinguish the PRT molecule from its analogues. The ^{1}H NMR spectrum of PRT (Figure 2.3) shows a three-proton doublet at δ1.03 (J=7 Hz, CH_3), a three-proton doublet at δ1.45 (J=0.8 Hz, CH_3), a three-proton singlet at δ1.49(CH_3), a three-proton singlet at δ2.16 ($OCOCH_3$), a one-proton doublet at δ3.65(J=3.5 Hz), a one-proton doublet of doublets at δ3.96 (J=3.5, 5 Hz), a one-proton singlet at δ6.43, (isolated ethylenic proton), and a one-proton singlet at δ9.75 (CHO). These signals account for 17 protons. The three remaining protons are spread between δ1.6 to 2.3 (Wei *et al.*, 1973). PRT can be further characterized by its infrared spectrum. The infrared (IR) spectrum of PRT has bands at 2945 cm^{-1} (methyl stretching); 1735 cm^{-1}, 1720 cm^{-1}, and 1680 cm^{-1} (three C=O); 1620 cm^{-1} (ethylenic unsaturation; 1460 cm^{-1}, 1435 cm^{-1}, and 1380 cm^{-1} (methyl

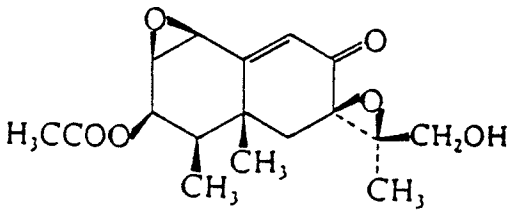
Figure 2.2 The chemical structures of molecules closely related to PRT: a) eremofortin A, b) eremofortin B, c) eremofortin C, d) eremofortin D and e) eremofortin E.


H3CCOO
O
O
O
CH3
CH3
CH3

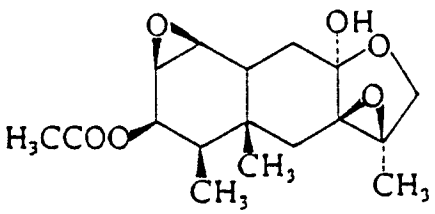
a


HO
O
O
CH2
CH3
CH3

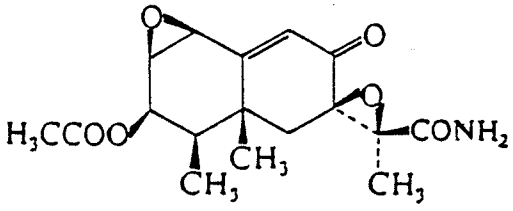
b


H3CCOO
O
O
O
CH2OH
CH3
CH3

c

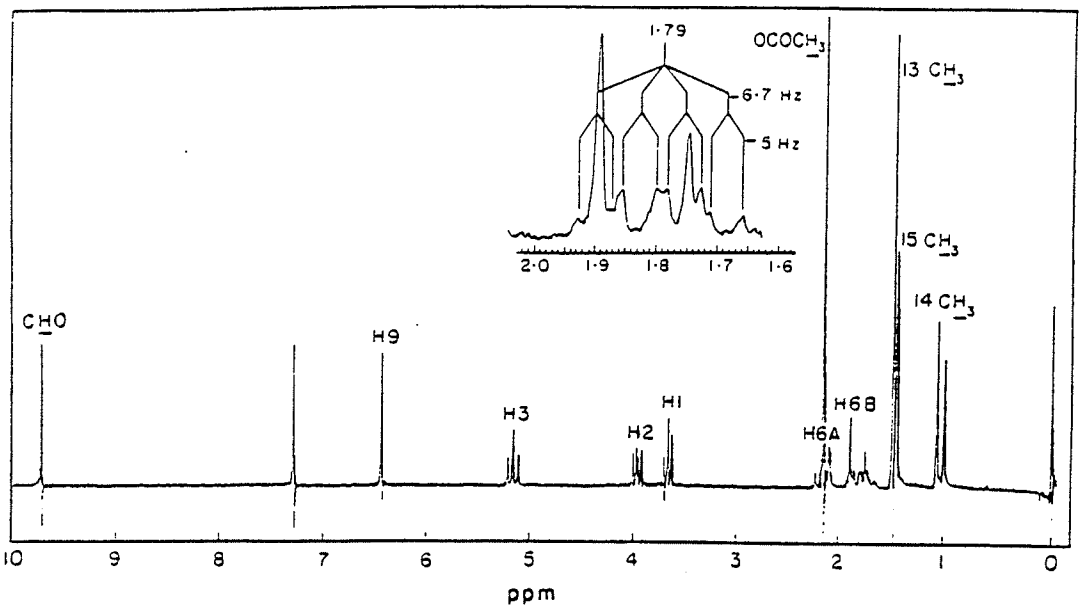

OH
O
O
O
H3CCOO
CH3
CH3
CH3

d


O
O
O
H3CCOO
CONH2
CH3
CH3
CH3

e

Figure 2.3 The 100 MHz ^1H NMR of PRT in $CDCl_3$, with trimethylsilane (TMS) as the internal standard.


1·79
OCOCH3
6·7 Hz
5 Hz
2·0
1·9
1·8
1·7
1·6
13 CH3
15 CH3
14 CH3
CHO
H9
H3
H2
H1
H6A
H6B
10
9
8
7
6
5
4
3
2
1
0
ppm

bending); 1245 cm^{-1} (C-O-C). The absence of any band in the region of 3400 cm^{-1} indicates the absence of free hydroxyl groups (Wei *et al.*, 1973).

2.2.2 Biosynthesis

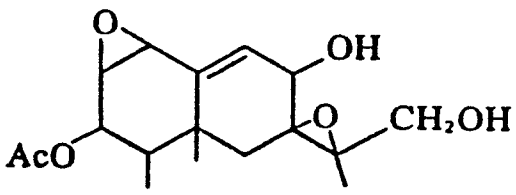
The biosynthetic origin of PRT has been investigated (Chalmers *et al.*, 1981) and it was hypothesized that PRT probably arises from acetate *via* an isoprenoid biosynthetic pathway, comprising mevalonate, dimethyl pyrophosphate, and farnesyl pyrophosphate. Subsequent cyclization initially yields a germacrene intermediate and a second cyclization proceeds *via* an eudesmane carbocation to result in the final proposed structure of PRT.

More recently, Engel and Teuber (1989) reported that acetyl-CoA (coenzyme A) plays a major role in the synthesis of this toxic secondary metabolite. PRT is synthesized more specifically *via* malonyl-CoA. Furthermore, Chang *et al.* (1985) and Li *et al.* (1985) suggested that eremofortin C (EC), another metabolite closely related to PRT, is the direct precursor of the toxin. A decrease in the amount of EC is always associated with a rapid increase in PRT production (Moreau *et al.*, 1980a). An oxidase enzyme responsible for this conversion, is secreted by species of *Penicillium*. Chang *et al.* (1985) have also reported the isolation, purification and partial characterization of this enzyme.

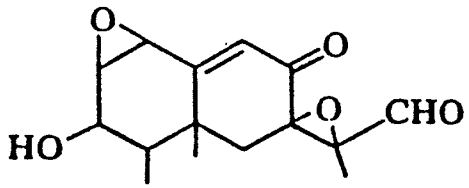
2.2.3 Chemical Reactions and Related Forms

Other altered, but closely related forms of PRT, have also been chemically derived. Tetrahydro-PR (Figure 2.4a), for example, results from the reduction of PRT with an excess of sodium borohydride in ethanol. The resulting compound has an R_F

Figure 2.4 The chemical structures of a) tetrahydro-PR and b) PR alcohol.



a



b

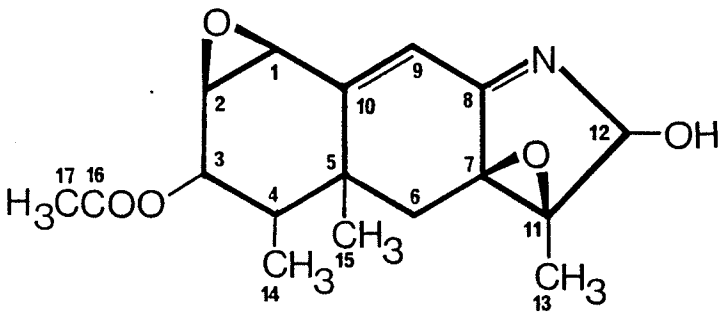
value of 0.55 in methanol-chloroform (15:85, v/v) on TLC plates, and this reduced product shows neither fluoresence nor UV absorption in the 247 nm region. Reversely, with an excess of PRT, sodium borohydride reduces the aldehyde group on the toxin molecule only to form eremofortin C (Scott, 1984), a compound found also to occur in *P. roqueforti* (PR) cultures.

Another closely related form, PR alcohol (Figure 2.4b) is the alkaline hydrolysis product of PRT (Wei *et al.*, 1975). This product has a lower R_F value (0.37) in methanol-chloroform (4:96, v/v) as assessed by TLC, and shows a λ_{max}^{MeOH} at 247 nm. Purified crystals of PR alcohol melt at 113.5-115°C. The IR spectrum lacks the carbonyl bend at 1735 cm^{-1} shown by the parent compound (Wei *et al.*, 1973).

PRT also readily undergoes a reaction with aqueous methanolic ammonia to form its analogue, PR imine (Figure 2.5). This compound exhibits a lower R_F value (0.18) in methanol-chloroform (4:96, v/v on silica gel TLC) than PRT (0.63) in the same solvent. The pale yellow PR imine crystals melt at 85-88°C and show λ_{max}^{MeOH} at 247 nm. This significant reaction is reported by Scott and Kanhere (1979) to also occur readily in blue-veined cheese. Moreau *et al.* (1980b) have isolated PR imine from PR cultures. However, to date no PR imine has been isolated from cheese.

Conversion of PR analogues back to the PRT form has also been shown under natural conditions, such as culture medium, and under controlled laboratory conditions. Moulé *et al.* (1977a) have shown a 10% conversion of PR imine to a PRT-like compound as a result of the natural hydrolysis of PR imine. Also, oxidation of eremofortin C, another secondary metabolite of PRT, was found to provide a high yield of PRT, under non-acidic conditions (Li *et al.*, 1985). Other chemical transformations have been demonstrated (Wei *et al.*, 1975) under controlled conditions such as the

Figure 2.5 The chemical structure of PR imine.


O
N
OH
O
H_3CCOO
CH_3
CH_3
CH_3
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17

acetylation of PR alcohol to PRT.

2.3 Biological Activity

Studies of the relationship between the chemical structure and biological activity suggest that the aldehyde group in position C-12, associated with the epoxy group of the PRT molecule constitutes the toxin active site (Wei *et al.*, 1973). Although *P. roqueforti* produces a number of other toxic metabolites: mycophenolic acid (Engel *et al.*, 1982), roquefortine, isofumigaclavine A, ferrichrome, and penicillic acid (Medina *et al.*, 1985), PRT is the most acutely toxic compound secreted by this species, lethal to both mice and rats.

2.3.1 Acute Toxicity

In mice, LD_{50} values for the acute toxicity of PRT range from 1 to 5 mg/kg by intraperitoneal injection (Scott, 1984), and between 16 and 50 mg/kg by oral administration (Arnold *et al.*, 1978). In rats, the LD_{50} values are slightly higher, at about 11 to 15 mg/kg by intraperitoneal injection (Scott, 1984). Reaction of PRT with L-leucine or L-γ-alanine, and conversion to the imine form were found to greatly reduce its acute toxicity (Arnold *et al.*, 1978). The LD_{50} value of PR imine is 100-200 mg/kg body weight by intraperitonial injection, although the actual role that the PR imine form may play as a toxic compound has not been fully investigated. Apparently the loss of the aldehyde functional group diminishes the toxicity of molecules such as PR imine and the eremofortins (Moulé *et al.*, 1977a). Moulé *et al.* (1977a) further concluded that PRT along with PR alcohol are the only biologically active molecules. However, a possible reformation of PRT from PR imine (Moulé *et al.*, 1977a) makes

this analogue important in overall toxicity.

In acute toxicity tests involving the intraperitoneal injection of 1.5 mg of PRT, affected animals developed difficulty breathing, motor incoordination, flaccid paralysis, and an inability to support their body weight (Polonelli *et al.*, 1978). Studies on the inhibitory effects of PRT on the protozoan *Tetrahymena pyriformis* resulted in an ID_{50} value (the dose of mycotoxin which decreased the count of *Tetrahymena pyriformis* by 50% compared to the mean control count) of about 3.80 μg/ml of PRT (Nishie *et al.*, 1989).

2.3.2 Chronic Toxicity

Histological examination of rats fed on 2 mg of PRT over a period of 52 days has indicated that this toxin exhibits carcinogenic activity. Examined rats were found to develop uterine sarcoma, liver necrosis and cirrhosis, as well as squamous epithelioma. (Polonelli *et al.*, 1982). Unfortunately, long-term effects of PRT have not been fully elucidated. More recently, however, Hradec and Vesely (1989) found PRT to have one of the highest carcinogenic activities, compared with other mycotoxins, in the initiator tRNA acceptance assay. This test analyzes the carcinogenic and mutagenic effects of molecules, in which case PRT tested positive on both counts, with a stimulation of tRNA acceptance of 59%, followed closely by aflatoxin B_1, a known carcinogen. In addition to its carcinogenicity, PRT has been found to be mutagenic to *Salmonella typhimurium*, *Neurospora crassa*, and *Saccharomyces cerevisiae* (Betina, 1989a).

2.3.3 Mechanisms of Toxicity

The observed biological effects by PRT are attributed to the induction of short-term alterations in liver metabolism (Moulé *et al.*, 1978), more specifically to the inhibition of protein and RNA syntheses. PRT also inhibits the activities of DNA polymerases: α, β, and γ (Lee Wu *et al.*, 1984), mitochondrial HCO_3^--ATPase activity (Hsieh *et al.*, 1986), and mitochondrial respiration and oxidative phosphorylation in animal cells (Wei *et al.*, 1985). Further investigation showed that PRT induces DNA-protein cross-links in chromatin of liver nuclei (Moulé *et al.*, 1980). It is suggested that the presence of the aldehyde group in the PRT molecule is required for the induction of cross-linking, in which case methylene bridges between nucleic acid and protein are presumably involved in the complex formation (Moulé *et al.*, 1980). Hence, the transcription process in the liver cells of the animal is impaired by interference of PRT in both the activity of RNA polymerases themselves and the elongation process (Betina, 1989a).

Although eremofortin C, with the reactive aldehyde group altered does not exhibit significant toxicity in animals (Chang *et al.*, 1991), Arnold *et al.* (1978), have shown that the imine analogue has a similar but diminished toxic effect as that of PRT, despite the lack of the aldehyde group in position C-12. Furthermore, Wei *et al.* (1976) showed that PR imine exhibited a 63% inhibition of protein synthesis in Ehrlich Ascites tumor cells, as compared with a 98% inhibition by PRT. In addition, PR imine inhibited 97% of nucleic acid synthesis, while PR imine exhibited a 74% inhibition.

2.4 Production of PRT

Many experiments have been conducted to determine optimum yields of PRT production by *P. roqueforti*. In most cases sucrose (15%) yeast extract (2%) (YES) was the medium chosen for time-course studies of *P. roqueforti* strains (Piva *et al.*, 1976; Scott *et al.*, 1977; Wei *et al.*, 1978 and Polonelli *et al.*, 1978). Production of PRT at 25° C in this medium peaked after 21 days of *P. roqueforti* growth; the estimated yield was dependent upon the strain used. Scott *et al.* (1977) reported yields after 21 days of 7.70 mg of toxin/100 ml for strain 596A, and Wei *et al.* (1978) reported toxin production of up to 30 mg/100 ml for ATCC 6989 and 29 mg/100 ml for ATCC 6987. However, Medina *et al.* (1985) obtained an average of 41.73 mg/100 ml for the strains ATCC 6989, ATCC 6987, and NRRL 849.

Timing of the harvest is very critical since levels can fall from a maximum to zero in only 1-2 days (Wei *et al.*, 1978), which might explain the differences observed in the yields of the toxin. PRT production occurs within pH 4.5-9 with an optimum at pH 5.5 (Polonelli *et al.*, 1978). Still (1973) found the highest concentrations of PRT (1 g/l at 21-22° C at 17-20 days of termination for strain WB 849) and further devised and reported a typical method for the production and extraction of the toxin. In this method filtered mycelia is extracted with chloroform, and column chromatography is used to purify the toxin. Several reports are given on the isolation and purification of PRT (Still *et al.*, 1972; Wei *et al.*, 1973; Scott, 1984).

2.5 Instability of PRT in Cheese

Since *P. roqueforti* cultures are deliberately introduced into cheeses such as Roquefort, Stilton, Danish Blue, Cabrales and Gorgonzola (Arnold *et al.*, 1978), there

is a particular toxicological concern about contamination by the secondary metabolite PRT. However, tests performed on whole and mouldy fractions of retail cheese samples have not yielded any presence of PRT (Lafont *et al.*, 1975; Polonelli *et al.*, 1978).

The ability of *P. roqueforti* strains to form PRT in synthetic and semi-synthetic media has been further described in numerous studies. Scott *et al.* (1977) showed that four strains of *P. roqueforti* isolated from blue cheese produced PRT. Wei and Lui (1978) tested different *P. roqueforti* strains from the American Type Culture Collection for the production of PRT. All strains were able to produce toxin at 24°C. Moreover, levels of PRT excreted by *P. roqueforti* isolates from Cabrales cheese ranged from 0.91 to 6.47 mg toxin/100 ml of YES culture broth (Medina *et al.*, 1985). However, no relationship between the origin of the isolate and the ability to synthesize PRT was found. It has been reported that conditions during cheese production favour growth of *P. roqueforti*, but not the production of PRT (Medina *et al.*, 1985).

Scott and Kanhere (1979) reported that PRT is neither stable in blue cheese nor in solvent extracts (methanol-water or chloroform) of blue cheese. In these cases PRT reacts with neutral and basic amino acids forming PR imine. Furthermore, PRT in blue cheese may react with ammonia and ammonium salts forming PR imine (Medina *et al.*, 1985). High reactivity of PRT with the above components may account for the absence of PRT in cheese.

Compounds responsible for the instability of PRT in blue cheese and extracts include amino acids (found in blue cheese in concentrations of up to 30 g/100 g total protein), such as glycine, L-cysteine, L-lysine, and L-leucine; amines such as tyramine, tryptamine, and histamine, which are known to be present in some blue cheeses in

individual concentrations of up to 2.3 mg/g; ammonia, which, during ripening, can reach levels of 6% of the amino acids; and casein and intermediate breakdown products (Scott and Kanhere, 1979). Yields of PR imine formed following addition of 10 and 20 mg/kg of PRT to Stilton cheese were 54 and 56% respectively, while yields of PR imine formed from the addition of 10 mg/kg of PRT to Roquefort cheese ranged between 8 and 15%. Furthermore, it has been shown that PR imine was unstable in blue cheese (Scott and Kanhere, 1979).

Continued experimentation seems necessary in order to determine the actual health risks involved concerning the interaction of the two forms. Although investigations to examine the inherent presence of PR imine in blue cheese samples have been conducted and proven negative (Scott and Kanhere, 1979), the detection limits of the TLC method used to quantitatively determine these compounds were generally only 0.13-0.5 mg/kg. A more sensitive method would be an asset to quantify these compounds, and could prove of great significance if, in fact, PR imine is converted to PRT *in vivo* in mice (Moulé *et al.*, 1977a). Although a limited number of studies indicate a lower acute toxicity for PR imine, the role that the PR imine analogue may play as a toxic compound in cheese requires further investigations.

2.6 Detection Methods

The most commonly used methods of detection of PRT and PR imine are physico-chemical methods. However, Nuñez *et al.* (1981) devised a microbiological method to quantify the production of PRT by 55 strains isolated from Cabrales cheese using *Bacillus megaterium* NRRL B-1368, the most sensitive micro-organism to PRT. This was accomplished by comparison of inhibition zones of produced toxin with that

of toxin standards. The method of quantitation stemmed from a procedure first introduced by Ueno and Kubota (1976), where toxins were tested for DNA-attacking ability in the *rec* assay using the recombination-deficient mutant of *Bacillus subtilis* M45 (rec^-) and the parent strain H17 (rec^+), by measuring the difference between the inhibition zone of the parent strain and that of the mutant, as caused by the toxin (Ueno and Kubota, 1976).

2.6.1 Thin-layer Chromatography

The most widely used method of detection and analysis of PRT and its imine form has been thin layer chromatography (TLC), employing a variety of absorbents and solvents. The most commonly used absorbent for detection of PRT is silica gel with a solvent mixture of methanol-chloroform (4:96, v/v) (Gorst-Allman and Steyn, 1984). With these conditions, PRT has a characteristic R_F value of 0.63 and PR imine a value of 0.18 (Wei *et al.*, 1973). However, the apparent sensitivity of the TLC assay is effected by environmental changes, particularly humidity. This led to the development of several alternative solvent systems, such as chloroform-2-propanol (10:1 or 4:1, v/v), toluene-ethyl acetate-formic acid (5:4:1 or 6:3:1, v/v/v) and toluene-ethyl acetate (30:70, v/v) saturated with water (Betina, 1985).

Detection methods using these solvents can vary. PRT can be detected by its green fluorescence under long-wave UV light following exposure of the chromatogram to short wave UV light for about 0.5 minute. An alternative method is to spray the chromatograms with 50% sulfuric acid after which PRT and PR imine appear as yellow spots. In these methods (Wei and Lui, 1978; Scott and Kanhere, 1979) which are used for semi-quantitative determination, a visual confrontation is made between the

intensity of the toxin spot in the solution to be assayed and that of a solution of known titre.

2.6.2 High-Performance Liquid Chromatography

For detection of minute amounts of toxin, high-performance liquid chromatography (HPLC) has been a more sensitive method to employ. Moreau *et al.* (1979) described a method for the detection of PRT using normal phase HPLC. The solvent system used was n-hexane and tetrahydrofuran (75:25, v/v), at a flow rate of 1.5 ml/min. PRT had a mean retention time of 7.4 min, showing good reproducibility with coefficients of variation from 1.6 to 2.8% representing the combined errors of HPLC injection, resolution, and detection. A linear relationship over a 10-5000 ng range of PRT was demonstrated, and quantitative analysis of this toxin above 10 ng was possible. However, application of this method for PRT detection in cheese was unsuccessful and Medina *et al.* (1985) concluded that an HPLC system is not suited for such analyses because of interference by the sample matrix.

Gorst-Allman and Steyn (1984) demonstrated a reversed-phase HPLC system to detect PRT. The solvent system used was a 65:35 (v/v) mixture of acetonitrile and water at a flow rate of 1.5 ml/min., which resulted in a mean retention time of 2.86 min for PRT. The toxin was detected using a 247 nm UV absorbance detector, but a sensitivity limit of only 50 ng was achieved. More recently, Danieli *et al.* (1980) also introduced a reversed-phase system using a micro-HPLC apparatus. This reversed-phase chromatography was performed using an acetonitrile and water solvent mixture in the ratio 65:35 (v/v), at a flow rate of 8 µl/min. With a mean retention time for PRT of 5.5 min, the quantitation was found to be satisfactory for toxin values of about

0.2 ng. In addition, other secondary metabolites of *P. roqueforti* including penicillic acid and roquefortine, and the eremofortins A, B, and C have been assessed by reversed-phase HPLC (Frisvad and Thrane, 1987; Moreau *et al.*, 1979). Investigations involving eremofortin C transformation to PRT have also employed normal phase HPLC (Li *et al.*, 1985). However, no HPLC method has been developed to detect and quantify PR imine.

2.6.3 Immunochemical Methods

In the last few years there has been an increase in interest to use immunoassay techniques for the analysis of mycotoxins. The reasons for this resides in the unique advantages of antibody-based methods: specificity, sensitivity and simplicity coupled with high speeds of sample through-puts (Morgan, 1989).

Recently, Wei and Chu (1988) produced and characterized a polyclonal antibody against PRT, to improve upon previous detection methods.

Prior to injection of PRT into the vertebrate for the production of an antibody-producing hybrid, the toxin must first be conjugated to a protein, such as bovine serum albumin (BSA). This is necessary because the toxin alone is a hapten, and needs to be coupled covalently to a larger molecule (Coleman *et al.*, 1989), such as a protein, in order to gain immunogenicity. The three most common methods of conjugating mycotoxin derivatives containing a carboxylic group or other related functional groups to BSA are: 1) the water soluble carbodiimide method, 2) the mixed anhydride method, and 3) the reductive alkylation technique (Erlanger, 1980). The method used depends upon the chemical structure and chemical modifications necessary to allow an appropriate reaction between hapten and protein.

An antibody against PRT was produced by Wei and Chu (1988) after immunizing rabbits with an immunogen prepared by conjugating PRT to BSA by a reductive alkylation method. In this method, the binding of PRT to BSA involves the formation of a Schiff base between the aldehyde group of the toxin and the amino group of the lysine residues in BSA (Shaw *et al.*, 1984). The free sulfhydryl group of the cysteine residue in BSA is also weakly involved in the binding of PRT to BSA. The molar ratio of hapten to carrier was determined to be 1:26.

The antibodies produced were found to be most specific against PR imine with a relative binding of 100%, and had a high cross-reactivity with PRT where the relative binding was 71%. Significant cross-reactivity of 50 and 30% was also observed with tetrahydro-PR and eremofortin C, respectively. PR alcohol and eremofortins A, B, and D showed minimal binding. Hence, the produced mixture of antibodies was not specific to PRT. In this study, side chain esters (-OAc group in the C-3 position), were found to greatly diminish antibody specificity, since the antibody was able to recognize this group, present in both the PRT and its imine form. Removal of this group resulted in virtually no cross-reactivity (Wei and Chu, 1988). A competitive radioimmunoassay (RIA) was used to determine the antibody specificity against PRT, using tritiated tetra-hydro PRT. The detection level for PRT by RIA was approximately 1 to 2 ng per assay. However, no PRT was found in blue cheese samples assessed by this method.

Amongst the reasons for the low specificity of the developed polyclonal antibodies may be the quality of the PRT-BSA conjugate. The toxin was conjugated to protein *via* its aldehyde group (a toxic site) having the acetoxy group in the C-3 position exposed to an immunogenic response. This triggered the production of

antibodies with specificity towards the part of the toxin molecule which is identical in PRT, PR imine, eremofortin C and tetrahydro-PR. Furthermore, polyclonal antibodies, have a number disadvantages. The resulting population of antibodies is a very heterogeneous mixture of high antigen cross-reactivities. In addition, polyclonal antibodies are produced in limited supply and the same combination of specific antibodies is impossible to reproduce in a new animal (Goding, 1983).

To overcome the problems associated with polyclonal antibodies, Kohler and Milstein (1975) established a method for the production of monoclonal antibodies. This development has made it possible to immortalize and propagate individual antibody-forming cells, thus enabling the generation and characterization of homogeneous reagents, specific for many antigens and eliminating much of the cross-reactivity associated with polyclonal antibodies.

Further work related to immunochemical analyses of PRT, perhaps involving monoclonal antibodies and alternate conjugation routes exposing the toxic site of the molecule, may prove beneficial to optimize sensitivity and specificity for the detection of not only PRT, but also its analogue, PR imine, in cheese; the latter of these compounds has not yet been accurately assessed. Sensitive methods are necessary in order to fully determine its presence in cheese and assess any toxicological significance.

3. MANUSCRIPT I

DETERMINATION OF *PENICILLIUM ROQUEFORTI* TOXIN BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

3.1 Abstract

A method for the detection and quantification of *Penicillium roqueforti* toxin (PRT) using reversed-phase high-performance liquid chromatography has been established. The limit of quantitation of this method was 3 ng of PRT, while the limit of detection was 2 ng of toxin. The precision of the analysis based on numerous runs was good. Retention times for PRT were highly reproducible with the average coefficient of variation about 1.6%. Analysis of PRT in liquid and solid samples showed no interference of the sample matrix. The accuracy of the method was 98.6%, with PRT mean recoveries of 96.8% and 100.4%, for the spiked culture medium and blue cheese extracts, respectively.

3.2 Introduction

Penicillium roqueforti toxin is a toxic fungal metabolite first isolated from mouldy grains and corn silage (Wei *et al.*, 1973) and then subsequently from cultures of *P. roqueforti* including some of those which are used in blue-veined cheese production (Medina *et al.*, 1985; Polonelli *et al.*, 1978). PRT is lethal for rats and mice with LD_{50} values of about 6-15 mg/kg by intraperitoneal administration (Scott, 1984). In addition, PRT was found to be carcinogenic for rats (Polonelli *et al.*, 1982; Hradec and Vesely, 1989) as well as mutagenic for *Salmonella typhimurium* (Levin *et al.*, 1982).

A number of thin-layer chromatographic methods are available for the detection of PRT in a variety of media (Betina, 1985). These methods are mainly used for identification and semi-quantitative determination of the PRT. More recently a normal-phase high-performance liquid chromatographic (HPLC) technique has been developed to measure the concentration of this toxin and other *P. roqueforti* metabolites in culture broths (Moreau *et al.,* 1979). It has been used to follow the transformation of eremofortin C into PRT with a detection limit of 10 ng for the latter toxin (Moreau *et al.*, 1980a; Chang *et al.*, 1985; Li *et al*, 1985). In addition, reversed-phase HPLC analysis of PRT has also been briefly reported (Gorst-Allman and Steyn, 1984). This technique, however, allows for detection of the toxin at a level of more than 50 ng. Although most of the above methods were successfully used for the detection of PRT in culture broths, determination of this toxin in cheese imposed problems due to its reactivity with proteins and amino acids as well as being influenced by the sample matrix (Medina *et al.*, 1985; Wei *et al.*, 1975).

In this paper we describe a method for the detection and quantitative

determination of PRT using reversed-phase HPLC. This method is more sensitive than chromatographies reported earlier. The feasibility to employ this method for the determination of PRT in culture broths and blue cheese was also investigated.

3.3 Experimental

3.3.1 Materials

Reference PRT was obtained from Sigma (St. Louis, MO, USA). High-performance thin layer chromatography (HPTLC) silica gel 60 F-254 aluminium plates were purchased from BDH (Toronto, Canada). A PRT-producing strain of *P. roqueforti* (ATCC No. 10110) was obtained from the American Type Culture Collection (Rockville, MD, USA) while Danish blue cheese was purchased from a local supplier. All solvents were of HPLC grade.

3.3.2 Chromatography

The HPLC method was developed on a Waters ALC 204 liquid chromatograph. The system consisted of a model 6000A solvent delivery system, a model 440 absorbance detector equipped with a 254 nm filter and a U6K universal liquid chromatography injector.

Chromatographic analyses were carried out on a pre-packed LiChrosorb reversed-phase C_{18} column (240 mm x 4.0 mm I.D.) of particle size 10 μm (E. Merck, Darmstadt, Germany). The signal from the detector was recorded by an SP4290 integrator (Spectra Physics) set at an attenuation of 8. A volume of 20 μl was injected for all samples. Mobile phases consisting of methanol-water in the ratios of 70:30 (v/v) and 65:35 (v/v) were used for HPLC analysis. All runs were carried out at a flow rate

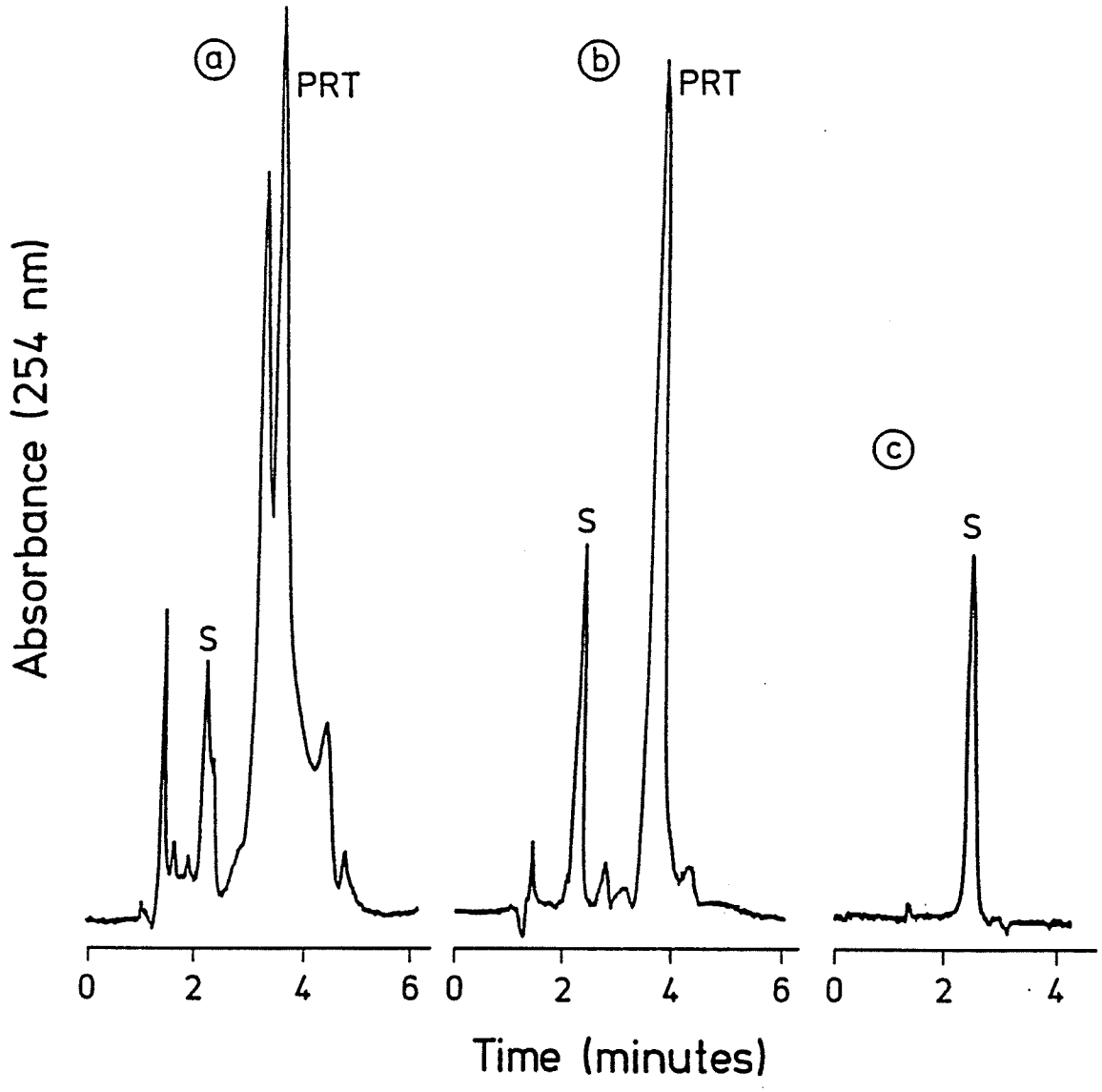
of 1.0 ml/min.

The presence of PRT in samples prior to reversed-phase HPLC analyses was assessed by analytical thin-layer chromatography (TLC) using chromatoplates of silica gel F-254 with methanol-chloroform (5:95, v/v) as the eluent (Wei *et al.*, 1973).

3.3.3 Standard Preparation

The reference PRT (Sigma) was further purified by normal-phase TLC. Toxin was dissolved in chloroform (0.5 μg/μl) and spotted on the TLC plate. The plate was developed using a mixture of methanol and chloroform in the ratio of 5:95 (v/v). The PRT spot, having an R_F value of 0.80, was located under short-wavelength UV light (254 nm) and clearly marked. The silica containing the PRT was carefully scraped from the plate, transferred to a vial and resuspended in 2.0 ml of methanol in order to extract the toxin. The resulting suspension was filtered through Whatman No. 4 paper. The PRT was extracted from silica two more times with a total of 2.0 ml of methanol. The three filtrates were combined and evaporated to dryness at room temperature under a stream of nitrogen, yielding a crystalline PRT standard used in further studies. Purity of the PRT standard was first determined using a diode-array spectrophotometer (Hewlett-Packard). The PRT crystals dissolved in 1.0 ml of methanol exhibited a single peak at 248 nm corresponding to its absorption maximum described by other workers (Polonelli *et al.*, 1978). In addition, the ^1H NMR spectrum (300 MHz) of the purified toxin was measured in $CDCl_3$ using a NMR spectrometer (Model AM 300, Bruker Spectrospin, Canada). The spectrum was identical to the PRT spectral data obtained by Wei *et al.* (1975). Finally, the effectiveness of the purification procedure was assessed by reversed-phase HPLC (Figure 3.1). The

Figure 3.1 Chromatograms of PRT standard a) 200 ng of an impure mixture containing PRT, b) 60 ng of PRT after TLC purification and c) 20 µl of methanol. Eluent: methanol-water (65:35, v/v); flow rate, 1.0 ml/min; peak S=methanol.


ⓐ
PRT
S
ⓑ
PRT
S
ⓒ
S
Absorbance (254 nm)
0
2
4
6
0
2
4
6
0
2
4
Time (minutes)

chromatography yielded a single peak indicating a high purity of the PRT standard. The concentration of the toxin dissolved in methanol was measured spectrophotometrically at 248 nm (ε=9000) (Polonelli, 1978).

Standard curves were prepared by making a series of dilutions of PRT in methanol ranging in concentration from 0.15 to 30 ng/µl. Samples were analyzed chromatographically as described above and the peak height and peak area were plotted against the corresponding concentration.

3.3.4 Sample Preparation

To assess the suitability of reversed-phase HPLC for the determination of PRT in different samples, analysis of this toxin in culture broth (preparation 1) and cheese (preparation 2) was conducted.

Preparation 1. PR toxin was produced using the strain of *P. roqueforti* ATCC No. 10110, according to the procedure of Wei *et al.* (1973). The mould was grown for 14 days at room temperature in a culture medium containing yeast extract and sucrose (YES, Difco). Then, the mycelium was removed from the culture broth by paper filtration and the broth (100 ml) was extracted with chloroform to yield a crude sample containing PRT. The presence of PRT was confirmed by TLC as described earlier in this paper. Crude samples from two separate fermentation batches were evaporated to dryness, each dissolved in 1.0 ml of methanol, and used for HPLC analysis.

Preparation 2. Purified PRT standard (0.5 mg) dissolved in 1.0 ml of methanol, was added to 40 g of Danish blue cheese to give a final concentration of 12.5 mg/kg. The cheese was immediately homogenized in a Waring blender with 250 ml of methanol-water (55:45, v/v) and 150 ml of hexane for 5 min at high speed and then

divided into two equal portions. Each portion was centrifuged at 1000 *g* at 4° C for 10 min. After removing the hexane layer, the methanol-water part was vacuum-filtered through Whatman No. 3 paper and the resultant filtrate was extracted with two 60-ml volumes of chloroform. The two chloroform extracts from each portion were pooled and evaporated to dryness using a rotary evaporator. The dried extract was dissolved in 1.0 ml methanol, 10 µl were withdrawn, diluted 40-fold with methanol, and immediately analyzed by HPLC as described earlier. The remaining extract was stored at -5° C for 24 days and the PRT content was periodically analyzed by HPLC. Cheese used for this experiment was free of PRT as assessed by TLC. Part of the cheese was extracted exactly as described above, and the extract was used as a control sample.

3.3.5 Accuracy Assessment

The accuracy of the HPLC method for PRT quantitation was determined by an external standard method. Culture medium and blue cheese samples free of PRT were extracted with chloroform, evaporated to dryness, dissolved in methanol and spiked with PRT over the concentration range from 1.0 to 8.3 ng/µl (20 to 166 ng/injection). Recoveries of PRT from extracts were assessed by HPLC using peak height measurements. A two-sample *t*-test was used to estimate the significance of the differences between known and measured values of PRT.

3.4 Results and Discussion

The PRT retention times for two solvent systems containing methanol and water are shown in Table 3.1. The average retention time for PRT using methanol-water in a 70:30 (v/v) ratio was 3.31 min based on three separate trials over four days.

TABLE 3.1 - *Reproducibility of retention time for PRT as analysed by reversed-phase HPLC*

Trial No.	Solvent[a] Ratio	n[b]	Retention Time (min)			C.V. (%)
			Range	Mean	S.D.	
1	70:30	24	3.15-3.44	3.26	0.11	3.37
2	70:30	23	3.31-3.39	3.34	0.02	0.60
3	70:30	32	3.25-3.41	3.32	0.05	1.51
4	65:35	67	3.60-3.84	3.70	0.05	1.35
5	65:35	17	3.55-3.84	3.69	0.12	3.25
6	65:35	27	3.92-3.96	3.93	0.01	0.25
7	65:35	41	3.75-3.89	3.84	0.03	0.78

[a]Methanol/water (v/v)
[b]Number of injections

Coefficients of variation (C.V.) ranged from 0.06 to 3.37%. Methanol-water in a 65:35 ratio (v/v) as the eluting solvent, resulted in an average retention time of 3.79 min for PRT, based on four trials over a four day period. C.V.s with this solvent ranged from 0.25 to 3.25%. The PRT retention times for both solvent systems were highly reproducible. However, the methanol-water in ratio 65:35 (v/v) allowed for longer retention of the toxin on the column, and hence, better resolution of the PRT peak from the initial solvent peak.

The precision and reproducibility of the peak height and the peak area were analyzed by injecting a volume (20 µl) containing either 55 or 28 ng of the purified standard, over a 2-day period. The C.V.s for the peak area measurements were 13.2 and 13.7%, respectively. Reproducibility of peak heights for the same injected amounts were better, with C.V.s of 6.58 and 3.22%, respectively (Table 3.2). Consequently, further determination of PRT by HPLC was based on the peak height measurements.

Total PRT recoveries and results of a Student *t*-test are shown in Table 3.3. The accuracy of the method for PRT determination was 98.6%, with mean recoveries of 96.8 and 100.4%, for spiked culture medium and blue cheese extracts, respectively. No significant difference (at a 95% confidence level) was found between the means of the standard PRT and the recovered PRT from spiked culture medium or cheese samples over the concentration range tested. The only exception was the spiked cheese extract at the lowest concentration level of 20 ng PRT where the *t*-value was slightly higher (4.39) than the tabulated value (4.30).

The limit of detection of the method was determined with the integrator set at an attenuation of 8. The highest sensitivity setting of 1 resulted in a very noisy

TABLE 3.2 - *Reproducibility of HPLC peak area and peak height for PRT using 65:35 methanol-water (v/v) solvent system*

PRT Injected (ng)	n[a]	Peak area or height			C.V. (%)
		Range	Mean	S.D.	
Area					
55.0	20	125952-218709	163445	21628	13.2
28.0[b]	12	60017-92814	72088	9860	13.7
Height					
55.0[c]	23	5525-6512	5899	388	6.58
28.0[d]	6	3661-4023	3831	123	3.22

[a]Number of injections

TABLE 3.3 - *Accuracy of reversed-phase HPLC to detect PRT in culture medium and blue cheese samples*

Amount of Std PRT[a] (ng)	Culture Medium Samples			Blue Cheese Samples		
	Mean PRT Recovered[a] (ng)	Student t-value[b]	Total Recovery (%)	Mean PRT Recovered[a] (ng)	Student t-value[b]	Total Recovery (%)
166.0	158.4 ± 3.96	2.24	95.4	151.6 ± 5.60	3.84	91.3
125.0	111.7 ± 7.83	3.14	89.4	126.6 ± 18.5	0.15	101.3
84.0	81.1 ± 6.35	0.78	96.5	84.0 ± 9.70	0.00	100.0
62.0	64.7 ± 1.83	2.02	104.4	60.1 ± 15.0	0.22	96.9
50.0	45.5 ± 0.66	3.24	91.0	50.0 ± 7.00	0.00	100.0
20.0	20.8 ± 0.65	1.50	104.0	22.6 ± 0.79	4.39	113.0

[a]based on triplicate injections
[b]two-sample t-test made at a 95% confidence level where $t_{\alpha=0.05} = 4.30$

baseline that interfered with the interpretation of the chromatogram, and hence, a more appropriate setting of 8 was chosen. With the parameter at this setting, PRT could be measured at a concentration as low as 3 ng per run (Figure 3.2a). Furthermore, at an attenuation setting of 4, an amount of 2 ng of toxin could be detected (Figure 3.2b). This sensitivity was better than those reported earlier by other HPLC methods (Moreau *et al.*, 1979; Gorst-Allman and Steyn, 1984).

In order to carry out the quantitative analysis, it was necessary to study the linearity of the detector response (peak area and peak height) with respect to concentrations of PRT. The linearity was evaluated by injecting 20 µl of increasing concentrations of the toxin standard, so that the injected amounts of PRT ranged from 3 to 600 ng. Regression analysis of the resulting calibration curves, obtained from three trials each ranging from 3 to 70 ng of PRT, indicated that the relationship between both peak height or peak area and the amount of PRT injected was linear over the tested concentration range, with correlation coefficients of 0.93 and 0.92, respectively (Appendix I). For a broader range of PRT concentrations (3 to 600 ng) the correlation coefficient for peak area and the amount of PRT injected was 0.97.

The selectivity of the developed reversed-phase HPLC method was assessed by the determination of PRT in samples each having a different matrix: *P. roqueforti* culture medium, and blue-veined cheese. Figure 3.3 shows chromatograms of crude PRT extracts obtained from two different fermentation batches. Both of these indicate the presence of the toxin produced by *P. roqueforti* during the fermentation process. Presence of the toxin was verified by spiking the extracts with standard PRT. Distinct PRT peaks in both chromatograms indicate that other extractable components do not interfere with the detection of the toxin. With the aid of the calibration curve (peak

Figure 3.2 Chromatograms of PRT standard a) 3.0 ng at attenuation 8 and b) 2.0 ng at attenuation 4. Eluent: methanol-water (65:35, v/v); flow rate, 1.0 ml/min; peak S=methanol.

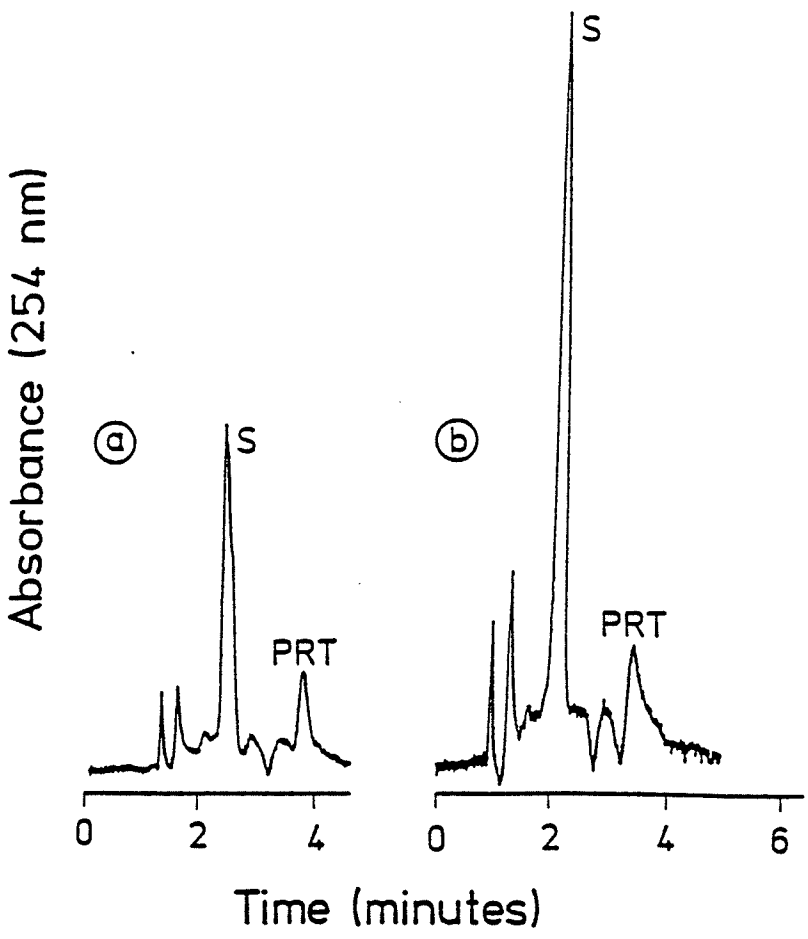
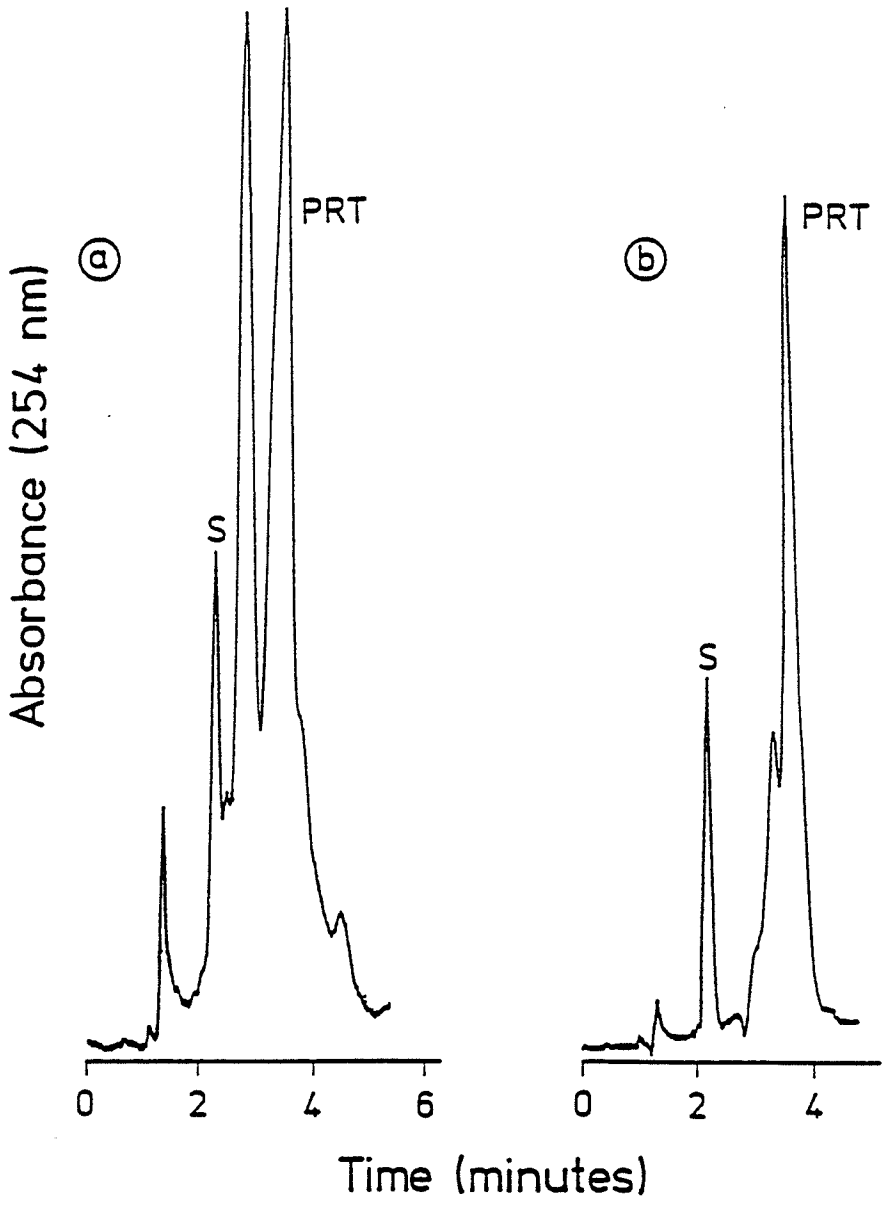

Absorbance (254 nm)
ⓐ
S
PRT
ⓑ
S
PRT
0
2
4
0
2
4
6
Time (minutes)

Figure 3.3 Chromatograms of crude PRT extracts obtained from two separate fermentation batches of *Penicillium roqueforti*, 100 ml of YES culture medium. Eluent: methanol-water (65:35, v/v); flow rate, 1.0 ml/min; peak S = methanol.


Absorbance (254 nm)
ⓐ
PRT
S
ⓑ
PRT
S
0
2
4
6
0
2
4
Time (minutes)

height *versus* concentration), a PRT concentration of 0.6 mg/ml, and 2.3 mg/ml was detected in the first (Figure 3.3a), and in the second (Figure 3.3b) extract, respectively.

A typical chromatogram of PRT recovered from cheese is shown in Figure 3.4a, while Figure 3.4b shows a chromatogram of the extract obtained from cheese prior to the addition of the toxin. Also in this case, the PRT was distinct (Figure 3.4a) and detection of the toxin was not influenced by other components present in the extract obtained from the cheese matrix (Figure 3.4b). Numerous analyses of PRT in the methanol extract of cheese stored at -5° C for 24 days resulted in the average retention time of 3.97 min, with a C.V. of 2.5%. This indicates good reproducibility of this parameter. Although the retention time was slightly higher than that of the standard PRT (3.79 min), presence of the toxin was verified by spiking the sample with the standard.

The determination of PRT allowed for the assessment of the recovery of toxin added to cheese, and subsequent stability analysis of the recovered PRT in methanol extract stored for 24 days (Table 3.4). Although the extraction procedure for cheese was designed to be simple and as rapid as possible, a mean recovery of only 48.6% PRT was obtained, as determined with the aid of the peak height calibration curve. This is not unexpected since the instability of PRT in blue cheese and its low yield of recovery has been reported by other researchers (Scott and Kanhere, 1979). The toxin forms *P. roqueforti* imine with ammonia and reacts with neutral and basic amino acids present in cheese. It has been reported that the aldehyde group of the toxin is the moiety that reacts with the amino group of the amino acids forming Schiff bases (Scott and Kanhere, 1979). Moreover, PRT may react with the ϵ-NH_2 group of lysine (Shaw *et al.*, 1984) and sulfhydryl group of cysteine (Rice *et al.*, 1976).

Figure 3.4 Chromatograms of a) crude PRT recovered from PRT-spiked blue-veined cheese (0.5 mg/40 g) and b) a control cheese extract. Eluent: methanol-water (65:35, v/v); flow rate, 1.0 ml/min; peak S = methanol.

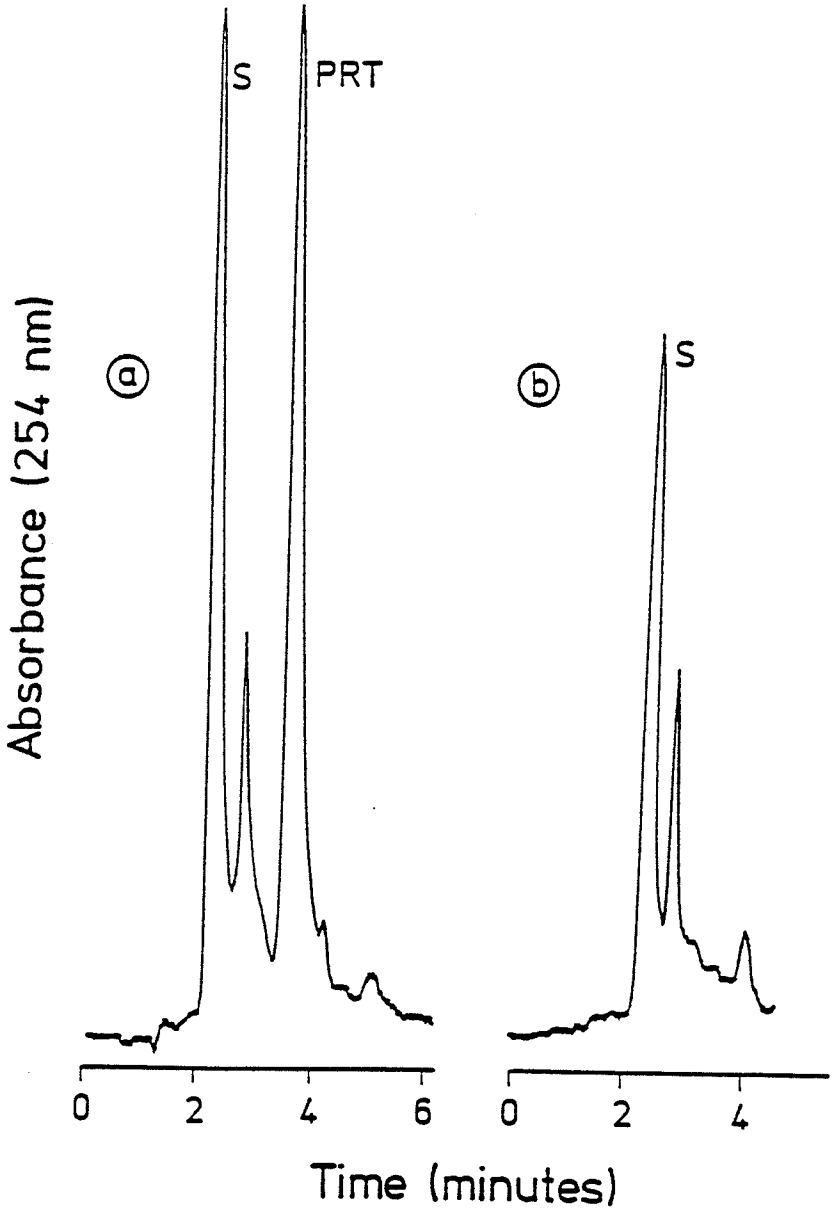

S
PRT
S
ⓐ
ⓑ
Absorbance (254 nm)
0
2
4
6
0
2
4
Time (minutes)

TABLE 3.4 - *Stability of PRT in methanol extract of blue cheese as determined by reversed-phase HPLC*[a]

Time after Extraction (days)	Amount of PRT in extract[a]	
	µg / µl	%
0	0.218	43.6
1	0.217	43.4
4	0.219	43.8
5	0.194	38.8
11	0.135	27.0
17	0.125	25.0
20	0.122	24.4
24	0.119	23.8

[a]based on triplicate runs and analysis performed using HPLC peak height
[b]PRT was added to cheese at 12.5 mg/kg and extracted as described in Experimental

Studies on the stability of PRT in methanol extract show that the toxin was relatively stable over approximately 4 days. However, prolonged storage at -5°C for up to 24 days resulted in a 55 % decrease in the amount of PRT present in the cheese extract (Table 3.4). Subsequent loss of PRT may be due to its reactivity with compounds such as tryptamine or other primary amines extracted from blue cheese that are present in concentrations of up to 2.3 mg/g (Scott and Kanhere, 1979; Rice *et al.*, 1977). This is supported by the fact that the standard PRT in pure methanol (0.01 μg/ml) was stable at -5°C for at least one month.

The developed reversed-phase HPLC method can be used for the determination of PRT produced by the *P. roqueforti* in culture broth extracts. Moreover, because of its specificity and sensitivity, the HPLC method is suitable to detect the toxin in blue cheeses.

3.5 Acknowledgements

The authors are indebted to Dr. A.A. Frohlich for the valuable technical advice and helpful comments on the manuscript. Financial support for this study from the Natural Science and Engineering Research Council of Canada is gratefully acknowledged.

3.6 Conclusion

The developed reversed-phase HPLC procedure proved to be a fast and relatively simple method for the determination of PRT in a variety of media. To further increase the sensitivity and specificity involved in determining PRT, an attempt was made to develop a monoclonal antibody against PRT. The following chapter describes an account of the conjugation of PRT with regards to the details of the organic chemistry involved, as well as the modifications that would be necessary in order to successfully obtain an adequate conjugate for subsequent monoclonal antibody production against PRT.

4. MANUSCRIPT II

CHEMICAL MODIFICATION AND CONJUGATION OF *PENICILLIUM ROQUEFORTI* TOXIN

4.1 Abstract

Attempts were made to prepare a *Penicillium roqueforti* toxin (PRT)-protein conjugate, in order to develop an immunoassay for the determination of PRT and its analogue, PR imine. The initial step in the conjugation was the modification involving the alkaline hydrolysis of PRT, which resulted in the desired PR alcohol. In addition, numerous procedures were used to further succinylate this compound. However, the production of the PR-hemisuccinate was not possible. Therefore, the formation of the protein conjugate was not feasible.

4.2 Introduction

Numerous attempts have been made to determine PRT in a variety of blue-veined cheeses, using such techniques as high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), and most recently immunochemical methods. However, only a few assays have been proposed to detect PR imine, an analogue of PRT. It is known that PRT undergoes conversion to PR imine in blue cheese, although the actual fate of these toxins has not yet been evaluated, due to the lack of an appropriate detection method that would allow discrimination between both toxins. Several TLC methods were used in the past to determine PRT and PR imine concomitantly occurring in cheese. However, a drawback of TLC is the low level of sensitivity and resolution, which depends on the clean-up procedure prior to analysis. To overcome these problems, alternative methods are needed. One attractive alternative would be to utilize immunochemical techniques which are specific and rapid, compared to other commonly used physicochemical detection methods.

Immunoassays provide a unique means for detecting and determining the amount of a particular constituent in a complex mixture, even if the constituent is present in low concentrations (Daussant and Bureau, 1984). The specificity of the antibody/antigen reaction, and the high sensitivity of immunological methods have been exploited recently by food scientists in the area of food safety (Hitchcock, 1984). Since 1963, considerable effort has been devoted to developing immunochemical methods for the analysis of mycotoxins (National Research Council of Canada, 1985).

Polyclonal antibodies have been produced against PRT (Wei and Chu, 1985). These antibodies, although sensitive, exhibit a high degree of cross-reactivity to PRT and its imine form; hence their application to discriminate between both toxic forms

was not feasible. Polyclonal antibodies have been the cornerstone of immunoassays because of their high affinities and low cost of production. These antibodies however, are a heterogeneous mixture of immunoglobulins and hence they are not very specific (Goding, 1983). Recently, monoclonal antibodies have begun to replace polyclonal antibodies because of improved specificity, ease of use, production of an unlimited supply, and the advantages of worldwide assay standardization.

Therefore, the production of a highly specific monoclonal antibody to PRT and also to PR imine, with the subsequent development of a suitable screening method such as enzyme-linked immunosorbent assay (ELISA), would be a valuable probe to assess the presence of PRT and PR imine in cheese, and confirm or verify the previously obtained negative results. By producing monoclonal antibodies against both PRT and its imine form, it may be possible to study the interconversion of these two toxins.

As mentioned earlier, PRT is a hapten and as such needs to be conjugated with a protein in order to gain immunogenicity. In addition, the final quality of antibodies against the hapten is effected by the quality of a protein-hapten conjugate. Anti-hapten antibodies are usually produced against that part of the hapten molecule which is furthest removed from the point of conjugation. To produce specific antibodies to PRT and PR imine, it is necessary to conjugate these toxins with protein carriers in such a way that the portion of the molecule, the toxic site, which differs in both toxic forms, is exposed to trigger a specific immune response.

It is proposed that conjugation of PRT to a protein carrier would be carried out *via* the acetoxy moiety (C-3 position), the site furthest removed from the toxic aldehyde group, by the mixed anhydride procedure. However, prior to conjugation, the

acetoxy moiety must be replaced by a carboxyl group which can then react with the amino group of the protein. The chemical modification of PRT would then consist of two phases: hydrolysis of the acetoxy moiety and succinylation of the hydroxyl group (Figure 4.1). Wei *et al.* (1975) have reported the conversion of the toxin to its alcohol form where a substitution reaction replaces the acetoxy moiety with a hydroxyl group. Subsequent conversion of the PR alcohol to the half ester of succinic acid may be possible by adapting procedures used for the succinylation of other mycotoxins.

The most suitable methods include the preparation of a hemisuccinate of roridin A, a macrocyclic trichothecene (Martlbauer *et al.*, 1988), as well as the formation of a hemisuccinate of the trichothecene mycotoxin diacetoxyscirpenol (Pauly *et al.*, 1988). In addition, Ohtani *et al.* (1988) reported an improved preparation of a T-2 hemisuccinate to replace the method proposed by Chu *et al.* (1979). Lau *et al.* (1981) used a slightly varied procedure to prepare and characterize the hemiglutarate of aflatoxin B_{2a}.

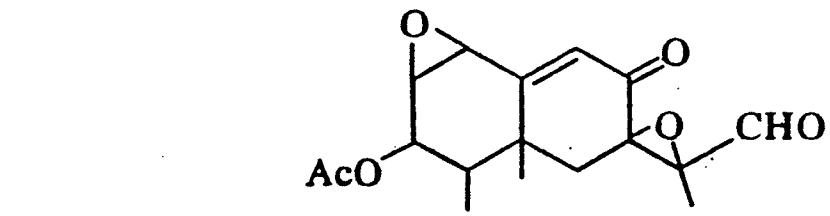
This paper presents the chemical modification of PRT in an attempt to prepare a specific conjugate with a protein carrier.

4.3 Experimental

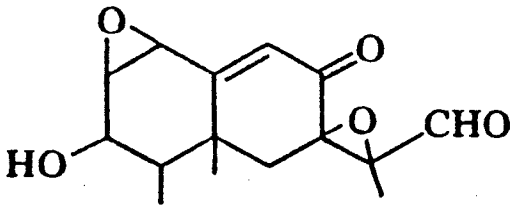
4.3.1 Materials

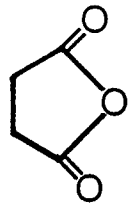
Reference PRT, succinic anhydride and 4-N,N-dimethylaminopyridine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Normal phase silica gel TLC plates as well as reversed-phase C_{18} silica TLC plates were purchased from Whatman (USA). All solvents were of HPLC grade.

Figure 4.1 Schematic diagram for the proposed chemical modifications of PRT: a) the alkaline hydrolysis of PRT to PR alcohol and b) the succinylation of PR alcohol to form the hemisuccinate.

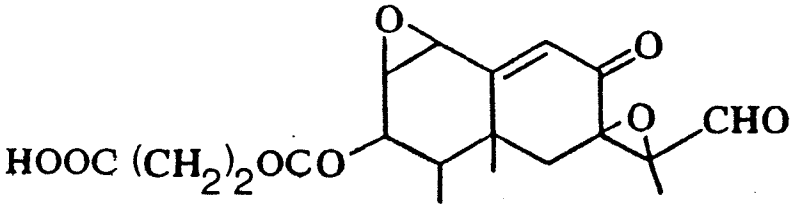


KOH





Succinic anhydride



4.3.2 Detection Methods

Thin-layer chromatography (TLC) - Reverse phase TLC plates (RP-TLC) were used to assess the presence of PRT and chemically related compounds at various stages of modification using a methanol-water mixture (70:30, v/v). In addition, the presence of these compounds was confirmed by normal phase TLC (NP-TLC) using a mixture of methanol-chloroform (5:95, v/v) as the eluent.

Nuclear Magnetic Resonance (NMR) - Alkaline hydrolysis and succinylation of PRT as well as purity of this toxin were monitored by NMR (Model AM 300 Bruker Spectrospin, Canada). The ^{1}H NMR spectrum (300 MHz) was used to monitor all compounds that arose during the process of PRT modification, and evaluate the presence of specific functional groups. In addition, the $^{13}CNMR$ spectrum was analyzed for various samples in order to confirm the obtained results.

HPLC - Reversed-phase HPLC as described previously (Siemens and Zawistowski, 1992) was employed to evaluate the purity of the standard PRT.

4.3.3 Standard Preparation

The reference PRT was further purified by NP-TLC. The resulting PRT crystals when dissolved in 1.0 ml of methanol exhibited a single peak at 248 nm, corresponding to its absorption maximum as described by Polonelli *et al.* (1978). Moreover, the measured ^{1}H NMR spectrum (300 MHz) of the purified toxin corresponded to the spectral data obtained by Wei *et al.* (1975). Additionally, $^{13}CNMR$ spectral analysis of PRT confirmed its purity. The PRT purity was assessed using reversed-phase HPLC (Siemens and Zawistowski, 1992). Analysis resulted in a single peak having a retention time of 3.8 min using methanol-water (65:35, v/v) as the eluent.

4.3.4 Alkaline hydrolysis of PRT

Alkaline hydrolysis of PRT was based on the procedure by Wei *et al.* (1973). Adjustments were made to accommodate a limited amount of PRT. The final procedure was as follows: PRT (1.5 mg) was dissolved in a 0.02 N solution of potassium hydroxide in methanol-water (4:1, v/v) (300 µl) and allowed to react at 40°C for 1.5 hours. At this time, the reaction mixture, as monitored by RP-TLC using methanol-water (70:30, v/v) as the eluent, revealed that most of the toxin was converted to one major product having an average R_F value of 0.75; the R_F of PRT was observed at 0.51. The resulting product showed an R_F value of 0.40 which was lower than that of PRT, using NP-TLC silica plates (Wei *et al.*, 1983). The mixture, after heating, was diluted with water and extracted with chloroform. The chloroform layer was then dried over anhydrous sodium sulfate and evaporated to an oil.

This procedure was devised after analyzing the yield and purity of product formed under various conditions. A range of heating times at 40°C were assessed. PRT (1.0 mg) was mixed with 300 µl of the KOH mixture as noted above and allowed to react. A 10 µl sample was removed at 0.5 hour intervals and analyzed by RP-TLC and NP-TLC to assess the presence of PRT and PR alcohol.

The resulting oil was dissolved in chloroform and spotted on a RP-TLC plate for further purification. The plate was developed, and the PR alcohol, having an R_F value of 0.78 was located by excitation under short wavelength UV light for 0.5 min, followed by long wavelength UV exposure. The spot was marked and carefully scraped from the plate, transferred to a vial and resuspended in 2.0 ml of methanol in order to extract the PR alcohol. After filtering (Whatman no. 3 paper), the PR alcohol was extracted twice from the silica using a total of 2.0 ml of methanol. The filtrates were

combined and evaporated to dryness at room temperature under a stream of nitrogen, yielding pale yellow crystals.

The presence of PR alcohol was confirmed by ^{1}H NMR and $^{13}CNMR$ spectral analyses.

4.3.5 Preparation of PRT-hemisuccinate

Succinylation of PRT was performed according to modified procedures described by other workers (Ohtani *et al.*, 1988; Pauly *et al.*, 1988; Martlbauer *et al.*, 1988).

Procedure 1 - This procedure was based on methods of Ohtani *et al.* (1988), Pauly *et al.* (1988), and Martlbauer *et al.* (1988). Purified PR alcohol (1.0 mg) and succinic anhydride (20 mg) were mixed in pyridine (300 µl), and the mixture was heated by refluxing at 80° C for 2 hours. The reaction was terminated by the addition of distilled water (500 µl) and the reaction mixture was extracted twice with 1 ml of chloroform. The organic layer was subsequently washed with an equal volume of distilled water and treated with anhydrous sodium sulfate and filtered. The chloroform layer was evaporated to dryness under a stream of nitrogen, yielding a brownish oil. The derivative exhibited a major spot at the same R_F value as PR alcohol on NP-TLC plates developed in methanol-chloroform (5:95, v/v). The resulting brownish oil was dissolved in 500 µl of chloroform, applied to a micro silica gel column (0.5 x 10.0 cm) packed with 1.0 g silica gel and eluted with methanol-chloroform (5:95, v/v). Fractions (500 µl) were collected and analyzed by UV spectrophotometry at 247 nm and those absorbing at this wavelength were pooled and dried over a stream of nitrogen to yield slightly yellowish crystals. These crystals were further analyzed by ^{1}H NMR in $CDCl_3$ to assess the presence of the desired carboxyl moiety at the C-3

position of the molecule.

Procedure 2 - This experiment was identical to that employed in procedure 1, except that a single parameter, the amount of succinic anhydride, was increased to 25 mg. Reaction time, temperature and volume of pyridine remained the same. The purification, and TLC and NMR assessment protocols were as described previously.

Procedure 3 - A slight adjustment was made to the protocol presented in procedure 1. The effect of heating time and temperature, as well as the amount of pyridine were investigated. This was performed in two parts. Part A involved the chemical modification of 1.5 mg of PR alcohol with 20 mg of succinic anhydride in 500 µl of pyridine. This mixture was incubated as in procedure 1 at 80°C for 2 hours. Part B involved the same mixture of reagents described above, except that it was allowed to react overnight at room temperature, and the refluxing step was omitted.

Purification in each part was performed by absorption chromatography using micro silica gel column as described earlier, and subsequently assessed by both TLC and ^{1}H NMR.

Further modifications which were investigated based on the same procedure, attempted to increase the production of the PR-hemisuccinate. These included increasing the amount of PR alcohol to 5 mg for the reaction, as well as utilizing alternate purification procedures such as C_{18} Sep-pak chromatography, preparative TLC, and preparative HPLC. Additional $^{13}CNMR$ spectral scans were also performed.

Procedure 4 - This procedure was based on the methods described by Lau *et al.* (1981) and Gendloff *et al.* (1986). A solution of 5 mg of PRT in 10 ml of dry tetrahydrofuran (THF) was mixed with 450 mg of succinic anhydride and 150 mg 4-N,N-dimethylaminopyridine. The mixture was refluxed for 45 min at 40°C, cooled,

and 1.5 ml of water was added. The THF was removed by rotary evaporation and 1.5 ml of 1 M HCl was added to the aqueous solution, followed by extraction 3 times with 4.0 ml portions of chloroform. The chloroform layers were combined and concentrated, washed 6 times with 500 µl portions of 0.1 M sodium acetate (pH 5.1) and 3 times with 500 µl portions of distilled water. The resulting chloroform solution was treated with anhydrous sodium sulfate, and loaded on a micro silica gel column (0.5x10.0 cm). The column was first washed with 10 ml of acetone-chloroform (20:80, v/v), and then eluted with 50 ml of acetone-chloroform-acetic acid (50:50:0.5, v/v/v). Fractions (1.0 ml) were collected and their absorbance assessed at 247 nm was analyzed. Fractions absorbing at this wavelength were pooled and dried under nitrogen for further analysis by TLC and ^{1}H NMR.

4.4 Results and Discussion

Reference PRT was successfully purified by RP-TLC. Figure 4.2 reveals the ^{1}H NMR spectrum of the purified product which is similar to the spectrum reported by Wei *et al.* (1975). However, some minor impurities are visible. The peak at δ7.25 is due to chloroform while the peak at δ1.55 is indicative of D_2O. The purity of the reference PRT standard was further confirmed by ^{13}CNMR analysis (Figure 4.3). As assessed by NMR analyses, purified PRT was a suitable starting material for the proposed modifications.

The chemical modification of PRT is outlined in Figure 4.1. The first stage of PRT modification, the alkaline hydrolysis, is a crucial step, as it provides the basis for further chemical alterations. When hydroxylation of PRT was performed according to Wei *et al.* (1973), TLC indicated incomplete conversion of PRT to PR alcohol. The

Figure 4.2 The 300 MHz ^1H NMR spectrum of 1.5 mg of PRT (in $CDCl_3$) following purification by RP-TLC; trimethylsilane (TMS) was used as the internal standard.

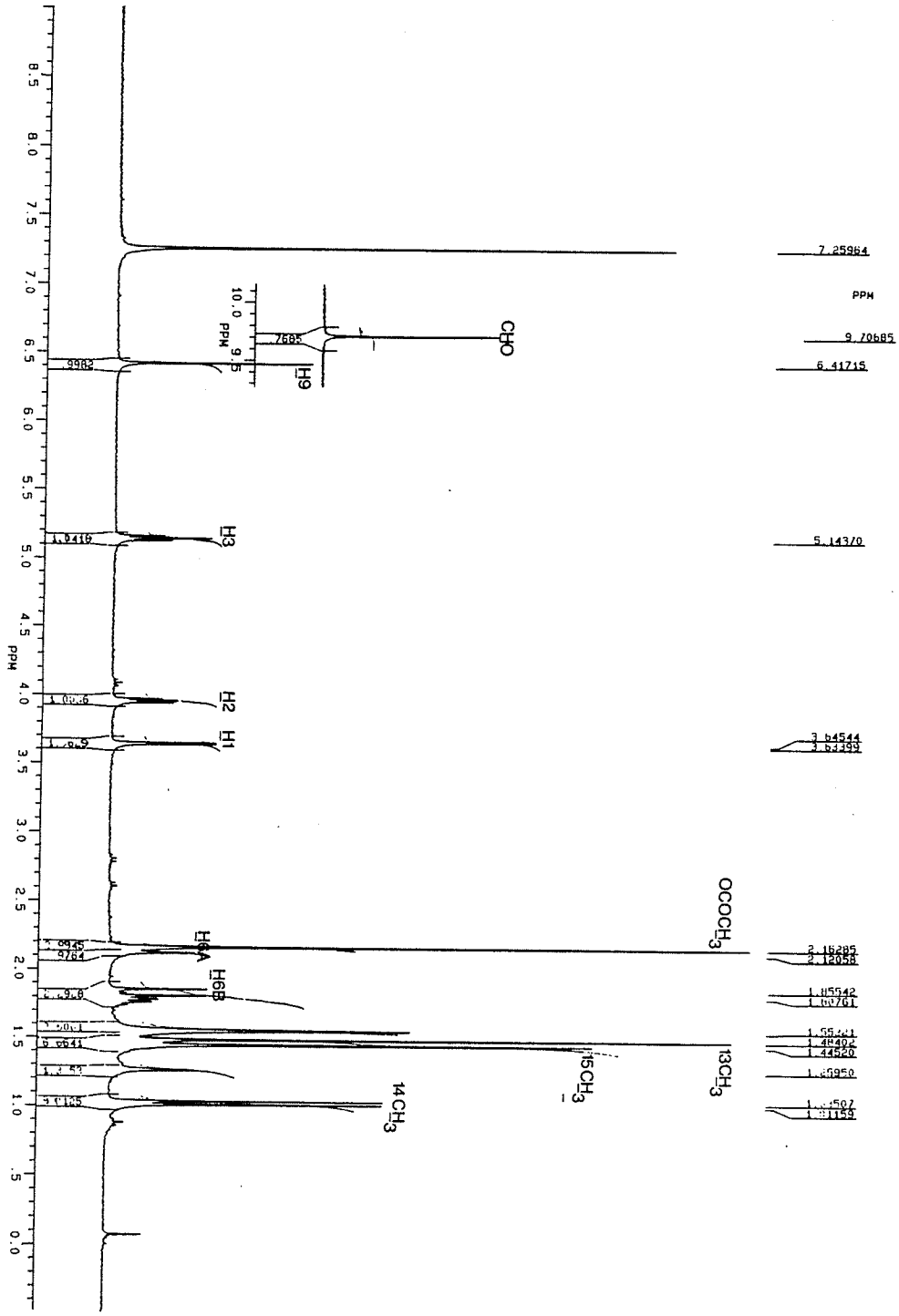
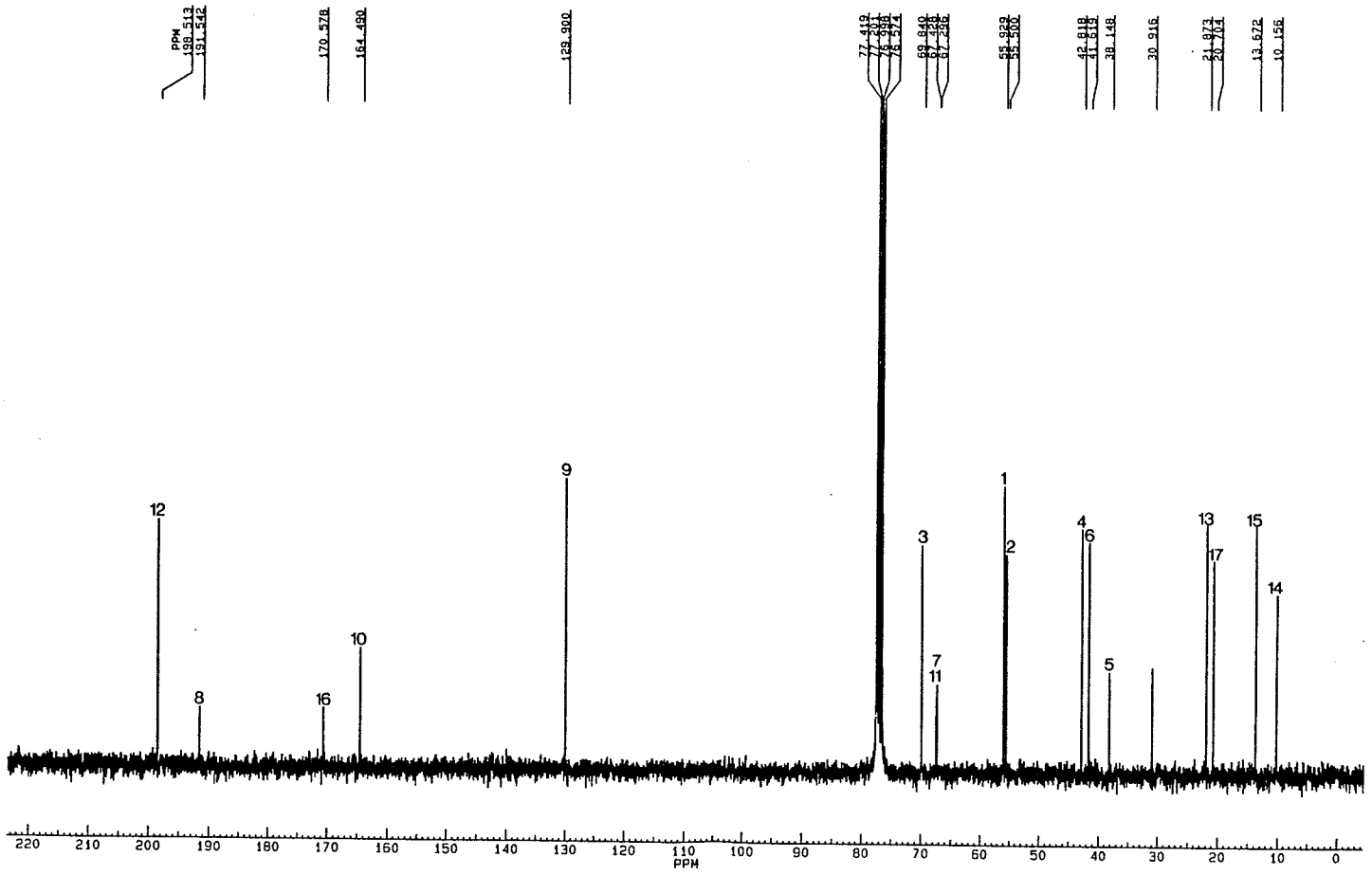

PPM
H9
CHO
H3
H2
H1
H4A
H4B
OCOCH3
13CH3
15CH3
14CH3

Figure 4.3 The 75.47 MHz ^{13}CNMR spectrum of 1.5 mg of PRT (in $CDCl_3$) following purification by RP-TLC.


PPM
191.542
170.578
164.490
129.900
77.419
77.201
76.984
76.574
69.840
67.368
67.256
55.928
55.500
42.818
41.519
38.148
30.916
21.873
20.764
13.672
12.156
12
8
16
10
9
3
7
11
1
2
4
6
5
13
17
15
14
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
PPM

amount of KOH in the reaction mixture was increased by 75% of that proposed by Wei *et al.* (1973), to supply an excess of the hydroxyl ion needed for the conversion of the PRT. This, however, resulted in several impurities, presumably breakdown products of PRT. Some resulting impurities of similar polarity to PR alcohol made it difficult to distinguish and isolate the PR alcohol spot. Heating up to 4 hours seemed to be the direct factor promoting increased breakdown of PRT. A 0.5 to 4 hour time course study revealed that there was still a considerable amount of PRT in the mixture between 0.5 and 1 hour of heating (Table 4.1). Further heating the reaction mixture anywhere from 2 to 4 hours confirmed the existence of interfering products. The highest conversion of PRT to PR alcohol with production of the lowest amounts of impurities, was observed after 1.5 hours of incubation at 40°C (Table 4.1, Figure 4.4).

The ^{1}H NMR spectrum (Figure 4.5) confirms a high purity of the synthesized PR alcohol. Disappearance of the three-proton singlet at δ2.16 upon alkaline hydrolysis indicates the removal of the acetyl group (C-3 position) originally observed in the PRT ^{1}H NMR spectrum (Figure 4.2). All other protons were unchanged after hydrolysis with the exception of the one-proton doublet of doublets at δ5.14 which was shifted up field to δ4.1 indicating that this proton was attached to the same carbon (C-3) as the acetoxy group. However, upon hydrolysis, the proton at the C-3 position became shielded so the field felt by the proton was diminished due to the replacement of the acetyl group with a hydroxyl group. This caused the chemical shift up field.

The presence of the hydroxyl group introduced into the toxin molecule is shown at δ1.6, as a small peak slightly above baseline (Figure 4.5). In general, a hydroxyl group appears as a slightly raised baseline in the range of δ1.0 to δ5.5, resulting in difficulties to properly assess its presence (Morrison and Boyd, 1983). The

TABLE 4.1 - *Alkaline hydrolysis of PRT as assessed by TLC*

Heating Time (hrs)	Amount of Toxin[a]	
	PRT	PR alcohol
0.5	++++	0
1.0	++	++
1.5	+	++++
2.0	+	+++
2.5	+	++
3.0	+	++
3.5	+	++
4.0	+	++

[a]symbol + indicates relative amount in the mixture

Figure 4.4 Typical reversed-phase silica gel thin layer chromatogram of a) PR alcohol produced after the alkaline hydrolysis of PRT (1.5 hrs incubation with KOH at 40° C), and b) 10 μg of standard PRT before reaction. Eluent: methanol-water (70:30, v/v).

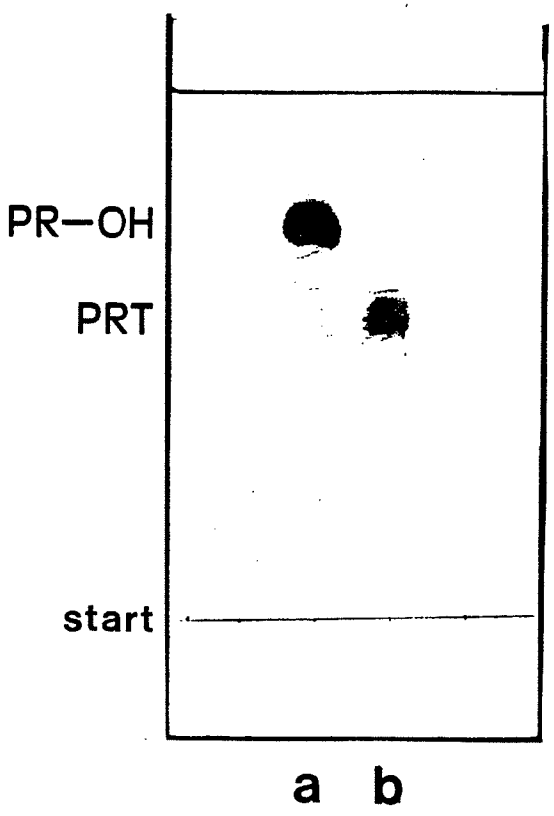
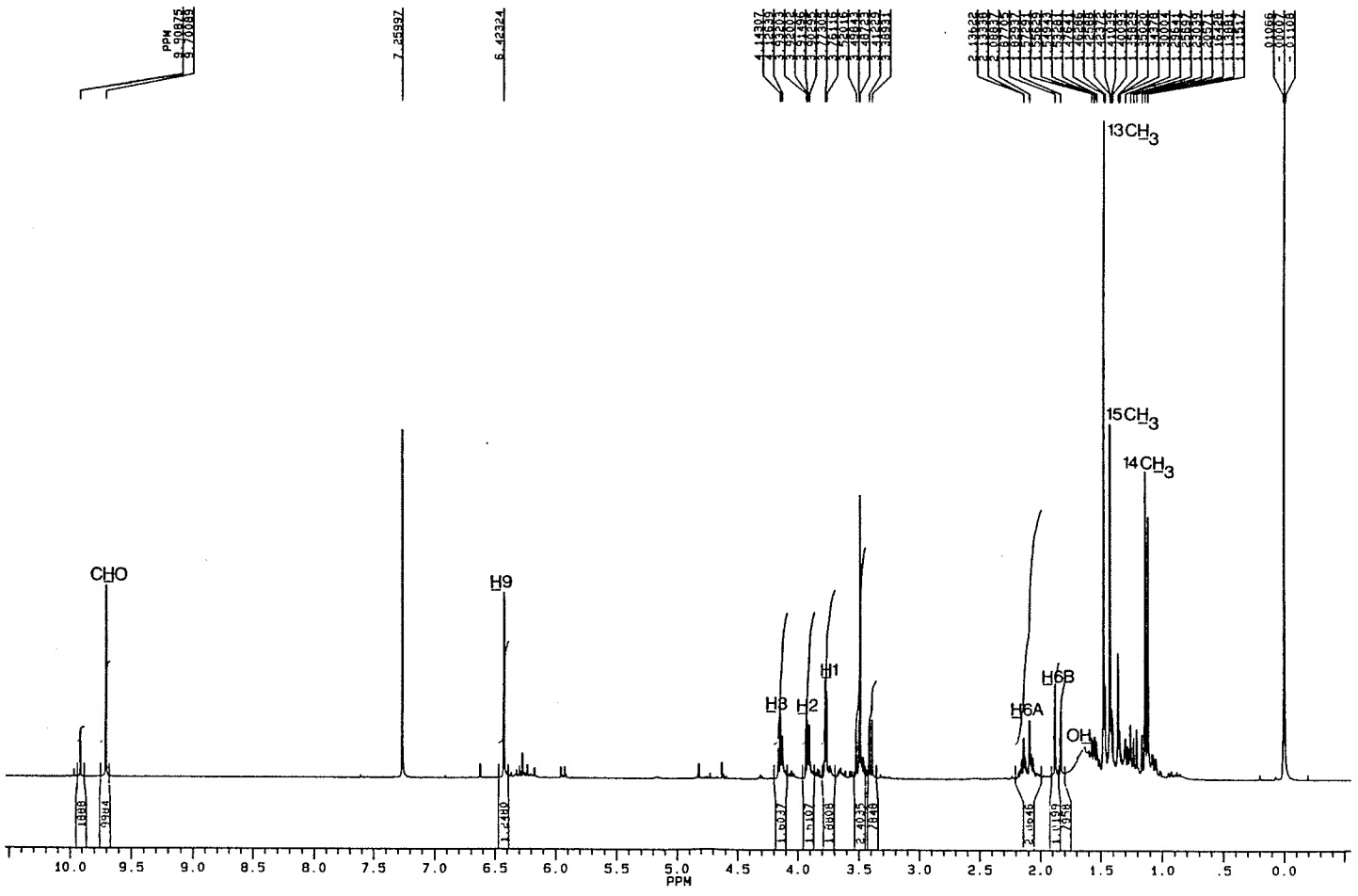

PR—OH
PRT
start
a
b

Figure 4.5 The 300 MHz ^{1}H NMR spectrum of PR alcohol (in $CDCl_3$) produced by the alkaline hydrolysis of 1.5 mg of purified PRT; TMS was used as internal standard.


CHO
H9
H3
H2
H1
H6A
H6B
OH
13CH3
15CH3
14CH3
PPM

variation in chemical shift of the hydroxyl proton is often due to a strong dependency on hydrogen bonding, as well as exchange effects, temperature, and concentration. Our sample was assessed at a relatively low concentration, and this may explain the reduced sensitivity of the response.

Figure 4.6 shows the ^{13}CNMR spectrum of the synthesized PR alcohol. These results confirmed proper modification of PRT. Carbons 16 and 17 comprising the acetyl group and seen in the parent compound at positions 173 and 20 ppm, respectively (Figure 4.3), disappeared upon hydroxylation (Figure 4.6).

Based on NMR data, one can conclude that hydroxylation of PRT was successful. The resulting PR alcohol was further used for succinylation. Numerous procedures were followed in an effort to achieve this goal. All were deemed unsuccessful in altering the PR alcohol to a hemisuccinate.

Succinylation according to Procedure 1, as outlined earlier, resulted in a product, which, when assessed by ^{1}H NMR (Figure 4.7), showed similarities to the starting material, PR alcohol. The secondary hydroxyl proton (δ1.6), originally visible in the ^{1}H NMR spectrum of the PR alcohol (Figure 4.5), was less distinquishable in the modified compound. This could be due in part to a reduction in concentration during the succinylation reaction and purification. Enlargement (insert in Figure 4.7) of this area (δ1.0 to δ2.0) seems to indicate the presence of the hydroxyl group visible as a rise in the baseline at about δ1.6.

Furthermore, a carboxyl functional group, which would be indicative of successful succinylation of the compound, is not visible on the spectrum. This group should appear somewhere between δ9.0 and δ10.0, although its chemical shift is strongly dependent on hydrogen bonding and temperature effects (Morrison and Boyd,

Figure 4.6 The 75.47 MHz ^{13}CNMR spectrum of PR alcohol (in $CDCl_3$) produced by the alkaline hydrolysis of 1.5 mg of purified PRT.

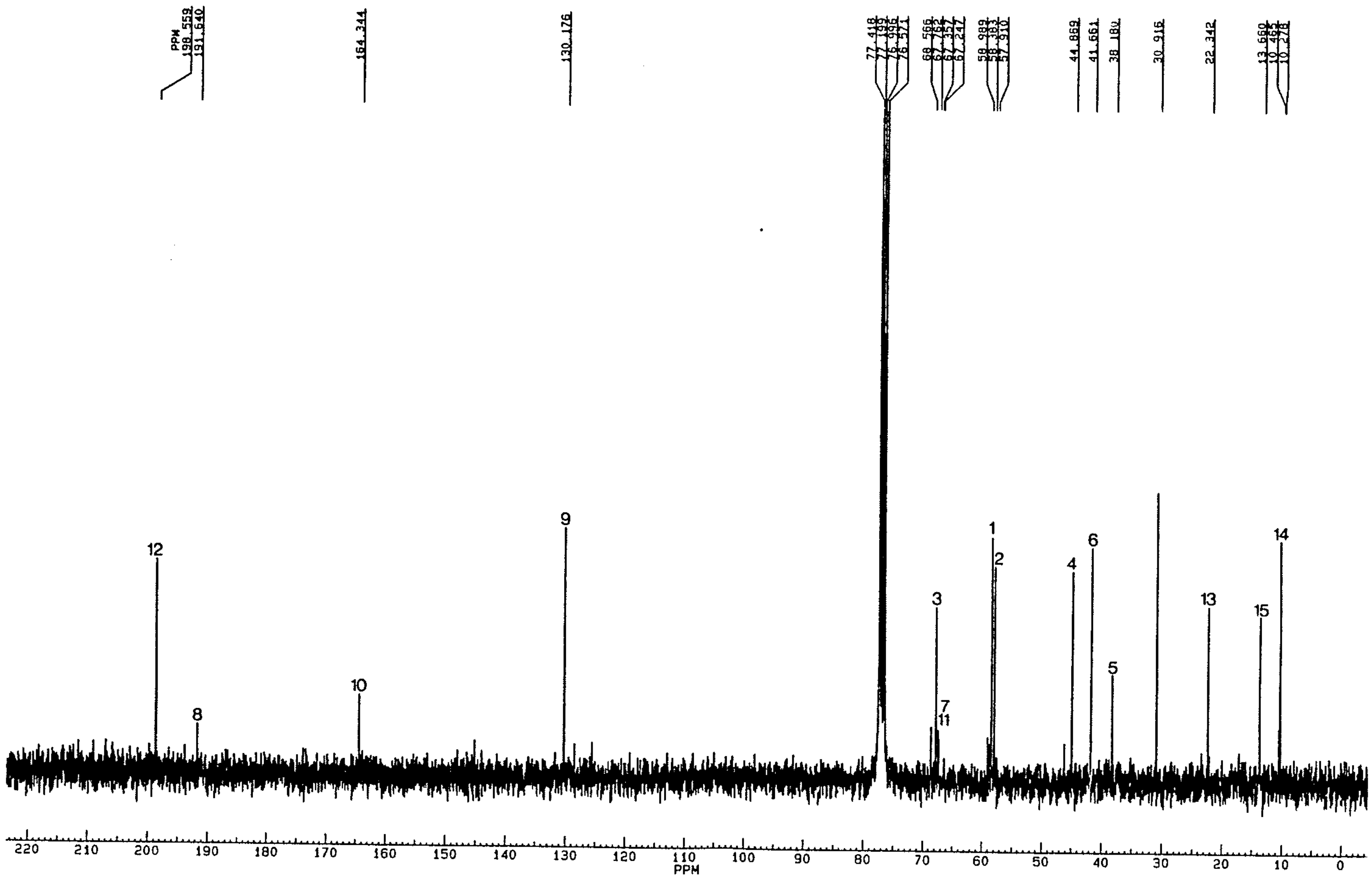
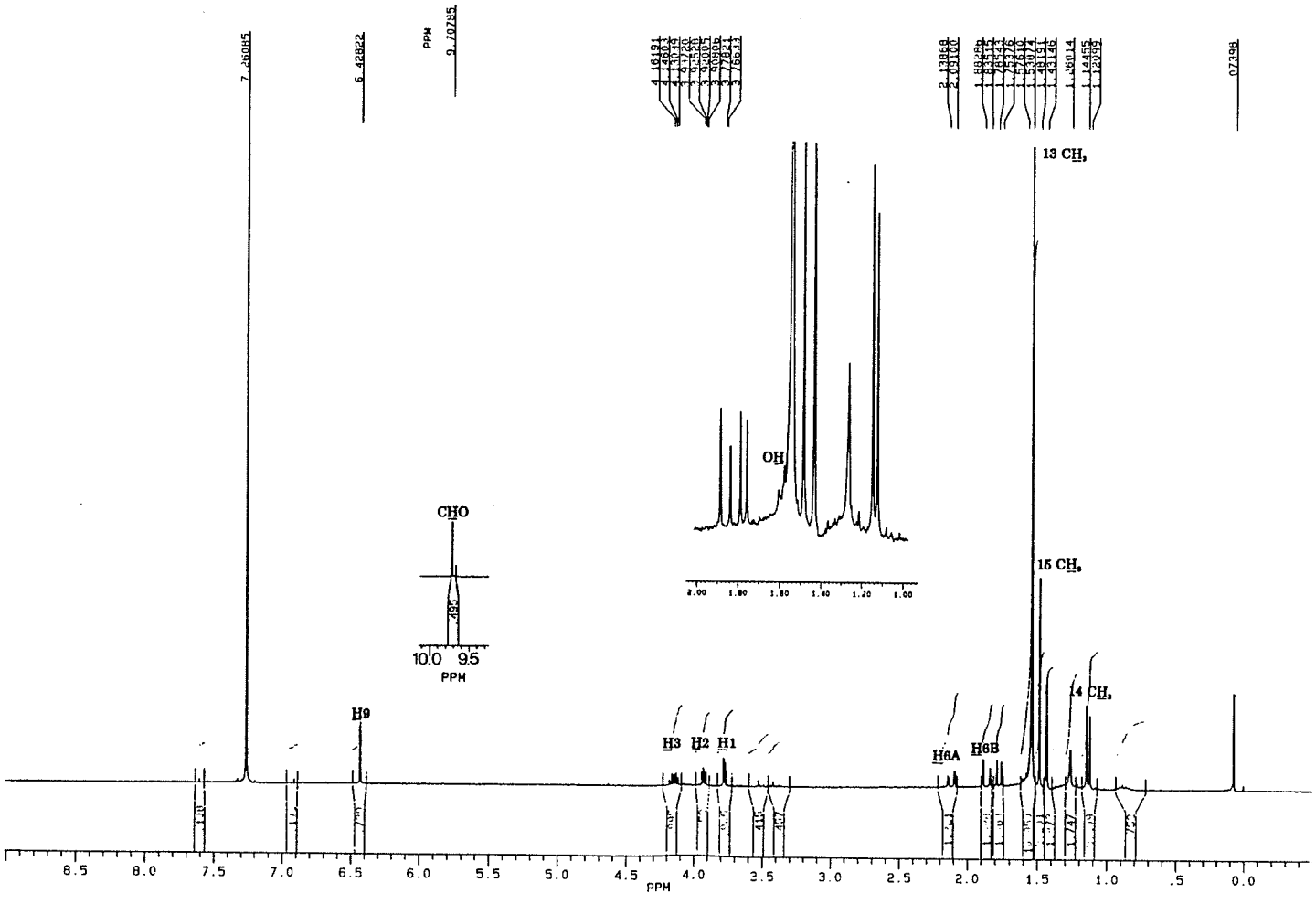

PPM
198.559
191.640
164.344
130.176
77.418
76.996
76.571
68.566
67.247
58.989
57.910
44.869
41.661
30.916
22.342
13.660
12
8
10
9
3
7
11
1
2
4
6
5
13
15
14
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
PPM

Figure 4.7 The 300 MHz ^1H NMR spectrum of the succinylation product (in $CDCl_3$) of 1.0 mg of PR alcohol using procedure 1 (TMS as internal standard). Expanded scale of signals between δ1.0 and 2.0 is shown in the insert.


7.26045
6.42822
9.70785
PPM
CHO
10.0 9.5
PPM
OH
13 CH3
15 CH3
14 CH3
H9
H3 H2 H1
H6A
H6B
8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 .5 0.0
PPM

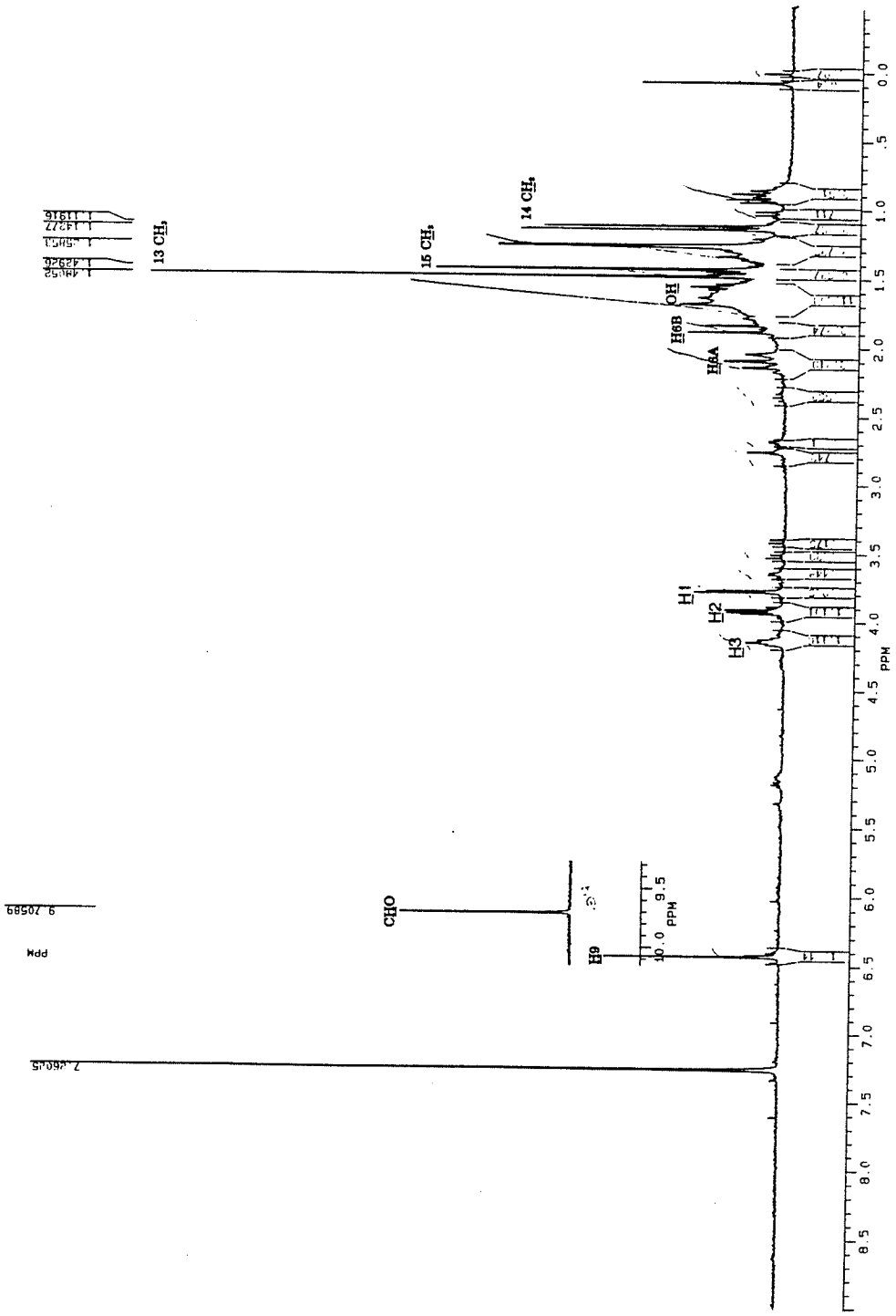
1983). No additional resonance proton, however, was seen in this region (Figure 4.7). Moreover, the one-proton doublet of doublets attached to the C-3 carbon remained at position δ4.1 (Figure 4.7), as it was in the PR alcohol (Figure 4.5). Had the organic synthesis been successful, then a shift up field due to the shielding of this proton in the C-3 position, as a direct result of an additional adjacent carboxyl proton, would have been observed.

Procedure 1 has been used successfully to produce the hemisuccinate of T-2 toxin (Chu *et al.*, 1979; Ohtani *et al.*, 1988). However, it was not suitable for the succinylation of PR alcohol. Although both toxins have several similarities in structure, some structural differences of PR alcohol may account for the results. In this respect, both toxins are double-ringed molecules containing acetoxy and hydroxyl side chains, although the hydroxyl group is present naturally in the T-2 molecule. In contrast to PR alcohol, T-2 contains no epoxy moiety in close proximity to the hydroxyl group.

An increase in the amount of succinic anhydride used to react with PR alcohol did not improve procedure 1. There appeared to be no change in the R_F value between that of the PR alcohol and the product as assessed by RP-TLC. Also, the ^1H NMR spectrum (Figure 4.8) showed a similar pattern to that seen in Figure 4.7. The hydroxyl proton is still visible at δ1.5 to δ1.6, and there appears no indication of a carboxyl moiety down field from the carbonyl group that would suggest successful succinylation.

Since anhydrous pyridine was used in these procedures as a solvent to provide suitable conditions for the desired organic synthesis, it was hypothesized that increasing the amount of pyridine may render the reaction milieu yet more fitting for

Figure 4.8 The 300 MHz ^1H NMR spectrum of the succinylation product (in $CDCl_3$) of 1.0 mg of PR alcohol using procedure 2 (TMS as internal standard).


13 CH3
15 CH3
14 CH3
OH
H6B
H6A
H1
H2
H3
CHO
H9
PPM

the proposed succinylation. However, an increased level of pyridine resulted only in the addition of an acetyl group as seen at δ2.16 of the ^{1}H NMR spectrum (Figure 4.9). A similar result was observed when the mixture was allowed to react at a slower rate at room temperature (Figure 4.10). Although an alternate conversion, perhaps even partial reversion to PRT was taking place, the revised procedure did not aid in the production of the hemi-succinate of PR alcohol.

Studying the effect of the temperature and time of the reaction on the succinylation of PR alcohol, revealed that the reaction was not dependent on these parameters.

Further methodology alterations included substituting pyridine with tetrahydrofuran, and employing an alternate reagent, 4-N,N-dimethylaminopyridine, according to the procedure of Lau *et al.* (1981). The latter reagent, although a widely used and versatile hypernucleophilic catalyst (Merck Index, 1968) proved unsuccessful in producing the succinylated PR alcohol as assessed by ^{1}H NMR (Figure 4.11). There was no indication of a carboxyl moiety, nor a chemical shift up field involving the C-3 proton.

Increasing the amount of PR alcohol used in the reaction mixture yielded only an increase in the concentration of the final product, shown by the intensity of the ^{1}H NMR peaks (Figure 4.12). Subsequent analysis by $^{13}CNMR$ (Figure 4.13) confirmed these results.

Furthermore, a control sample of succinic anhydride analysed by ^{1}H NMR (Figure 4.14) revealed that some of the the impurities noted in previous spectral data may have been due in part to unreacted and unremoved succinic anhydride.

Figure 4.9 The 300 MHz ^1H NMR spectrum of the succinylation product (in $CDCl_3$) of 1.0 mg of PR alcohol using procedure 3 at 80° C (TMS as internal standard).

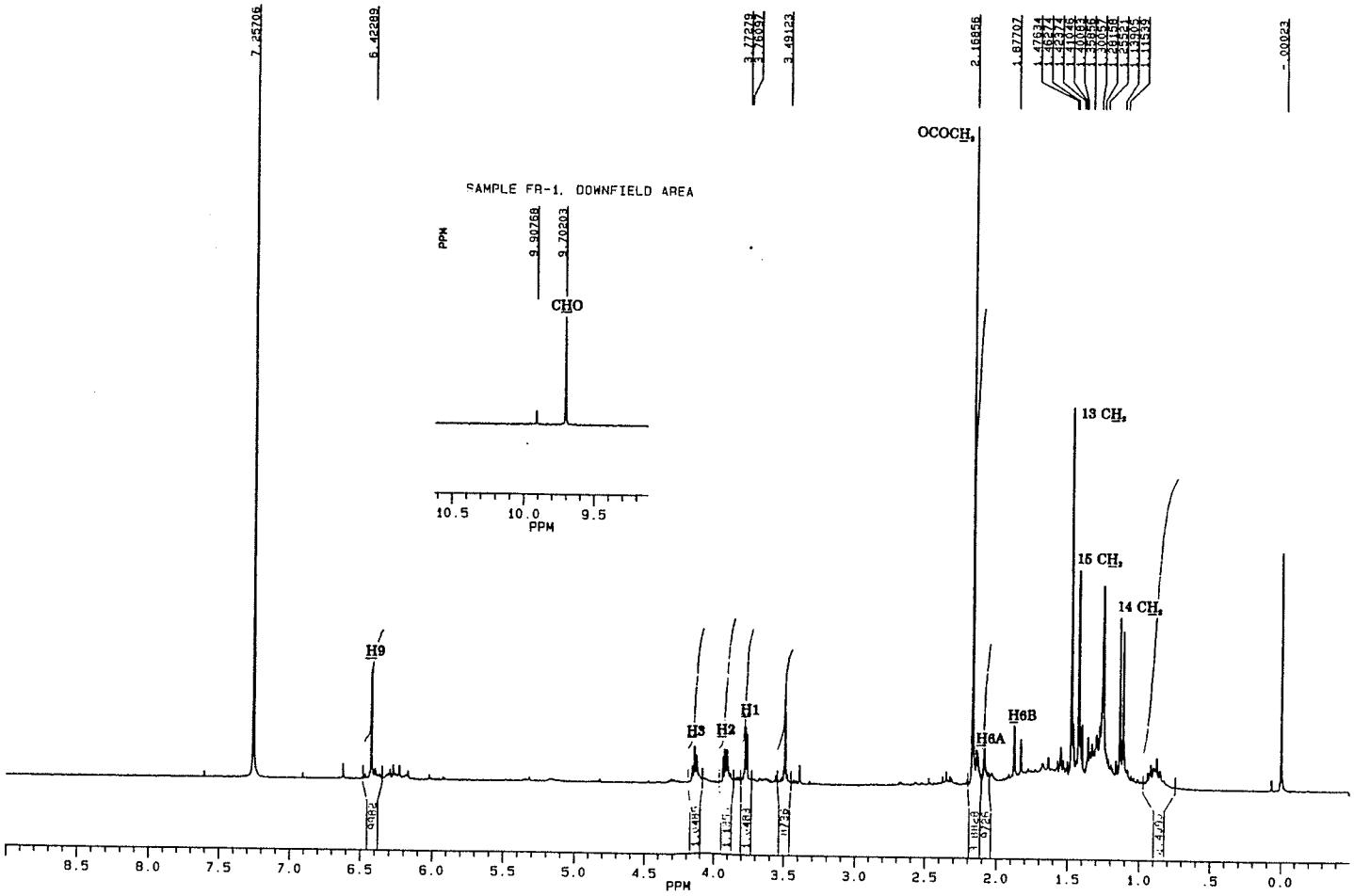

SAMPLE FR-1. DOWNFIELD AREA
PPM
9.90768
9.70203
CHO
10.5
10.0
9.5
PPM
7.25706
6.42289
3.77379
3.76097
3.49123
2.16856
1.87707
.00023
OCOCH3
13 CH3
15 CH3
14 CH3
H9
H3
H2
H1
H6B
H6A
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
0.0
PPM

Figure 4.10 The 300 MHz ^1H NMR spectrum of the succinylation product (in $CDCl_3$) of 1.0 mg of PR alcohol using procedure 3 at room temperature (TMS as internal standard).

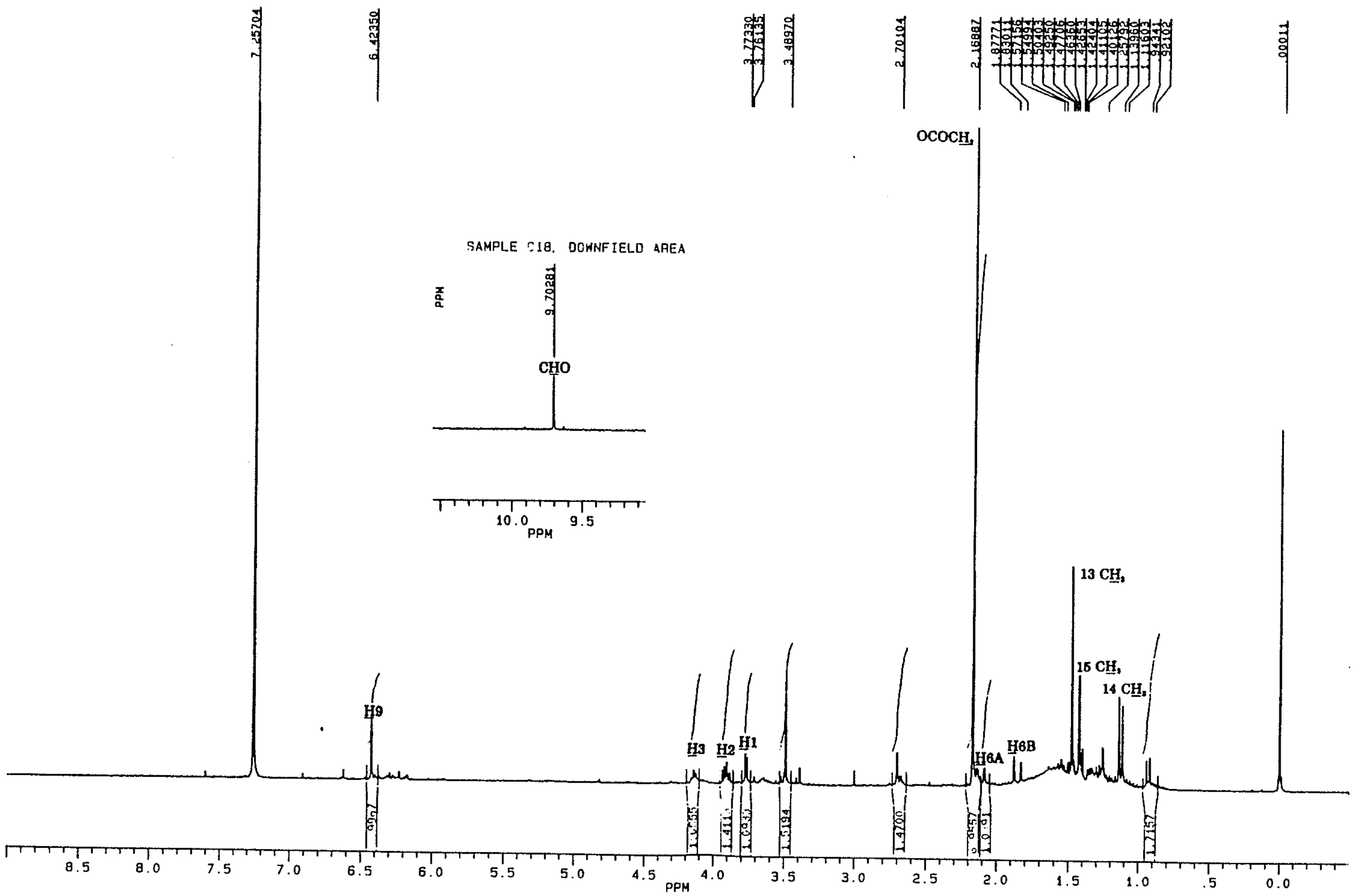

SAMPLE C18, DOWNFIELD AREA
9.70281
CHO
PPM
OCOCH3
H9
H3
H2
H1
H6A
H6B
13 CH3
15 CH3
14 CH3
PPM

Figure 4.11 The 300 MHz ^1H NMR spectrum of the succinylation product (in $CDCl_3$) of 1.0 mg of PR alcohol using procedure 4 (TMS as internal standard).

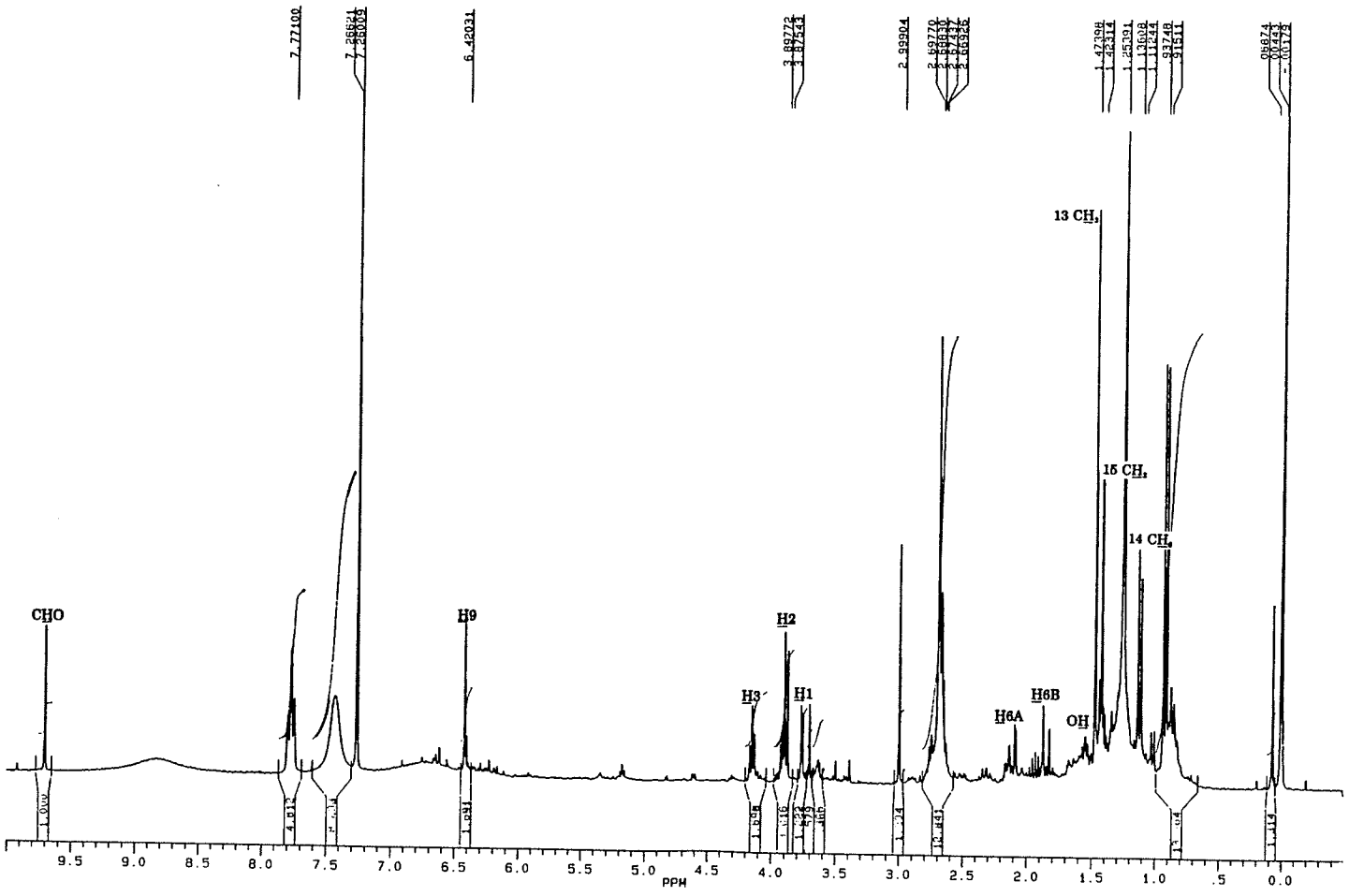

CHO
H9
H2
H3
H1
H6A
H6B
OH
13 CH3
15 CH3
14 CH3
PPM

Figure 4.12 The 300 MHz ^1H NMR spectrum of the succinylation product (in $CDCl_3$) of 5.0 mg of PR alcohol using procedure 4 (TMS as internal standard).

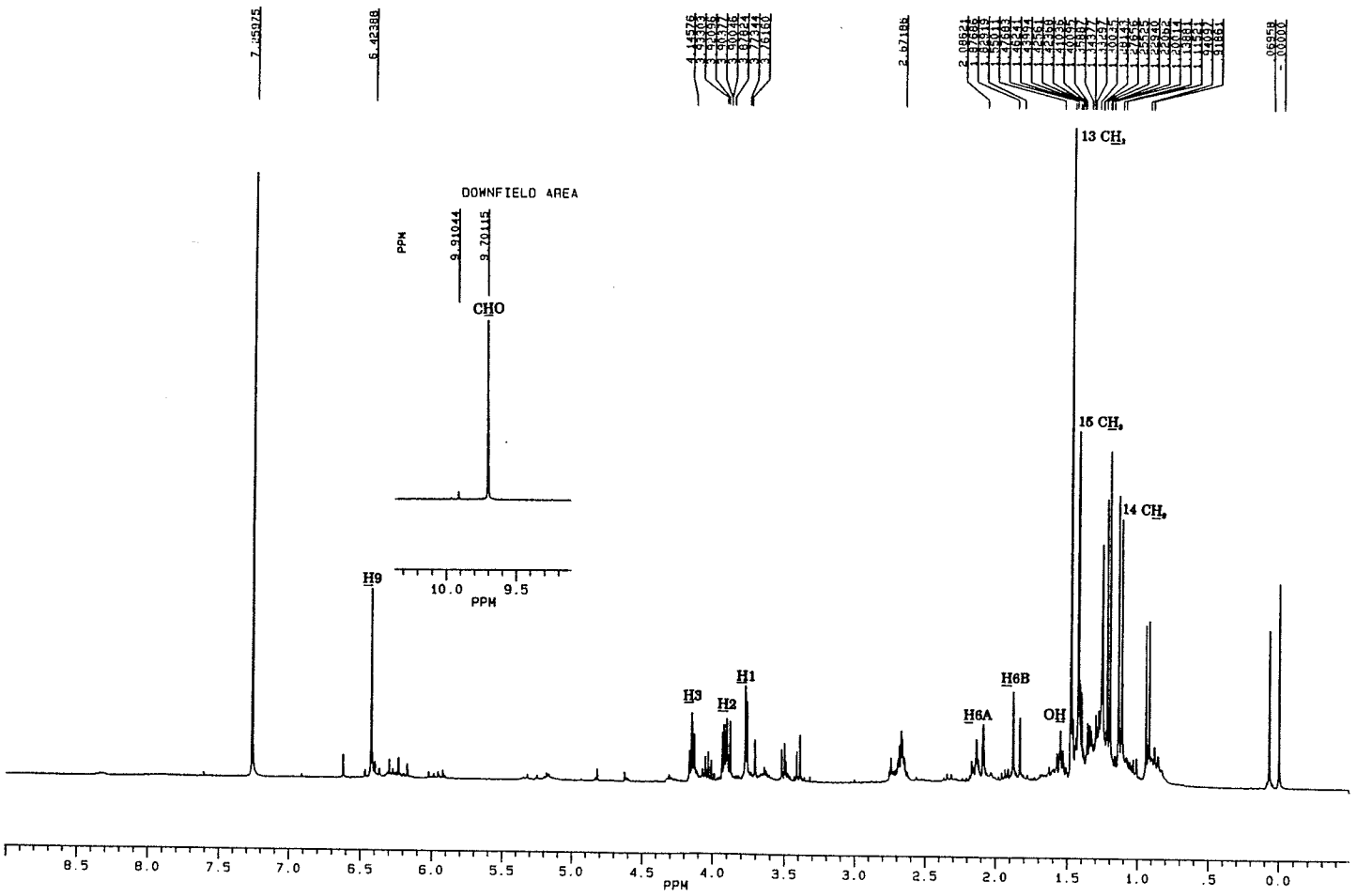

DOWNFIELD AREA
PPM
9.91044
9.70115
CHO
10.0
9.5
PPM
H9
H3
H2
H1
H6A
H6B
OH
13 CH3
15 CH3
14 CH3
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
0.0
PPM

Figure 4.13 The 75.47 MHz ^{13}CNMR spectrum of the succinylation product (in $CDCl_3$) of 5.0 mg of PR alcohol.

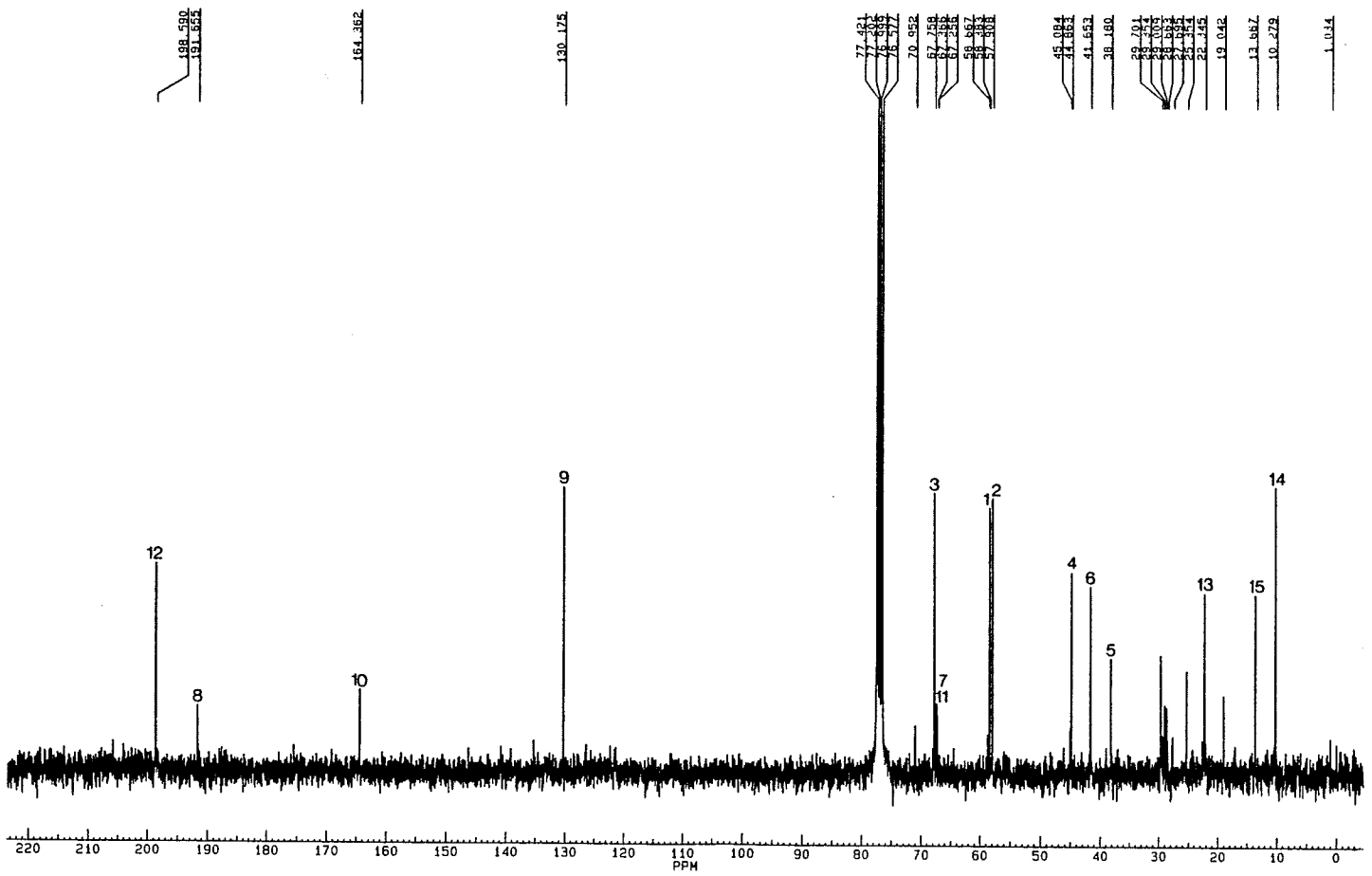
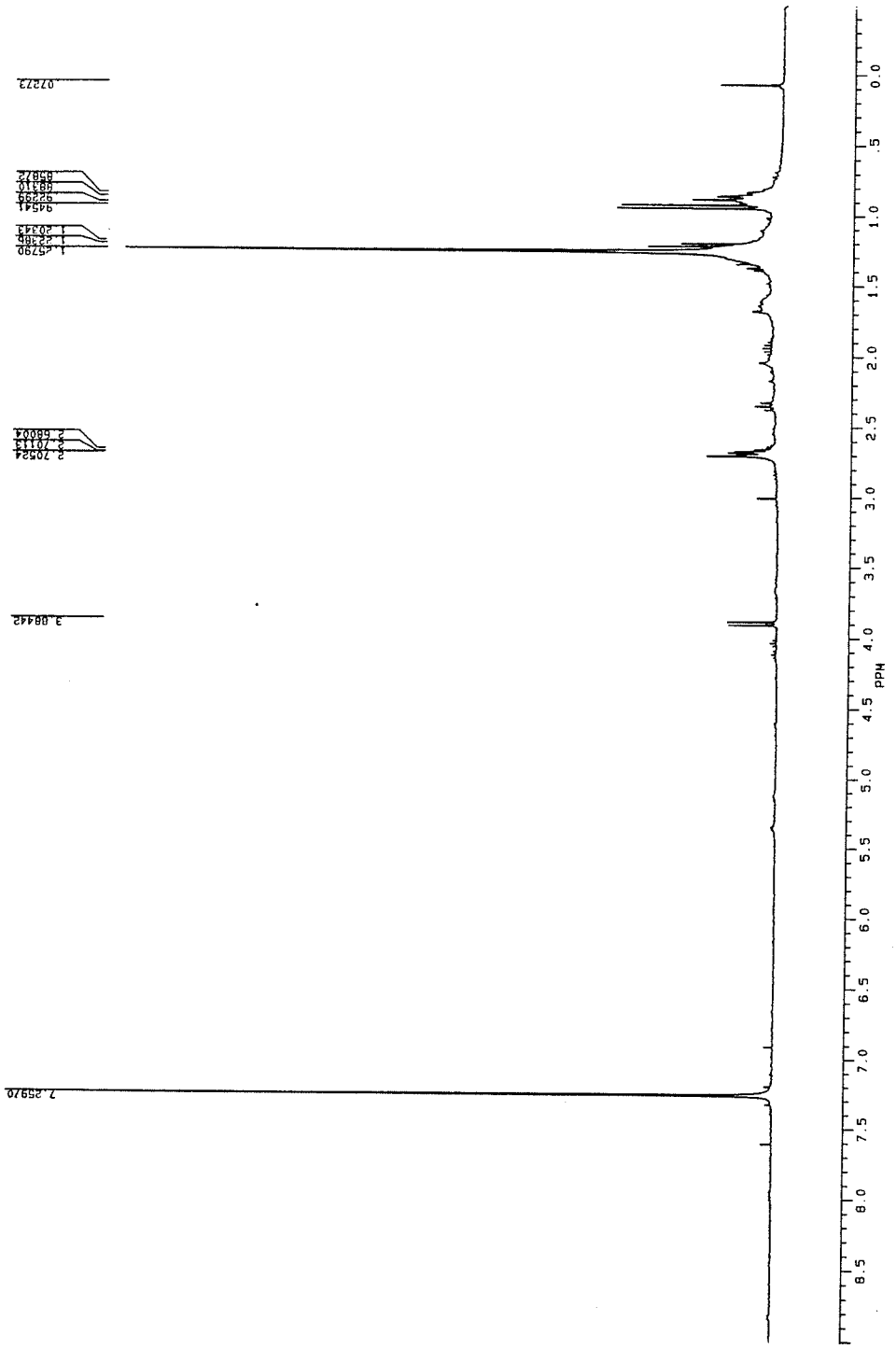

198.590
191.655
164.362
130.175
77.421
77.000
76.577
70.952
67.758
67.558
67.358
58.867
58.383
57.904
45.084
44.863
41.653
38.180
29.701
25.341
22.445
19.042
13.867
10.279
1.034
12
8
10
9
3
7
11
1
2
4
6
5
13
15
14
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
PPM

Figure 4.14 The 300 MHz ^1H NMR spectrum of a succinic anhydride control sample (in $CDCl_3$) using TMS as the internal standard.


7.25970
3.00442
2.70524
2.70113
2.68004
1.45790
1.42366
1.40341
0.94541
0.92239
0.89110
0.87872
0.87242
PPM

None of the modifications investigated contributed to the successful succinylation of PR alcohol. This may be attributed to the stearic hindrances inherent to the PRT molecule. For example, Baert *et al.* (1980) studied the structural and absolute configuration of PRT and reported that the epoxide group at carbons 1 and 2, the acetate group on carbon 3, and the methyl group on carbon 5 are mutually *syn.*, thereby minimizing the esterification of the 3β-hydroxide by ρ-bromobenzoyl chloride to synthesize a bromo derivative of PRT. Similarly, this configuration could explain the unsuccessful succinylation of the 3β-hydroxide group of PR alcohol.

4.5 Conclusion

Although the alkaline hydrolysis of PRT was successful, further succinylation of the resulting PR alcohol was not achieved possibly because of the various stearic hindrances.

As a result, the site specific conjugation of PRT to a protein carrier was also not possible and consequently, the development of a specific immunoassay to detect and discriminate PRT and PR imine as outlined earlier was not achievable.

An alternative method must therefore be employed to allow sufficient assessment of both PRT and its corresponding imine form. As outlined earlier, reversed-phase HPLC proved to be an extremely sensitive method for the detection and quantification of PRT. Hence, due to the unavailing proposed procedure, reversed-phase HPLC was further optimized and adapted for the concomitant determination of both PR toxins.

5. MANUSCRIPT III

REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF *PENICILLIUM ROQUEFORTI* TOXIN AND ITS IMINE FORM

5.1 Abstract

A method for the detection and quantitation of *Penicillium roqueforti* toxin (PRT), and its analogue, PR imine, using reversed-phase high-performance liquid chromatography has been developed. The quantitation limit of this method for PRT and PR imine was 1.5 and 1.0 ng, respectively. The retention times for PR imine (3.38 min) and PR toxin (5.86 min) were highly reproducible with coefficients of variation of amout 0.5% for both toxins. Reproducibility of the peak height was good with variation coefficients for PRT and PR imine of 5.1 and 4.5%, respectively. Analysis of PRT and PR imine in cheese sample extracts showed no interference of the sample matrix. Accuracy assessment of the method resulted in mean PRT and PR imine recoveries of 89.2 and 110.6%, respectively.

5.2 Introduction

PRT and PR imine are secondary metabolites of certain strains of *Penicillium roqueforti*, including those used in the production of blue-veined cheeses (Scott *et al.*, 1977; Polonelli *et al.*, 1978; Wei and Liu, 1978, Moreau *et al.*, 1980b). The chemical structures and interconversion of both toxins have been previously described (Wei *et al.*, 1978; Scott and Kanhere, 1979).

PRT is the most acutely toxic compound secreted by *P. roqueforti*. It is lethal to mice and weanling rats exhibiting a LD_{50} in the range of 1 to 5.8 mg/kg, and 11 to 14.5 mg/kg by intraperitoneal injection, respectively (Arnold *et al.*, 1978; Chen *et al.*, 1982). PRT impairs the transcription process in liver cells of both animals (Moulé *et al.*, 1976; Lee *et al.*, 1984) and inhibits the *in vivo* and *in vitro* synthesis of protein and nucleic acids (Moulé *et al.*, 1978). PRT was found to be carcinogenic for rats (Polonelli *et al.*, 1982) and mutagenic for *Salmonella typhimurium* (Ueno *et al.*, 1978). However, the acute toxicity of PRT is reduced in the presence of ammonium ions due to its conversion into the PR imine form (Moulé *et al.*, 1977b). This conversion is apparent in blue cheese where PRT reacts with ammonia and free amino acids (Wei *et al.*, 1975; Scott and Kanhere, 1979). Arnold *et al.* (1978) showed that PR imine is less toxic than PRT exhibiting intraperitoneal LD_{50} values ranging from 100 to 200 mg/kg in mice, although it has similar but diminished inhibitory effects on protein and nucleic acid syntheses (Arnold *et al.*, 1978). Furthermore, it has been suggested that some of the acute toxicity of PR imine may be attributable to PRT, resulting from the *in vivo* conversion of PR imine to PRT (Moulé *et al.*, 1977a; Arnold *et al.*, 1978). However, the role that PR imine plays as a toxicological compound has not been fully investigated, in part, due to the lack of a sensitive and specific assay.

A number of semi-quanitative thin-layer chromatography (TLC) methods have been used to detect PRT (Betina, 1985) and PR imine (Wei *et al.*, 1973; Scott and Kanhere, 1979) in a variety of media. Recently, several more sensitive high-performance liquid chromatography (HPLC) procedures, using both normal phase (Moreau *et al.*, 1979) and reversed-phase (Gorst-Allman and Steyn, 1984; Danieli *et al.*, 1984; Siemens and Zawistowski, 1992) columns have been developed to detect and quantify PRT. However, none of these methods have been devised for the determination of PR imine.

This report describes a reversed-phase HPLC method for the detection and quantitation of PRT and its PR imine analogue.

5.3 Materials and Methods

5.3.1 Standards

Crystalline PRT was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and was further purified as described previously (Siemens and Zawistowski, 1992). PR imine was prepared according to the method of Wei *et al.* (1973) and its purity was confirmed by ^{1}H NMR (Model AM300 Bruker Spectrospin, Canada) and TLC, using a standard PR imine, kindly supplied by Dr. P.M. Scott (Health Protection Branch, Ottawa, Canada).

Examination of PR imine by TLC revealed the lower R_F value (0.18) than that of the PRT molecule (0.63) on silica gel plates (Whatman, USA) in methanol-chloroform (4:96, v/v). The ^{1}H NMR spectrum (300 MHz) of the prepared PR imine comfirmed its purity, and showed that the aldehyde proton at δ9.75 had disappeared, with the generation of two one-proton singlets between δ5.0 and δ5.5 (Appendix II).

The resulting spectrum was similar to results reported previously (Wei *et al.*, 1973).

5.3.2 HPLC

The HPLC method was developed on a Waters ALC 204 liquid chromatograph. The system consisted of a model 6000A solvent delivery system, a model 440 absorbance detector equipped with a 254 nm filter and a U6K universal liquid chromatography injector.

Chromatographic analyses were carried out on a pre-packed μBondapak reversed-phase C_{18} column (300 mm x 3.9 mm ID) of particle size 10 μm (Waters, Division of Millipore, Toronto, Canada). Signals from the UV detector were recorded by a SP4290 integrator (Spectra Physics, San Jose, CA, USA) set at an attenuation of 8 (2^n where n=3 and n=0 denotes the highest noise to integrator detection ratio). The solvent system used for HPLC analysis was a mixture of acetonitrile and water at 60:40 (v/v). All solvents were of HPLC grade. Both PRT and PR imine were analyzed using the same solvent at a flow rate of 1.0 ml/min.

Standard curves were prepared by making a series of dilutions of PRT and PR imine in acetonitrile ranging in concentration from 0.05 to 2.5 ng/μl (1.0 ng to 50 ng per injection) for each toxin. Peak heights were integrated and monitored as a function of the amount (ng) of the compounds injected.

5.3.3 Sample Preparation

Application of the reversed-phase HPLC method for the determination of both PRT and PR imine in blue cheese was assessed by conducting an analysis of cheese samples spiked with PRT as follows. PRT standard (0.25 mg) dissolved in 1.0 ml of

methanol was added to 10 g of Danish Blue cheese to give a final concentration of 25.0 mg/kg. The cheese was immediately homogenized in a Waring blender with 250 ml of methanol-water (55:45, v/v) and 150 ml of hexane for 5 minutes at high speed. The mixture was centrifuged at 1000 *g* at 4°C for 10 min. After removing the hexane layer, the methanol-water portion was extracted with two 60 ml volumes of chloroform. The two chloroform extracts were pooled and evaporated to dryness using a rotary evaporator. The dried extract was dissolved in 1.0 ml of acetonitrile, and 10 µl were withdrawn, diluted, and immediately analyzed by HPLC as described earlier.

The presence of both toxins in cheese samples spiked with PRT (25.0 mg/kg) and stored at 4°C for 10, 30, 60 and 120 min prior to extraction were analyzed by the HPLC method described above.

5.3.4 Accuracy assessment

The accuracy of the HPLC method for the quantitation of a mixture of PRT and PR imine was determined by an external standard method. Blue cheese samples were extracted with chloroform, evaporated to dryness, dissolved in acetonitrile and spiked with both PRT and PR imine standards over a concentration range of 0.22 to 1.7 ng/µl (4.4 to 34 ng per injection) for each compound. Recoveries of both toxins from the extracts were determined by HPLC using peak height measurements. A two-sample *t*-test was then used to estimate the significance of the differences between known and measured values of PRT and PR imine.

5.4 Results and Discussion

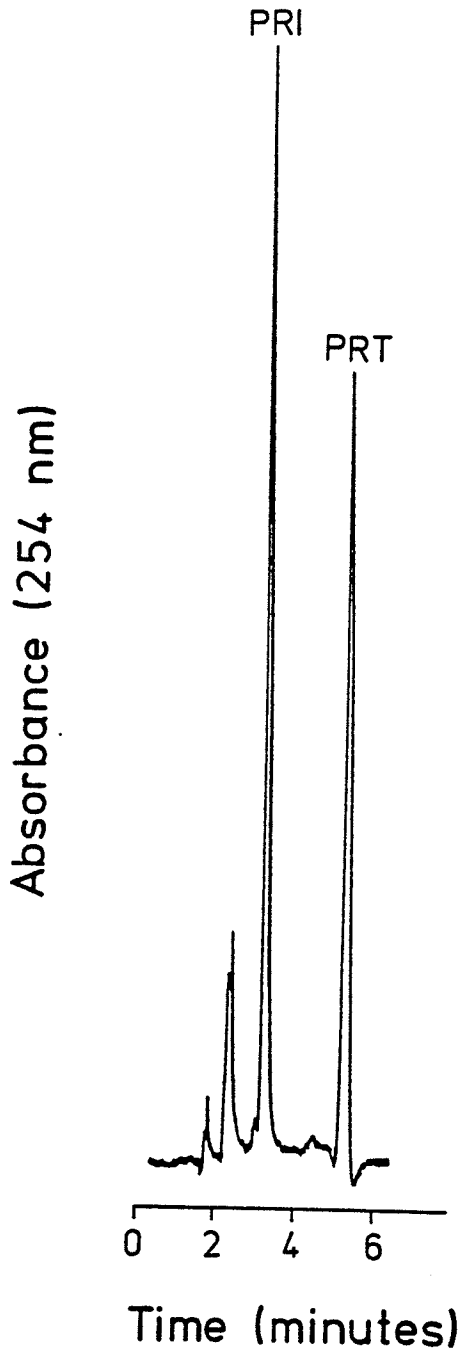
Since PRT and PR imine are oxygenated sesquiterpenoids differing principly in the number of ketone and hydroxyl groups, they display a different polarity which enables good separation by HPLC. Until now, only TLC analysis of spiked cheese samples containing both toxins has been carried out with a detection limit of 0.13-0.5 mg/kg for PR imine (Scott and Kanhere, 1979). In our experiments we used a reversed-phase HPLC method which enabled the toxins to be quantified at the ng level. Both PRT and PR imine have an ultraviolet absorption maximum in the region of 250 nm with molar extinction coefficients of 9000 (Polonelli, 1978). Hence, the chosen 254 nm filter was adequate to assess both compounds.

Optimum separation of PRT and PR imine was obtained using a 60:40 (v/v) mixture of acetonitrile and water at a flow rate of 1.0 ml/min. A typical chromatogram of the separation is shown in Figure 5.1. The capacity (k') and the selectivity factor (α) are also given in this figure. PRT and PR imine were well resolved with a selectivity factor of 3.18.

Retention times as determined for each compound either in a mixture or separately were highly reproducible. A total of 20 injections (20 µl) of each toxin ranging from 5 to 50 ng, gave a mean retention time of 3.38 min for PR imine and 5.86 min for PRT with coefficients of variation of about 0.5 % (Table 5.1).

Reproducibility of the peak height was also evaluated by a total of 10 injections of 50 ng of PRT and 20 ng of PR imine, over a 2-day period. The precision of the method, assessed by peak height reproducibility, was good, with variation coefficients for PRT and PR imine of 5.1 and 4.5%, respectively (Table 5.2).

Figure 5.1 Reversed-phase HPLC resolution of PR imine and PRT standards (20 ng each). Eluent: acetonitrile-water (60:40, v/v); flow rate, 1.0 ml/min.



Resolved Compound	Retention Time (min)	Capacity Factor (k')	Selectivity Factor (α)
PR imine	3.38	0.50	
PRT	5.86	1.59	3.18

TABLE 5.1 - *Reproduciblity of retention time for PRT and PR imine as assessed by reversed-phase HPLC*[a]

Compound[b]	Mean Retention Time (min)	Standard Deviation (min)	Variation Coefficient (%)
PRT	5.86	0.03	0.53
PR imine	3.38	0.02	0.51

[a]acetonitrile-water (65:35 v/v)
[b]based on 20 injections of each standard ranging from 5 to 50 ng

TABLE 5.2 - *Reproducibility of peak height for PRT and PR imine as assessed by reversed-phase HPLC*[a]

Compound	Mean Peak Height	Standard Deviation	Variation Coefficient (%)
PRT[b]	10820	547	5.1
PR imine[c]	10040	451	4.5

[a]acetonitrile-water (65:35 v/v)
[b]based on 10 injections of 50 ng of PRT
[c]based on 10 injections of 20 ng of PR imine

The sensitivity of the developed method was analyzed by injecting decreasing concentrations of the mixed standard toxins, to the lowest detectable level. At an attenuation setting of 4, an amount of 2.2 ng of each toxin was easily detectable, and quantifiable (Figure 5.2a). Adjusting the attenuation to 2, allowed as low as 1.0 ng of PR imine and 1.5 ng of PRT to be quantified (Figure 5.2b).

The linearity between the peak heights and the concentration of both toxins was determined by injecting 20 µl of increasing concentrations of the mixed PRT and PR imine standards. The amounts injected ranged from 1.0 to 50 ng for each compound. Regression analysis of the resulting data demonstrated a linear relationship for the two compounds over the tested range, with correlation coefficients of 0.99 and 0.98 for PRT (Appendix III) and PR imine (Appendix IV), respectively.

Total recoveries of PRT and PR imine from the spiked cheese extract and the results of the a Student *t*-test are shown in Table 5.3. The accuracy of the method for PRT and PR imine determination was 89.2 and 110.6%, respectively. No significant difference (at a 95% confidence level) was found between expected values for each compound and recovered amounts of toxins from the spiked cheese sample over the concentration range tested.

Figure 5.3 shows a chromatogram of PRT and PR imine recovered from a cheese sample spiked with 20 ng of each standard toxin. Distinct peaks corresponding to PRT and PR imine indicate that other components present in the cheese extract do not interfere with the detection of the toxins.

Furthermore, experiments were carried out to assess suitability of the developed reversed-phase HPLC method to monitor the conversion of PRT to PR imine in cheese. Blue cheese was spiked with PRT and stored for up to 2 hrs. The

Figure 5.2 Reversed-phase HPLC resolution of PR imine and PRT standards a) 2.2 ng of each at an attenuation of 4 and b) 1.0 ng of PR imine and 1.5 ng of PRT at an attenuation of 2. Eluent: acetonitrile-water (60:40, v/v); flow rate, 1.0 ml/min.

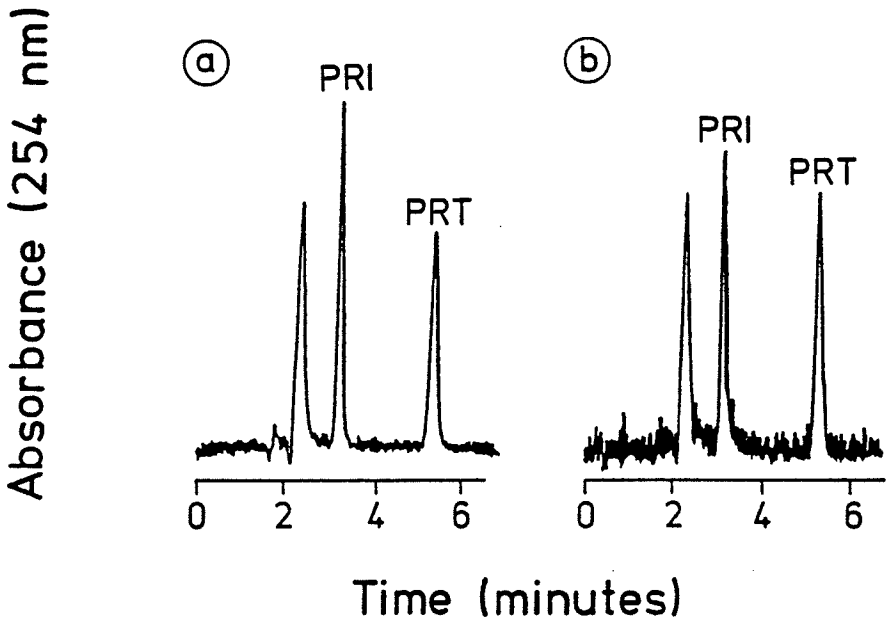
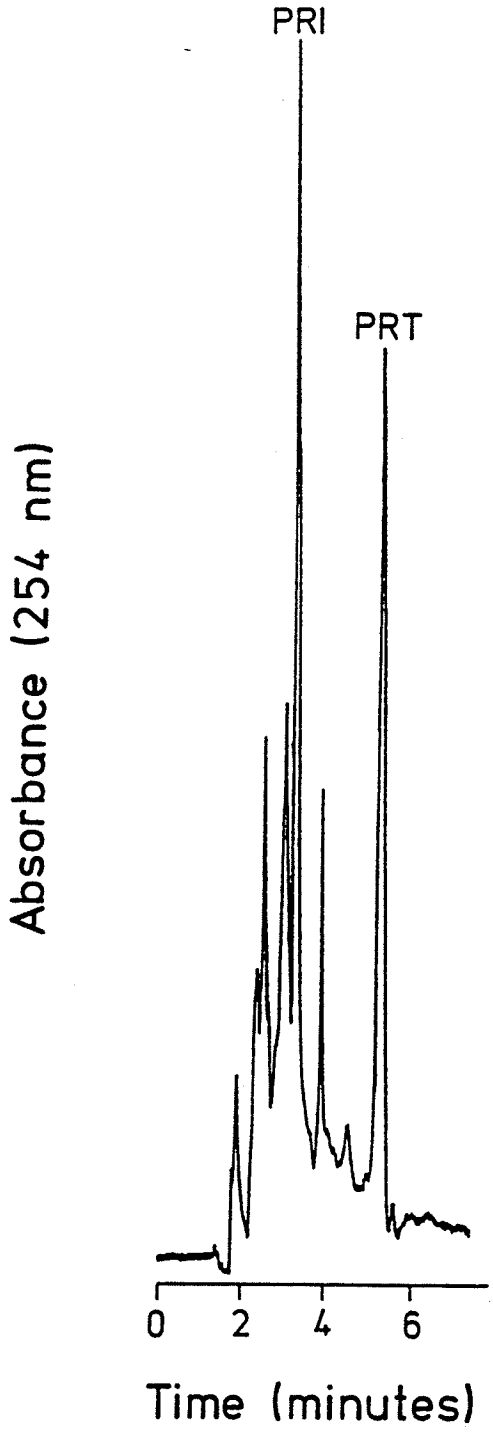

Absorbance (254 nm)
a
PRI
PRT
b
PRI
PRT
0
2
4
6
0
2
4
6
Time (minutes)

TABLE 5.3 - *Accuracy of reversed-phase HPLC to detect PRT and PR imine in blue cheese samples*

Expected Amount of Compound (ng)	PRT			PR imine		
	Observed Amount[a] (ng)	Student t-test[b] value	Total Recovery (%)	Observed Amount[a] (ng)	Student t-test[b] value	Total Recovery (%)
34.0	33.5 ± 0.1	1.57	98.5	39.5 ± 0.5	3.36	116.8
22.0	20.9 ± 0.1	0.41	95.0	20.3 ± 0.9	0.26	92.4
14.8	13.3 ± 1.2	1.67	90.0	13.8 ± 0.6	0.90	93.2
9.8	7.7 ± 1.1	2.52	78.6	8.3 ± 0.3	2.37	84.7
6.6	5.1 ± 0.6	3.24	81.8	7.9 ± 0.6	1.62	119.7
4.4	4.0 ± 1.6	0.70	91.0	6.9 ± 0.5	2.74	156.8

[a]based on triplicate runs
[b]two-sample t-test made at a 95% confidence level where $t_{\alpha=0.05}=4.303$

Figure 5.3 Reversed-phase HPLC resolution of PR imine and PRT recovered from spiked blue cheese extract. Eluent: acetonitrile-water (60:40, v/v); flow rate, 1.0 ml/min.


PRI
PRT
Absorbance (254 nm)
0
2
4
6
Time (minutes)

presence of both toxins in cheese, during the storage, was monitored by HPLC (Table 5.4). The results of the conducted analysis demonstrated that PRT was unstable in blue cheese. This confirms previous findings reported by other workers (Scott and Kanhere, 1979; Siemens and Zawistowski, 1992). After 10 minutes of storage, only about 48% of PRT remained in blue cheese. A rapid loss of PRT was associated with a parallel appearance of PR imine. No PRT was detected in cheese stored for 2 hrs while as much as 92.7% of PR imine was recovered after this time of storage (Table 5.4). It has been suggested by Scott and Kanhere (1979) that the conversion of PRT into PR imine is the direct result of the PRT reacting with ammonia and ammonium salts naturally occurring in blue cheese.

Our data show that the developed reversed-phase HPLC procedure is a simple and highly sensitive method suitable for concomitant detection and quantitative analysis of PRT and PR imine. The method will be applied to further analyze these toxins in blue cheeses.

5.5 Acknowledgements

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada. We thank Dr. Peter Scott of Health and Welfare Canada, Ottawa, Ontario, Canada, for providing PR imine toxin.

5.6 Conclusion

A novel and improved method for the concomitant determination of PRT and PR imine was developed. This method was applied to further examine each of these compounds in cheese samples.

TABLE 5.4 - *Conversion of PRT to PR imine in Danish blue cheese as assessed by reversed-phase HPLC*[a]

Compound	PRT or PR imine present[b] (%)			
	10 min[c]	30 min[c]	60 min[c]	120 min[c]
PRT[d]	48.1	29.2	2.6	0.0
PR imine	42.3	60.0	82.2	92.7

[a]acetonitrile-water (65:35 v/v)
[b]Based on triplicate experiments
[c]Storage time of PR toxin-spiked cheese at 4°C
[d]PRT was added to cheese at 25 mg/kg

6. MANUSCRIPT IV

OCCURRENCE OF PR IMINE, A METABOLITE OF *PENICILLIUM ROQUEFORTI*, IN BLUE CHEESE

6.1 Abstract

Analysis of blue cheese and blue cheese dressing for PR imine and PRT, metabolites of *Penicillium roqueforti*, was performed by reversed-phase HPLC. PR imine was found in 50 of 60 samples of blue cheese, in amounts ranging from 19.1 to 41.9 μg/kg of cheese, while PRT was not detected. Analysis of blue cheese dressing showed no evidence of any PR imine or PRT. Furthermore, the conversion of PR imine to PRT in the presence of bovine serum (*in vitro*) has been demonstrated.

6.2 Introduction

Many strains of *Penicillium roqueforti*, including those used in blue cheese production, have the ability to secrete a secondary metabolite, *P. roqueforti* toxin (PRT) (Medina *et al.*, 1985; Polonelli *et al.*, 1978).

PRT is lethal to mice and rats with LD_{50} values of about 1 to 15 mg/kg by intraperitoneal administration (Scott, 1984), carcinogenic for rats (Polonelli *et al.*, 1982), as well as mutagenic for *Salmonella typhimurium* (Ueno *et al.*, 1978). Furthermore, it has been shown that PRT impairs protein and nucleic acid syntheses (Moulé *et al.*, 1978).

Since *P. roqueforti* cultures are deliberately introduced into certain cheeses, the potential for mycotoxin production by this mould is of unique toxicological concern. Fortunately, it has been shown that PRT is unstable in blue cheese and undergoes a reaction with ammonia and ammonium salts as well as neutral and basic amino acids in the cheese to form PR imine (Scott and Kanhere, 1979).

PR imine does not exhibit significant toxicity in animals. The LD_{50} for mice is in the range of 100 to 200 mg/kg by intraperitoneal injection. However, this toxin has similar to PRT, but diminished, inhibitory effects on protein and nucleic acid syntheses (Arnold *et al.*, 1978). In addition, there is evidence that *in vivo* (mice), PR imine is reversibly converted to its more toxic counterpart, PRT (Moulé *et al.*, 1977a). This may pose a potential health risk for humans. PRT is unstable in cheese, and although tests performed on its whole and mouldy fractions have not yielded any indication for the presence of PRT (Lafont *et al.*, 1976; Polonelli *et al.*, 1978), the presence of PR imine in cheese has not been fully elucidated.

The aim of this work was to conduct a survey of retail blue cheese and blue

cheese dressing for the presence of PR imine toxin using a reversed-phase HPLC method. The conversion of PR imine into PRT in bovine serum (*in vitro*) was also investigated.

6.3 Materials and Methods

6.3.1 Materials

Samples of Danish blue cheese and blue cheese dressing, all of which had been manufactured in Canada, were obtained from various local retail outlets. Standard PRT was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and was further purified as described previously (Siemens and Zawistowski, 1992). PR imine was prepared according to the method of Wei *et al.* (1973) and its purity was confirmed by ^{1}H NMR (Model AM300 Bruker Spectrospin, Canada), and TLC, using a standard PR imine, kindly supplied by Dr. P.M. Scott (Health Protection Branch, Ottawa, Canada). Fetal bovine serum was obtained from Bocknek (Rexdale, Ontario, Canada). The total protein content of the serum was 36 mg/ml, as determined by the method of Lowry *et al.* (1951). All other reagents were of analytical grade, while solvents used for HPLC analysis were of HPLC grade.

6.3.2 Detection of Toxins

The presence of both PRT and PR imine in blue cheese, blue cheese dressing and bovine serum was determined by reversed-phase HPLC as described previously (Siemens and Zawistowski, 1992).

The HPLC system used was a Waters ALC 204 liquid chromatograph consisting of a model 6000A solvent delivery system, a model 440 absorbance detector equipped

with a 254 nm filter and a U6K universal liquid chromatography injector.

Chromatographic analyses were carried out on a pre-packed μBondapak reversed-phase C_{18} column (300 mm x 3.0 mm ID) of particle size 10 μm (Waters, Toronto, Canada). Signals from the UV detector were recorded as peak heights integrated by a SP4290 integrator (Spectra Physics) set at an attenuation of 8.

The solvent system used for the analysis consisted of acetonitrile and water (60:40, v/v), at a flow rate of 1.0 ml/min. Standard curves for PRT and PR imine were prepared by making serial dilutions of each in acetonitrile, in order to allow quantification of both toxins in the samples.

6.3.3 Blue Cheese Extraction

Cheese samples (10 g) were homogenized in a Waring blender with 65 ml of methanol-water (55:45 v/v) and 40 ml of hexane for 5 minutes at high speed. The mixture was centrifuged at 1000 *g* at 4° C for 10 minutes. After removal of the hexane layer, the methanol-water portion was extracted twice with 20 ml volumes of chloroform. The two chloroform extracts were pooled and concentrated using a rotary evaporator. The sample was then evaporated to dryness under a stream of nitrogen, dissolved in 1.0 ml of acetonitrile, diluted, and immediately analyzed by HPLC, as described earlier, for the presence of PR imine.

6.3.4 Blue Cheese Dressing Extraction

Samples of blue cheese dressing (10 g) were mixed thoroughly with 65 ml of methanol-water (60:40, v/v) and 70 ml of hexane. The mixture was centrifuged at 1000 *g* at 4° C for 10 minutes. After removal of the hexane layer, the methanol-water

portion was extracted twice with 25 ml volumes of chloroform. The two chloroform extracts were pooled and concentrated using a rotary evaporator. The sample was then evaporated to dryness under a stream of nitrogen. The dried extract was dissolved in 1.0 ml acetonitrile, diluted, and analyzed by HPLC.

6.3.5 Conversion of PR imine to PRT

Conversion experiments were carried out either in the presence of fetal bovine serum or phosphate buffered saline (PBS). Fetal bovine serum (5 ml) was diluted 5-fold with distilled water and spiked with either 62 or 125 μg of standard PR imine, to yield a final concentration of 2.5 or 5.0 μg PR imine/ml of serum diluent, respectively. The mixture was then extracted twice with 25 ml volumes of chloroform. The chloroform extracts were pooled, and concentrated using a rotary evaporator. The sample extract was further evaporated to dryness under a stream of nitrogen. The dried extract was dissolved in 1.0 ml acetonitrile and immediately analyzed by HPLC.

The spiking and extraction protocols were repeated for samples that were stored prior to extraction at room temperature for 1, 2, 3, 4 hours, and 2 days. Fetal bovine serum, free of PR imine, was extracted as above and used as a control sample.

Similar experiments were carried out using PBS (4.38g NaCl, 2.45g KH_2PO_4 and 8.10g Na_2HPO_4 in 1L of distilled water (pH 7.2)). PBS (25 ml) was spiked with either 62 or 125 μg of standard PR imine. The mixture was extracted similarly to the serum samples. This procedure was repeated for PBS samples that were spiked and stored at room temperature for 1, 2, 3, 4 hours, and 2 days. In addition, a PBS sample free of PR imine was also extracted and used as a control.

6.4 Results and Discussion

The analyses of 60 samples of Danish blue cheese and 6 samples of blue cheese dressing for the presence of PR imine and PRT was performed using reversed-phase HPLC. The detection limit of this method was 1.0 ng of PR imine and 1.5 ng of PRT. PR imine was detected in 50 cheese samples at levels ranging from about 19 to 42 µg/kg, with the mean content of 29.5 ng/g (Table 6.1). No PRT was detected in any of the analyzed cheese samples. Furthermore, analysis of blue cheese dressing showed no evidence of PR imine or PRT. The broad range of the PR imine content in the cheese samples tested signifies an uneven distribution of this toxin in cheese. This is reflected in the relatively large variation coefficient (23.1%) amongst samples. However, replicates within samples were highly reproducible. The retention times for PR imine averaged 3.38 minutes and were also highly reproducible with a coefficient of variation of 0.99 (Table 6.1). Figure 6.1a shows a chromatogram of a blue cheese sample in which PR imine was detected at a concentration of about 35 µg/kg. The presence of the toxin was confirmed by spiking the cheese sample with standard PR imine (Figure 6.1b).

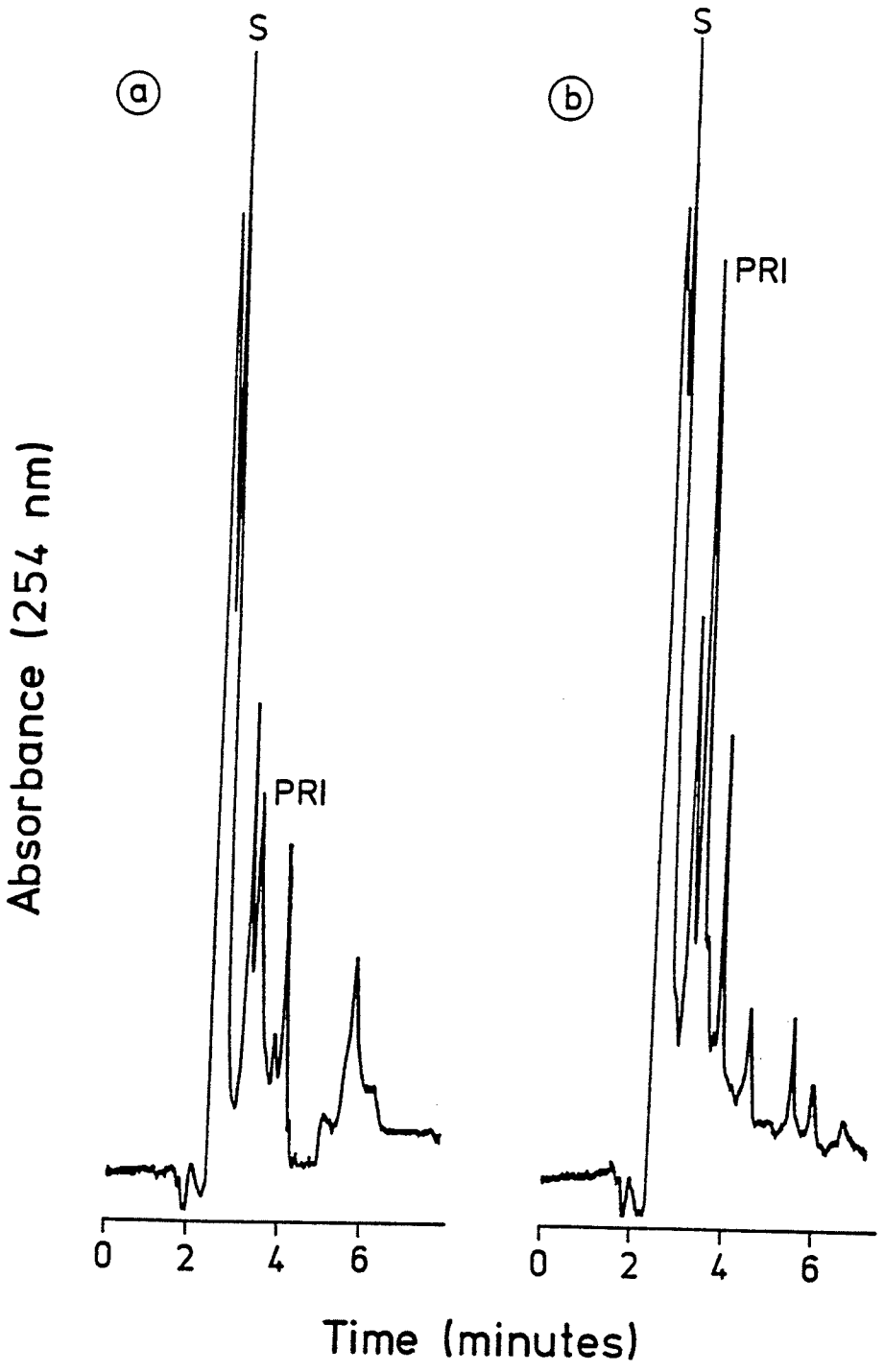
The occurrence of PR imine in cheese implies that this toxin may be a result of PRT conversion. It is most likely that PRT was initially produced in cheese by *P. roqueforti*, and was rapidly converted to PR imine in the presence of ammonium ions and amino acids (Scott and Kanhere, 1979). Thus, our findings are in disagreement with observations by Polonelli and coworkers (1978) who reported that conditions during cheese production favour growth of *P. roqueforti* mycelium but not the production of PRT. The assumption that PR imine is a metabolite directly excreted into blue cheese may be ruled out, since numerous *P. roqueforti* strains analyzed for

TABLE 6.1 - *Amount of PR imine found in Danish blue cheese as assessed by reversed-phase HPLC*[a]

	Peak Heights	Retention Time (min)	Amount of PR imine (ng)
Mean[b]	1856 ± 487	3.38 ± 0.03	29.5 ± 6.8
Range[b]	1112-2677	3.32-3.41	19.1-41.9
CV (%)[bc]	26.2	0.99	23.1

[a]acetonitrile-water (65:35 v/v)
[b]based on runs of 50 samples each in triplicate
[c]coefficient of variation

Figure 6.1 Reversed-phase HPLC resolution of a) PR imine extracted from 10 g cheese and b) the same extract with standard PR imine. Eluent: acetonitrile-water (60:40, v/v); flow rate, 1.0 ml/min; peak S=acetonitrile.


S
a
PRI
S
b
PRI
Absorbance (254 nm)
0
2
4
6
0
2
4
6
Time (minutes)

toxin production appeared to synthesize only PRT (Lafont *et al.*, 1976). Although PR imine was once isolated from *P. roqueforti* culture, it was further concluded that this compound resulted from the reaction of PRT with ammonium ions (Moreau *et al.*, 1980b). Furthermore, our results do not contradict reports on the absence of this toxin in blue cheese, since analyses of cheese were performed using TLC with low detection limits, generally 0.13-0.5 μg/g (Scott and Kanhere, 1979).

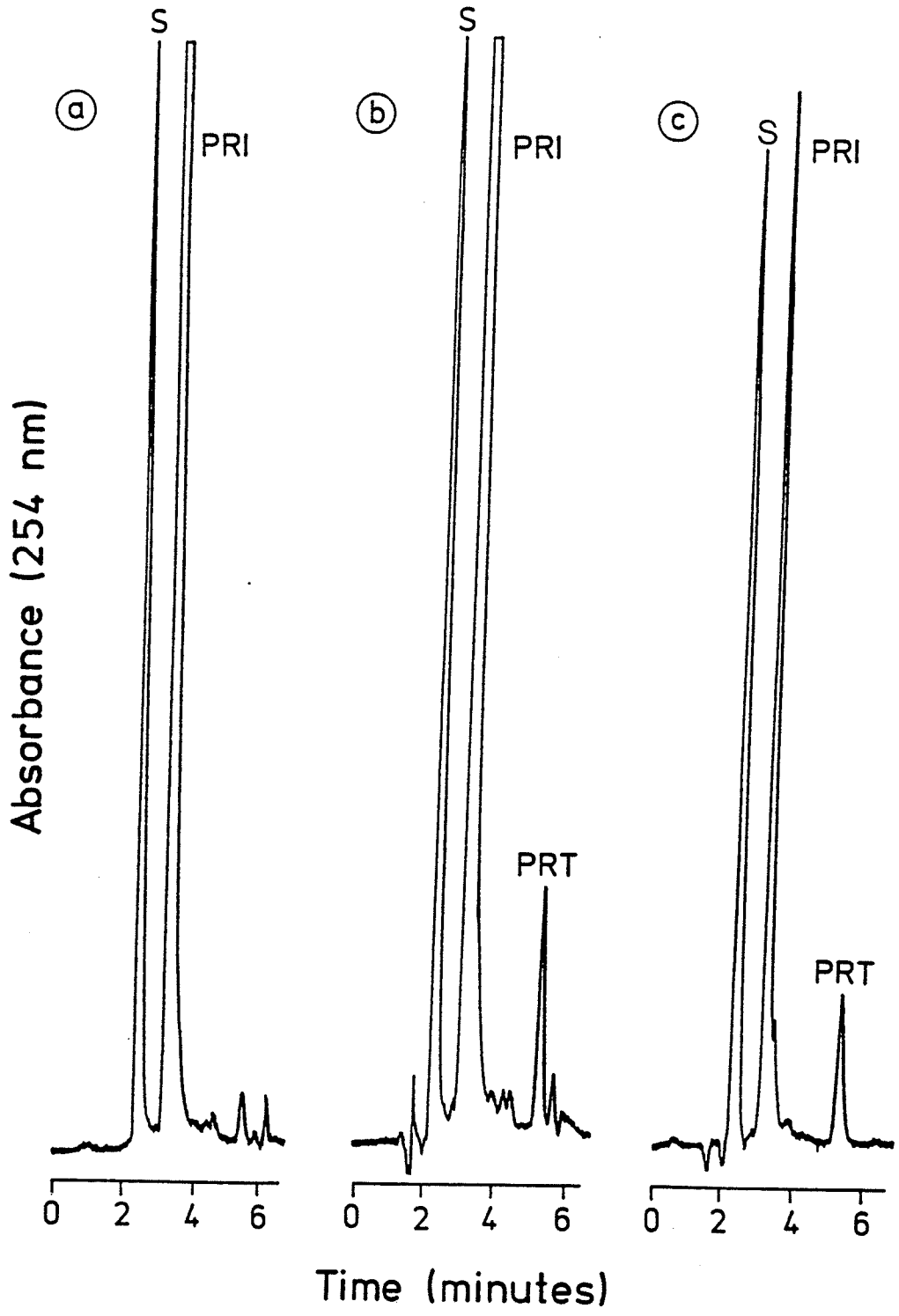
Detection of PR imine in blue cheese prompted us to investigate whether this compound could be converted back to the more toxic PRT in the presence of animal serum. The conversion of PR imine to PRT in bovine serum spiked with two concentrations of PR imine is shown in Table 6.2. Typical chromatograms of toxin conversion over time are shown in Figure 6.2. The conversion pattern for both concentrations were similar. No significant changes in the PR imine concentration had occurred, nor had it undergone any conversion to PRT, when the serum was extracted immediately upon spiking with PR imine. However, after 1 hour of storage at room temperature, the presence of PRT in the mixture was detectable. Storage exceeding 1 hour, yielded a more significant increase in PRT concentration. The maximum ratio of conversion of PR imine to PRT was observed after storage at room temperature for 2 hours for both serum samples spiked with different concentrations of PR imine. The average conversions of the 62 and 125 μg of PR imine were approximately 0.9 and 1.4%, respectively. At this time, the level of PR imine was also at a maximum. Longer storage time resulted in a reduction in the amount of both toxins. After two days about 6-7% of PR imine and 0-0.6% PRT of the original PR imine added to serum was detected. Instability of both PRT and PR imine has been reported previously and was attributed to ammonia, and neutral and basic amino acids

TABLE 6.2 - *Recovery of PR imine and conversion to PRT in extracts of serum spiked with PR imine*

Amount of PR imine added[b]	Storage Time	Amount Recovered in Extract (μg)[a]		% PRT present
		PR imine	PRT	
62.0 μg	0	35.39 ± 3.01	0.11 ± 0.19	0.2
	1 hr	42.60 ± 1.56	0.13 ± 0.11	0.2
	2 hrs	60.48 ± 10.3	0.57 ± 0.13	0.9
	3 hrs	28.91 ± 2.84	0.10 ± 0.17	0.3
	4 hrs	19.63 ± 3.44	0.07 ± 0.12	0.1
	2 days	3.90 ± 0.37	0	0
125.0 μg	0	114.4 ± 0.35	0	0
	1 hr	121.8 ± 0.92	0.17 ± 0.14	0.1
	2 hrs	130.5 ± 2.47	1.78 ± 0.45	1.4
	3 hrs	99.85 ± 3.75	1.57 ± 0.43	1.3
	4 hrs	51.12 ± 1.21	1.51 ± 0.14	1.2
	2 days	9.10 ± 0.36	0.71 ± 0.61	0.6

[a]based on two separate trials with triplicate injections for each storage time
[b]PR imine added to 25 ml serum (diluted 5-fold with distilled water)

Figure 6.2 Reversed-phase HPLC resolution of a) PR imine recovered from spiked serum (5.0 μg/ml dilute serum) after immediate extraction, b) PR imine and PRT present in an extract after 2 hours of storage and c) the two compounds after 2 days of storage. Eluent: acetonitrile-water (60:40, v/v); flow rate, 1.0 ml/min; peak S=acetonitrile.


a
b
c
S
PRI
PRT
Absorbance (254 nm)
0
2
4
6
Time (minutes)

(Scott and Kanhere, 1979). More recently Shaw *et al.* (1984) reported that PRT binds strongly to protein such as bovine serum albumin. The binding of PRT to BSA involves the formation of a Schiff base between the aldehyde group of the toxin and the ϵ-NH_2 group of the lysine and free sulfhydryl group of the cysteine residues in BSA. It is possible that the formation of the adduct between PRT and proteins may account for its significant reduction in the serum over time, since the protein content in the analyzed bovine serum was as high as 36 mg/ml, with the majority of this amount being albumin. However, the reduction of PR imine, which lacks the aldehyde group, may be due to its conversion to PRT.

To further support this hypothesis the conversion behaviour of PR imine was investigated in phosphate buffered saline free of protein and having pH (7.2) similar to that of bovine serum. Table 6.3 shows the results of toxin conversion in buffer spiked with two concentrations of PR imine. The concentration of PR imine decreased over time reaching 9-11% of the original PR imine content after two days in a manner similar to that obtained in the experiment carried out with bovine serum. However, the decrease in the amount of PR imine was associated with a simultaneous increase in the amount of PRT as a result of the conversion. After two days the PRT level accounted for about 54 and 78% of the 62 and 125 μg of PR imine added to serum, respectively. The mechanism of the conversion is not clear and needs to be elucidated.

In conclusion, our results indicate that the presence of PR imine naturally occurring in blue-veined cheese is not of great toxicological significance. The level of PR imine was minimal (equivalent to a maximum of 42 ppb in the cheese). In addition, more toxic PRT which may result from the conversion in animal serum is likely to be sequestered by the serum albumins. Nevertheless, additional analyses of

TABLE 6.3 - *Recovery of PR imine and PRT from phosphate buffered saline spiked with PR imine*

Amount of PR imine added[b]	Storage Time	Amount Recovered in Extract (µg)[a]		
		PR imine	PRT	% PRT present
62.0 µg	0	66.92 ± 0.47	0	0
	1 hr	66.31 ± 2.11	7.08 ± 0.11	11.4
	2 hrs	56.78 ± 0.72	9.27 ± 0.23	15.0
	3 hrs	50.18 ± 0.40	12.70 ± 0.02	20.5
	4 hrs	40.14 ± 0.81	15.25 ± 0.09	24.6
	2 days	5.65 ± 0.11	48.13 ± 1.32	77.6
125.0 µg	0	85.38 ± 0.62	0.80 ± 0.01	0.6
	1 hr	108.26 ± 3.32	7.87 ± 0.13	6.3
	2 hrs	85.35 ± 2.73	15.59 ± 0.90	12.5
	3 hrs	90.69 ± 2.42	22.46 ± 1.36	18.0
	4 hrs	76.81 ± 3.39	31.66 ± 2.95	25.3
	2 days	13.92 ± 0.18	67.74 ± 0.03	54.2

[a]based on two separate trials with triplicate injections for each time
[b]PR imine added to 25 ml of PBS

different blue-veined cheeses and more indepth *in vivo* investigations of the behaviour of PR imine need to be conducted in order to clarify the actual risk involved.

6.5 Acknowledgements

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7. GENERAL DISCUSSION

The chemistry and toxicity of PRT has been extensively discussed in the literature, since its discovery in 1972. The results presented in manuscripts I thru IV extends the information on the chemistry and the detection of this toxin.

A novel reversed-phase HPLC system was developed for the detection and quantitation of PRT and PR imine as outlined in manuscripts I and III. Although reversed-phase systems have been described previously (Gorst-Allman and Steyn, 1984; Danieli *et al.*, 1980), these methods suffer certain drawbacks. Gorst-Allman and Steyn (1984) achieved a relatively low detection limit of 50 ng of PRT per injection, while Danieli *et al.* (1980) presented a micro system, that may not be easily attainable. The current method, however, provides a simple system, with a 10-fold increase in the sensitivity, that allows both toxins, PRT and PR imine, to be well resolved. In addition, concomitant determination of PR imine and PRT by an HPLC method, has not been previously reported.

This reversed-phase HPLC system, could be applied to detect the toxins in spiked cheese samples. Cheese samples spiked with PRT resulted in a maximum PRT recovery of only 43.6%. This low yield, was due to its conversion to PR imine, most likely by reacting with ammmonia and ammonium salts, as well as neutral and basic amino acids in the cheese. These results on the stability of PRT support previous findings (Scott and Kanhere, 1979). However, although Scott and Kanhere (1979) report that PR imine was unstable in solvent and in cheese extract, investigations currently presented do not support these findings. In contrast, PR imine seemed to

be much more stable than PRT under any storage conditions.

The established method was comparable in sensitivity to an immunological detection assay reported earlier. The radioimmunoassay developed by Wei and Chu (1988), resulted in a detection limit of 1 to 2 ng of PRT per assay. Similarly, the reversed-phase HPLC procedure described allows the quantitative determination of 1.0 and 1.5 ng of PR imine and PRT, respectively. Therefore, it follows that the application of this method for the accurate assessment of the behavior of PRT and PR imine in cheese was possible.

A conversion of PR imine back to PRT at a rate of up to 10% has been reported to take place *in vivo* (Moulé *et al.*, 1977a). Current studies involving dilute animal serum spiked with PR imine, showed that the conversion of PR imine back to PRT also occurs *in vitro*.

Finally, the developed reversed-phase HPLC method proved adequately sensitive and led to the detection of the inherent presence of PR imine in Danish blue cheese samples at levels of up to 42 ppb. This may result from the conversion of PR toxin, secreted by *Pencillium roqueforti* used for the production of this cheese. Previous accounts of this compound in cheese have not been made and hence, may warrant further investigation. These preliminary results, therefore, demonstrate the value and practicality of the devised reversed-phase HPLC methodologies.

8. CONCLUSIONS AND RECOMMENDATIONS

A reversed-phase HPLC procedure has been developed for the determination of PRT and PR imine in cheese. The optimal resolution of the compounds was achieved in a system using an acetonitrile and water mixture as the eluent.

The developed method was also used to monitor the conversion of PRT to PR imine in cheese, and the instability of PRT was confirmed. Future studies may focus on the effect of storage conditions on the complex interconversion of PR imine in blue cheese.

PRT was not discovered in any cheese samples, although minute amounts of PR imine were found in the majority of the blue cheese samples analyzed. However, only Danish blue cheese was investigated, and hence the screening of different blue cheeses (eg. Stilton, Gorgonzola and Cabrales) for the presence of PR imine is necessary.

The reversed conversion of PR imine to PRT *in vitro*, in the presence of animal serum, was demonstrated. Whether this conversion imposes a health risk for the consumer needs to be assessed by further *in vivo* studies. Animal models involving rats or mice may be used to assess the toxic effect of PR imine.

The HPLC method was developed for the determination of both toxins as an alternative method to an immunochemical assay. It was not possible to develop the latter procedure due to the inability to prepare a site specific PRT-protein conjugate which is required for the production of monoclonal antibodies. The stearic hindrances of the molecule did not allow successful succinylation of the modified alcohol form of PRT, and hence, the desired conjugation could not be performed. Future studies,

however, may produce a useful conjugate. Perhaps chemical blocking of the interfering moieties would prove helpful in a reaction proceeding as proposed.

The obtained results provide new insight into the behaviour of PRT and its analogue PR imine, with regards to stability, and interconversions in various matrices. The development of the reversed-phase HPLC method is of great importance. This method, due to its high sensitivity and simplicity, should be employed in further studies to evaluate a toxicological significance of both PRT and PR imine in foodstuffs, as well as their involvement as potential health hazards.

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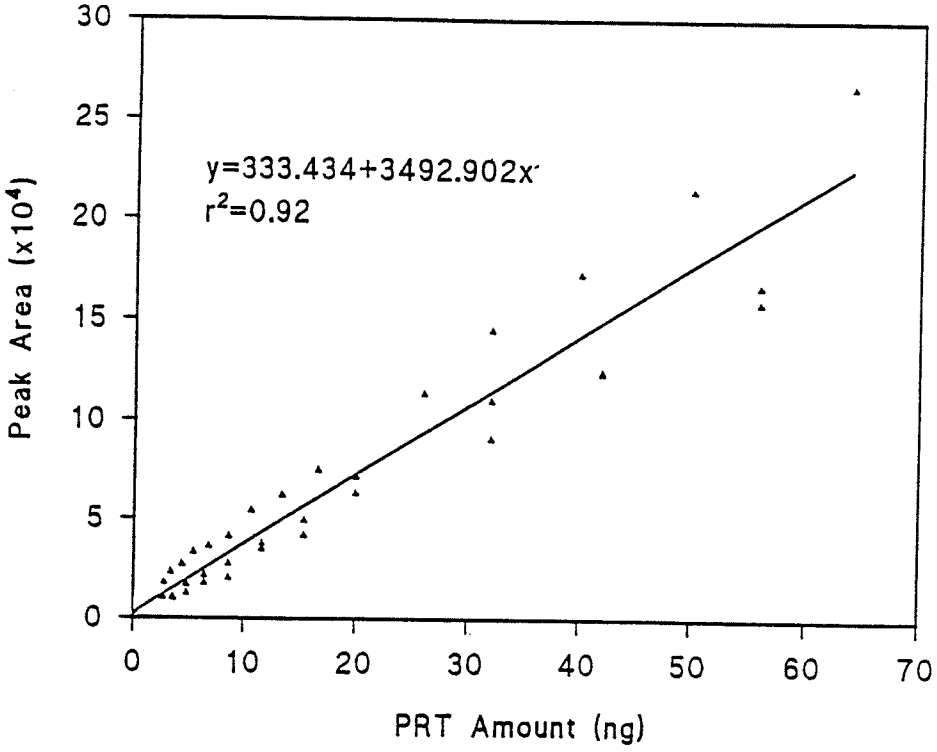
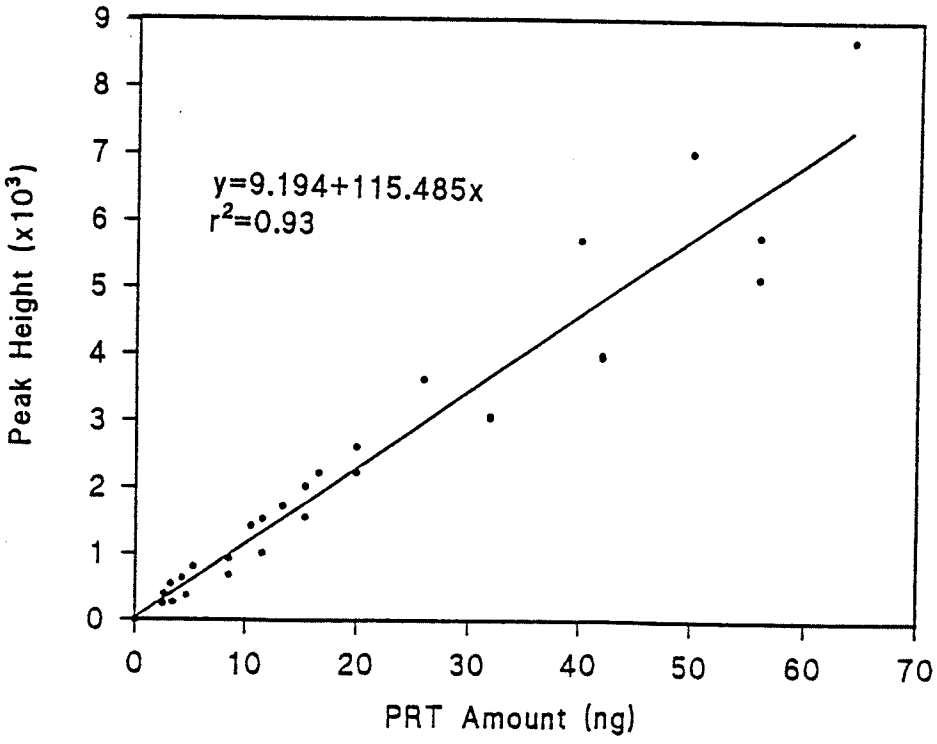
APPENDIX I - Correlation of PRT amount and a) HPLC peak area and b) HPLC peak height. Eluent: methanol-water (70:30, v/v).

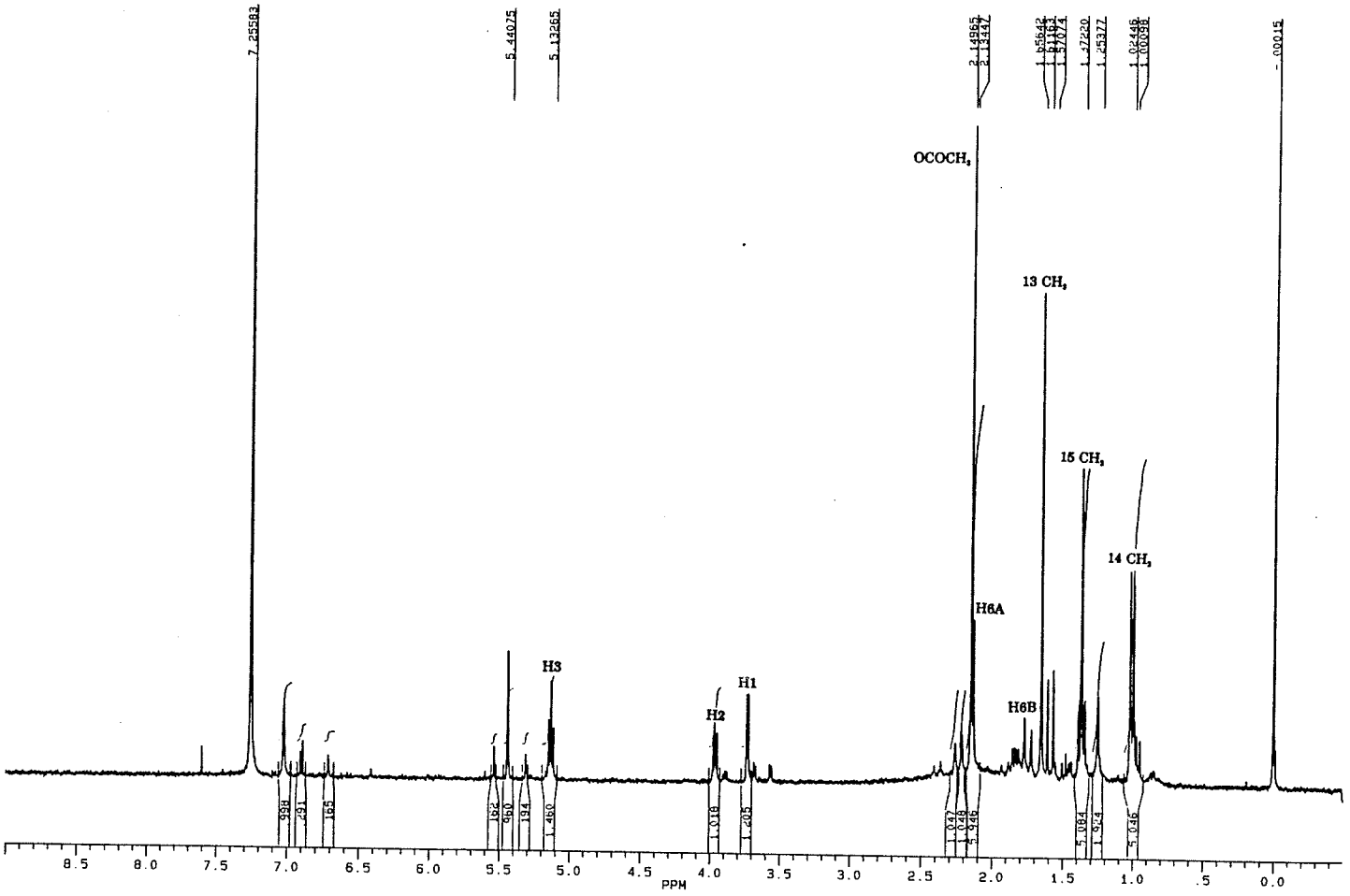
a


y=333.434+3492.902x
r²=0.92
Peak Area (x10⁴)
PRT Amount (ng)
0
5
10
15
20
25
30
0
10
20
30
40
50
60
70

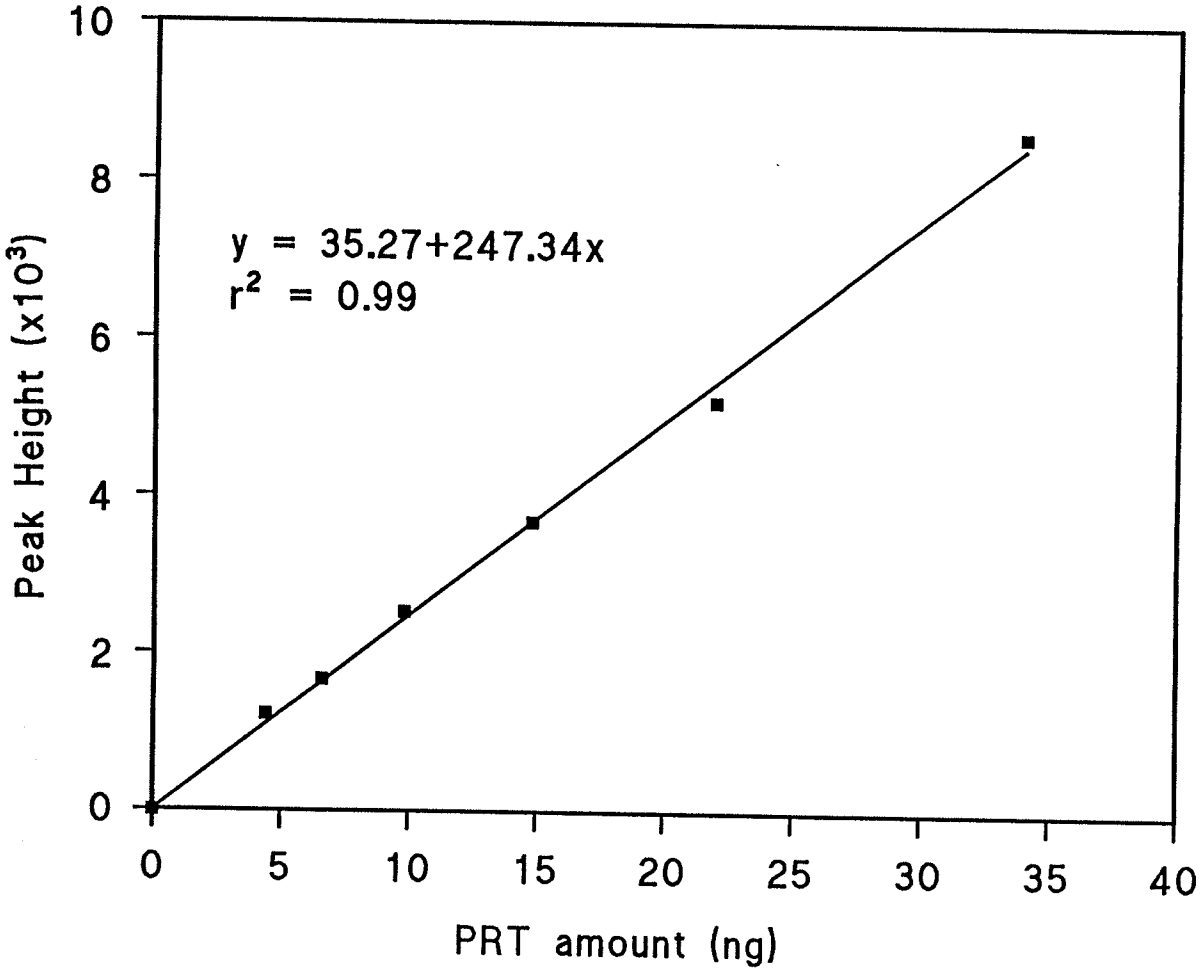
b


y=9.194+115.485x
r²=0.93
Peak Height (x10³)
PRT Amount (ng)
0
1
2
3
4
5
6
7
8
9
0
10
20
30
40
50
60
70

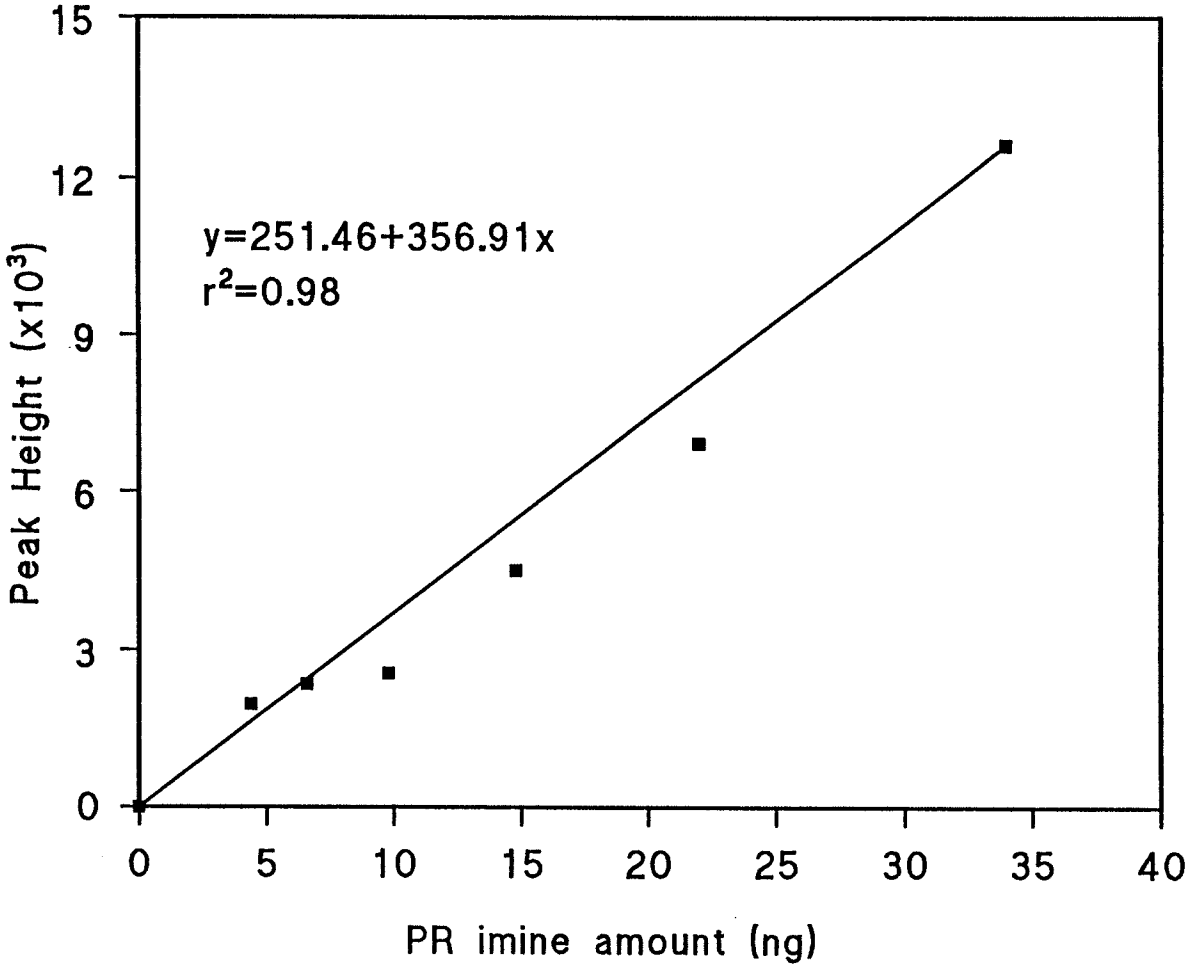
APPENDIX II - The 300 MHz ^1H NMR spectrum of PR imine (in $CDCl_3$). TMS is used as the internal standard.


7.25583
5.44075
5.13265
2.14965
2.13447
1.56442
1.51163
1.47074
1.47220
1.25377
1.02446
1.00098
.00015
OCOCH3
13 CH2
15 CH2
14 CH2
H6A
H6B
H3
H2
H1
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
0.0
PPM

APPENDIX III - Correlation of PRT amount and HPLC peak height. Eluent: acetonitrile-water (60:40, v/v).


y = 35.27+247.34x
r² = 0.99
Peak Height (x10³)
PRT amount (ng)
0
2
4
6
8
10
0
5
10
15
20
25
30
35
40

APPENDIX IV - Correlation of PR imine amount and HPLC peak height. Eluent: acetonitrile-water (60:40, v/v).


15
12
9
6
3
0
Peak Height (x10³)
y=251.46+356.91x
r²=0.98
0
5
10
15
20
25
30
35
40
PR imine amount (ng)