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FORMATION OF N-NITROSOPIPERIDINE IN SAUSAGES

OWNITEE PENPAIN

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
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จาก
บัณฑิตวิทยาลัย มหิดล

IN

FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY

1989

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Thesis

entitled

FORMATION OF N-NITROSOPIPERIDINE

IN SAUSAGES

Ownitee Penpain

Ownitee Penpain
Candidate

Songsak Srianujata, Ph.D.
Major Advisor

Kaew Kangsadalampai, Ph.D.
Co-advisor

Thirayudh Glinsukon, D.Sc.
Co-advisor

M. Chulasamaya

Monthree Chulasamaya,M.D.,Ph.D.
Dean
Faculty of Graduate Studies

Vichai Tanphaichitr

Vichai Tanphaichitr, M.D.,Ph.D.,F.A.C.P.
Director, Graduate Program in Nutrition
Faculty of Medicine, Ramathibodi Hospital

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FORMATION OF N-NITROSOPIPERIDINE
IN SAUSAGES

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for the degree of Master of Science (Nutrition)

on

May 18, 1989

Ownitee Penpain
Ownitee Penpain
Candidate

Songsak Srianujata, Ph.D.
Chairman

Kaew Kangsadalampai, Ph.D.
Member

Thirayudh Glinsukon, D.Sc.
Member

M. Chulasamaya
Monthree Chulasamaya,M.D.,Ph.D.
Dean
Faculty of Graduate Studies

Athasit Vejjajiva
Athasit Vejjajiva, M.B.,F.R.C.P.
Dean
Faculty of Medicien, Ramathibodi Hospital

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BIOGRAPHY

NAME	Ownitee Penpain
DATE OF BIRTH	December 6, 1959
PLACE OF BIRTH	Samutprakan, Thailand
INSTITUTIONS ATTENDED	Faculty of Agriculture Kasetsart University, Bangkok April, 1981. B.Sc. (Home Economics)
GRANT	National Research Council,Thailand

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ชื่อวิทยานิพนธ์ การเกิดเอ็น-ไนโตรโซปิเปอริดีนในไส้กรอก

ผู้วิจัย อรนิธี เพ็ญเพียร

ปริญญา วิทยาศาสตร์มหาบัณฑิต (โภชนศาสตร์)

คณะกรรมการควบคุมวิทยานิพนธ์

ทรงศักดิ์ ศรีอนุชาต Ph.D.

แก้ว กังสดาลอาไพ Ph.D.

ธีระยุทธ กลิ่นสุคนธ์ D.Sc.

วันที่สำเร็จการศึกษา 18 พฤษภาคม พ.ศ. 2532

บทคัดย่อ

การตรวจหาปริมาณเอ็น-ไนโตรซามีนชนิดระเหยได้, โซเดียม-ไนไตรท์, โซเดียมไนเตรท, ปิเปอรีน และ ปิเปอริดีนในไส้กรอกที่มีขายในกรุงเทพมหานครนั้น พบว่าจากไส้กรอก 50 ตัวอย่างตรวจพบเอ็น-ไนโตรซามีน 30 ตัวอย่าง เอ็น-ไนโตรซามีนที่ตรวจพบ ได้แก่ เอ็น-ไนโตรโซปิเปอริดีนและเอ็น-ไนโตรโซไพโรริดีน ปริมาณเอ็น-ไนโตรโซปิเปอริดีนและเอ็น-ไนโตรโซไพโรริดีนที่ตรวจพบอยู่ในช่วง 0.31-45.98 ppb และ 0.32-10.34 ppb ตามลำดับ สำหรับปริมาณโซเดียมไนไตรท์, โซเดียมไนเตรท, ปิเปอรีนและปิเปอริดีนในไส้กรอกมีค่าอยู่ในช่วง 11.74-156.69 ppm, 79.91-309.79 ppm,27.50-200.90 ppm และ 0.02-0.61 ppm ตามลำดับ

ในตัวอย่างไส้กรอกที่ได้ทดลองผลิตขึ้นโดยใส่ไนไตรท์และพริกไทยในปริมาณที่แตกต่างกัน พบว่า ตัวอย่างไส้กรอกที่ผลิตโดยมีไนไตรท์เฉพาะจากผงเพรคและใส่พริกไทยในระดับ 5, 12.5, 25 และ 50 กรัม/กิโลกรัม มีเพียงตัวอย่างเดียวคือใส่พริกไทย 50 กรัม/กิโลกรัม ที่ตรวจพบเอ็น-ไนโตรโซปิเปอริดีนในปริมาณ 1.98 และ 2.90 ppb หลังจากวันที่ผลิต สำหรับตัวอย่างไส้กรอกที่ผลิตขึ้นโดยมีไนไตรท์จากผงเพรคอย่างเดียว กับใส่ไนไตรท์เพิ่มขึ้นอีกในรูปของโซเดียมไนไตรท์ 300 ppm และใส่พริกไทยในปริมาณ 25 และ 50 กรัม/กิโลกรัม; มีการตรวจพบเอ็น-ไนโตรโซปิเปอริดีนในปริมาณ

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3.91,3.96 ppb และ 3.25,5.55 ppbในวันที่ 1 และ 7 หลังจากวันที่ผลิต ผลการทดลองนี้ชี้ให้เห็นว่า การใช้ไนไตรท์ในปริมาณที่สูงควบคู่ไปกับการใช้พริกไทย มีผลทำให้เกิดเอ็น-ไนโตรรซปิเปอริดีนขึ้นได้

นอกจากนี้ ความแตกต่างในการผลิต, พันธุ์ของพริกไทย เวลาและอุณหภูมิในการเก็บก็อาจมีผลต่อปริมาณเอ็น-ไนโตรรซปิเปอริดีนที่ตรวจพบด้วย

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Thesis Title Formation of N-nitrosopiperidine in Sausages

Name Ownitee Penpain

Degree Master of Science (Nutrition)

Thesis Supervisory Committee

Songsak Srianujata, Ph.D.

Kaew Kangsadalampai, Ph.D.

Thirayudh Glinsukon, D.Sc.

Date of Graduation 18 May B.E.2532 (1989)

Abstract

Sausages in Bangkok area were detected for nitrosamines, nitrite, nitrate, piperine and piperidine. From 50 samples of popular brands of sausages analysed 30 samples were positive for nitrosamines, NPIP and NPYR in particular. They were detected in the range of 0.31 to 45.98 ppb and 0.32 to 10.34 ppb respectively. Residual nitrite, nitrate, piperine and piperidine were in the range of 11.74 to 156.69 ppm, 79.91 to 309.79 ppm, 27.50 to 200.90 ppm and 0.02 to 0.61 ppm respectively. Sample sausages were prepared by varying the amount of nitrite and pepper. Sausages prepared with nitrite (normally present in prek powder) and with pepper of 5,12.5, 25 and 50 g/kg, only sample with pepper of 50 g/kg that NPIP was detected 1.98 and 2.90 ppb at day 1 and day 7 respectively. And sausages prepared with nitrite from prek powder and added nitrite in the form of $NaNO_2$ for

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additional 300 ppm, and with pepper 25 and 50 g/kg; NPIP was detected 3.91, 3.96 ppb and 3.25, 5.55 ppb at day 1 and day 7 respectively. It was speculated these experiment that excessive initial levels of nitrite and pepper used could be responsible for high level of NPIP found. Furthermore the differences in processing, origin of pepper, time and temperature of storage could affected in the differences of results found.

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List of Abbreviations

cm	centimetre
^{o}C	degree celsius
DEA	diethylamine
DMA	dimethylamine
DNFB	dinitrofluorobenzene
GC	Gas - Chromatography
HPLC	High Pressure Liquid Chromatography
hr	hour
id	internal diameter
kg	kilogram
l	litre
m	metre
M	Molar
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	milli Molar
mp	melting point
µg	microgram
NDMA	N-nitrosodimethylamine
NDEA	N-nitrosodiethylamine
NDPA	N-nitrosodipropylamine
NMOR	N-nitrosomorpholine
NPIP	N-nitrosopiperidine
NPYR	N-nitrosopyrrolidine

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NO_x	oxide of nitrogen
od	outer diameter
O.D.	Optical density
PIPR	piperine
PIPD	piperidine
PYR	pyrrolidine
ppb	parts per billion
ppm	parts per million
%	percent
S.D.	Standard deviation
TEA	Thermal Energy Analyzer
w	weight
v	volumn
VNAs	Volatile N-nitrosamines

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Chapter I

Introduction

The discovery of carcinogenicity of nitrosamines excited much interest and many reports have appeared concerning the carcinogenic, mutagenic, and toxic characters of nitrosamines. Nitrosamines have been shown to be carcinogenic in a wide range of species and to be capable of inducing tumors at different sites of the body. Of the known nitrosamines NDMA and NPYR are the two compounds which have been most frequently found to be present in cured meat products. Because the widespread use of nitrite as a preservative and color fixation, coupled with the natural occurrence of amines in foods, the extent of these compounds can be determined in cured meat products. As the previous preliminary survey of cured meat product in Thailand, the consistent occurrence of fairly high level of NPIP in suasages made by certain firms in Thailand have been observed (52). This finding seemed unusual as it was the first reported instance of NPIP in sausage made in Thailand. So it is interesting to study the formation of NPIP in sausage because nowaday, sausages become popular due to its high protein and convenience to eat. This study continues to determine the concentration of the important precursors of NPIP which are nitrite, nitrate, piperine, and piperidine and relation in forming NPIP in sausage. The result of this study can be used as a guideline for the prevention and

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reduction of NPIP in sausages. This study will bring benefits to consumers because NPIP is a nitrosamine which have been reported to be carcinogenic although no direct evidence of their carcinogenic effect in man is available. It will be highly desirable to maintain the concentration of this chemical in food at the lowest possible level.

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Chapter II

Purpose of this study

The aim of this study is firstly, to measure the level of NPIP in five popular brands of sausages produced in Thailand. Secondly, to study the formation of NPIP by measuring the level of residual nitrite, nitrate, piperine and piperidine. Then thirdly, to study the effect of precursors of NPIP in sausages. Sausages are prepared with and without nitrite, and also with and without pepper which are the origin of piperine and piperidine.

Specific Objective.

1. determine the level of residual nitrite, nitrate, VNAs, piperine and piperidine in five popular brands of sausages which produced in Thailand.

2. study the relationship between the amount of residual nitrite, nitrate, piperine and piperidine on NPIP formation.

3. study the added precursors of NPIP by processing sausages by varying the amount of nitrite and pepper.

4. study the effect of storage on NPIP formation by processed sausages.

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Chapter III

Literature Reviews

N-nitroso compounds are simple derivatives of secondary amines and have been known for more than 100 years, although their toxicological properties were not described until 1954. Magee and Barnes described liver necrosis induced in rats by exposure to high doses of dimethylnitrosamine. Later, long-term chronic feeding of the same compound to rats gave rise to liver tumors(1,2). Since N-nitroso compounds are easily prepared by reaction of secondary or tertiary amino compounds with nitrous acid, there is almost no limit to the variety of these compounds have been synthesized and tested by long-term administration of small doses to rats, mainly by Druckrey *et al.* and by Lijinsky and colleagues, as summerized in Lijinsky(3). The number of these compounds tested now exceeds 120, of which three-quarters were found to be carcinogenic, some very potently so. Most of these tests have been carried out in rats, but two compounds, dimethylnitrosamine and diethylnitrosamine, have been tested in 20 species or more and have induced tumors in all of them. It is improbable that any species is refractory to N-nitroso compounds. The information available about the carcinogenicity of N-nitroso compounds suggests that they are most effective by the oral route. In the mid-1960s, it became apparent that some of these compounds could exist in the human food

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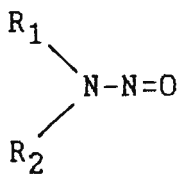
supply, and in the mid-1970s, this observation was extended to include the human work place and urban environment. Today, N-nitroso compounds are recognized as pollutants of specialized environments and occuring at low levels; precursors of N-nitroso compounds occur for more extensively and at greater concentrations and lead to the formation of N-nitroso compounds in the air, water, soil, food, and human GI tract.

1. Chemistry

1.1 Structure

N-nitroso compounds are divided into the nitrosamines, derived from dialkyl, alkaryl, diaryl, or cyclic secondary amines; and the nitrosamides, derived from N-alkylureas, N-alkylcarbamates and simple N-alkylamides. Most tested nitrosamines and nitrosamides have proved to be strong carcinogens(4).

The general formula of nitrosamines can be described as follows:



R_1 and R_2 can be either alkyl or aryl groups or, in some cases, alicyclic. For certain cyclic nitrosamines R_1 and R_2 can be replaced by a cyclic ring

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as in the case of nitrosopiperidine and nitroso-pyrroridine (Fig. 1). Besides the nitrosamines there are many other carcinogenic N-nitroso compounds which could be present or formed in food. These include nitroso sarcosine, a nitroso amino acid, nitrosomethylurethane and many others. The structural formulas of several common N-nitroso compounds are presented in Fig 1.

1.2 General Properties and Reactions.

N-nitrosamines are characteristically yellow, orange-yellow liquids or solids at room temperature. The only common feature of all nitrosamines is the presence of the N-N=O functional group. Consequently a wide range of chemical and physical properties exist for the various nitrosamines depending on the substituents on the amine nitrogen. The simple low molecular weight dialkyl-nitrosamines such as dimethylnitrosamine are liquide which are water soluble. Many of the higher molecular weight nitrosamines are soluble in organic solvents and in food liquids. Nitrosoproline is a solid at room temperature and is water soluble.

Nitrosamines are generally formed in the laboratory by the reaction of nitrous acid with the corresponding secondary amines. Pensabene *et al.*(5) compared the preparation of nitrosamines from secondary amines by reaction with sodium nitrite and nitrosyl tetrafluoroborate. The authors prefered nitrosyl tetrafluoroborate with ether-soluble amine as they

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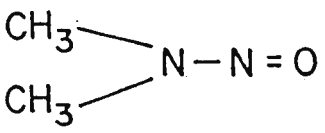
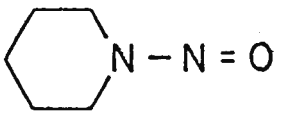
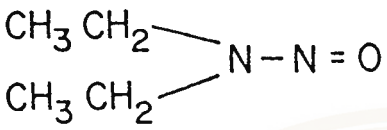


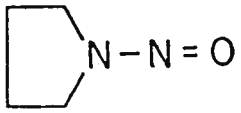
N – nitrosodimethylamine



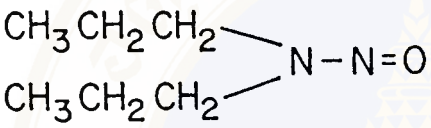
N – nitrosopiperidine



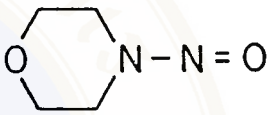
N – nitrosodiethylamine



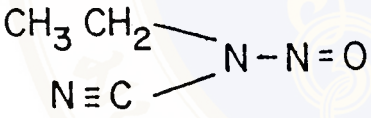
N – nitrosopyrrolidine



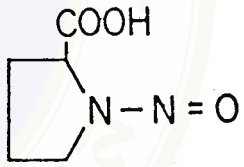
N – nitrosodipropylamine



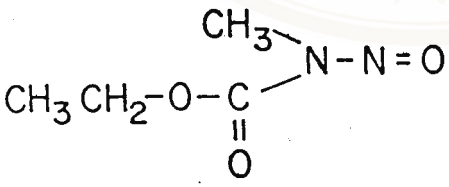
N – nitrosomorpholine



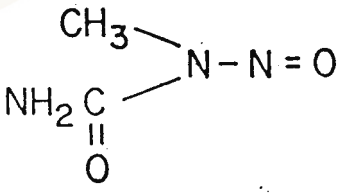
Ethylnitrosocyanamide



N – nitrosoproline



Methylnitrosourethane



Methylnitrosourea

Figure 1 Structures of vairous N-nitroso compounds.

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claimed the reaction proceeded without the formation of secondary reaction products. N-nitrosamide have commonly been synthesized by reaction the corresponding amide with nitrous acid. The formation of N-nitroso-N-methylurea, one of the more widely studied N-nitrosamides, is illustrated in Fig. 2.

Nitrosamines are generally considered a more stable class of compounds than N-nitrosamides. Nitrosamines are sensitive to ultraviolet radiation which splits the nitroso group. The cleavage of nitrosamines can also be accomplished by strong acid. Nitrosamines can be either oxidized or reduced by the appropriate reagents. Both lithium aluminum hydride and diborate have been used to reduce nitrosamines to be corresponding hydrazines. Peroxytrifluoroacetic acid has been used to oxidize nitrosamine to nitramine. Under the influence of acid, aryl-N-nitrosamines undergo the Fisher-Hepp rearrangement and the nitroso group migrates from the nitrogen to the para position of the aromatic ring(6).

2. Formation of N-Nitroso Compounds.

Nitrosamines are formed by chemical reactions between nitrosating agents and nitrosatable amines. The chemistry of N-nitrosation has been studied extensively. For nitrosation to occur, nitrite is usually first converted to nitrous acid. The HNO_2 is then converted to an active nitrosating species, e.g., nitrous anhydride(N_2O_3), nitrosyl thyocyanate(ON-NCS), nitrosyl-

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halinde (NOX),or nitrous acidium ion ($H_2NO_2^+$)(4). In acid solution, nitrite is converted to nitrous acid which then forms nitrous anhydride as follows:

$$NO_2^- + H^+ \longrightarrow HNO_2$$

$$2HNO_2 \rightleftharpoons N_2O_3 + H_2O$$

When secondary amines are treated with nitrous acid, nitrosation occurs and a nitrosamine is formed as illustrated in step a, (Fig. 3). Since nitrosamines from secondary amines are quite stable, the reaction goes on further and the nitrosamine can be considered a final product. When a primary aromatic amine is treated with nitrous acid, and unstable nitrosamine formed which tautomerizes to diazohydroxide (step b, Fig.3). The same reactions occur with aliphatic primary amines, but the unstable diazonium ion loses nitrogen to form a carbonium ion as shown in step c, Fig.3. The carbonium ion can undergo addition, elimination, and rearrangement reactions(step e, Fig.3) to produce a variety of deamination products.

The subject of nitrosamine formation in foods relates more to the reactions of nitrous acid with secondary rather than primary amines. However, since the nitrosation step (step a, Fig.3) is the rate controlling step in the sequence of reactions of nitrous acid with primary and secondary amines, much of the kinetic and

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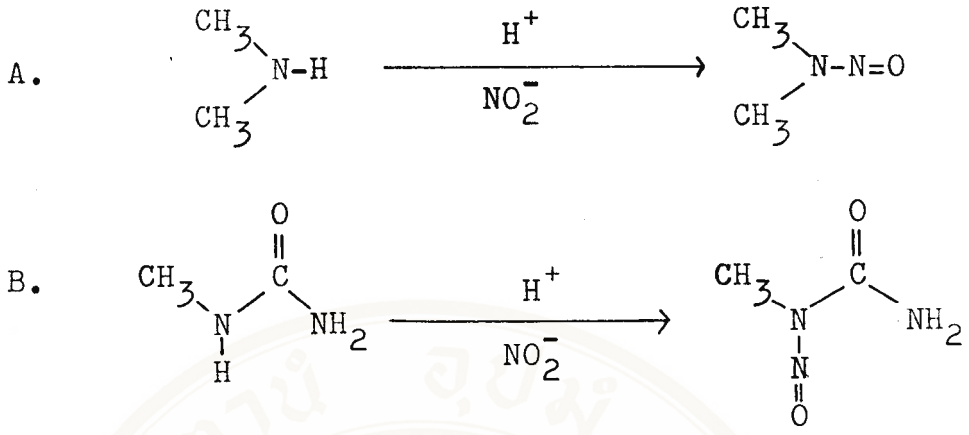

Figure 2 Example of nitrosamine (A) and nitrosamide (B) formation.

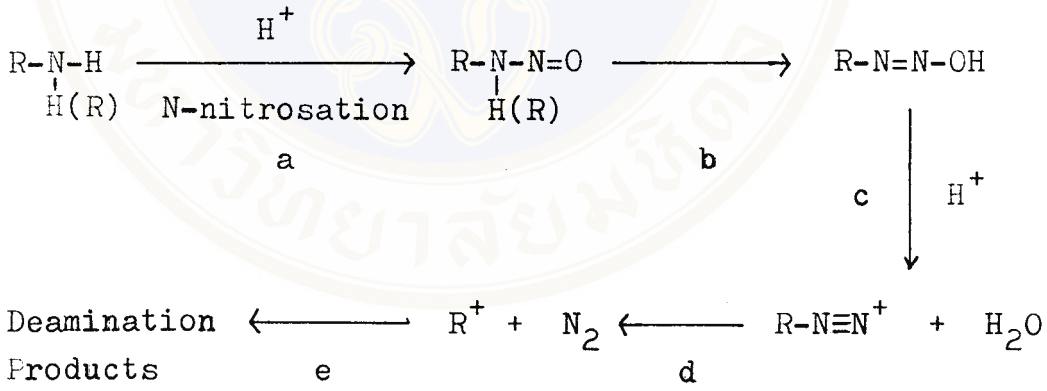

Figure 3 Reaction sequence in nitrosation, diazotization and deamination.

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mechanistic information, which has been gathered from diazotization and deamination studies of primary amines, applies to nitrosation reactions for secondary amines, Kalatzis and Ridd(7) established that the same rate equation and similar rate apply to both the nitrosamine formation from N-methylaniline and to the diazotization of amine. The rate equation was second order in terms of nitrous acid concentration and first order in terms of amine concentration as shown in Equation 1.

$$\text{Rate} = k(HNO_2)^2 \text{ (amine)} \qquad (1)$$

Nitrous anhydride was formed in a rapid equilibrium reaction and then was slowly attacted by the unprotonated amine. The reaction.scheme illustrated in Fig.4 is consistent with the kinetics which has been observed for secondry amines.

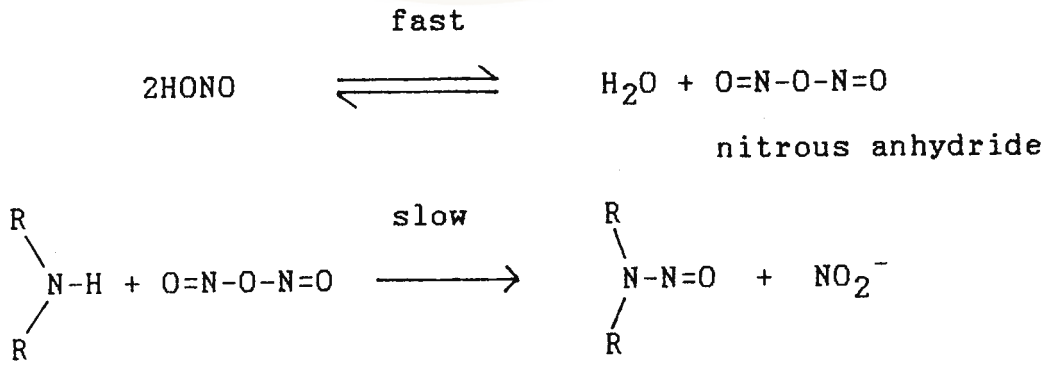

Figure 4 Formation of nitrous anhydride and subsequent nitrosation of secondary amine.

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Equation 1 describes the relationship between the rate of nitrosation and the concentration of reactions for the nitrosation of secondary amines. Since it is the free amine and not the protonated amine that is nitrosated, both pH and amine basicity will influence nitrosamine formation. Nitrous acid is weak acid (pK_a=3.35),pH will influence the actual concentration of nitrous acid and nitrite ion in a nitrosating system. Mirvish(7) studied extensively the relationships between pH, amine basicity, and rate of nitrosation. A summary of some of Mirvish's finding is shown in Table 1 and a curve depicting the pH optimum for the nitrosation of dimethylamine is shown in Fig. 5. The kinetics of the nitrosation of the five amines in Table 1 followed Equation 1 at the optimum pH which was about 3.0. At pH 3.0, the rate constants increased 1.85×10^5 times on proceeding from the weakly basic piperidine to the strongly basic piperazine. These results confirmed the finding of Sander *et al.* (9) regarding the inverse relationship between amine basicity and case of nitrosation.

Nitrous anhydride is not the only nitrosating species that needs to be considered in the nitrosation of amines, particulary in complex biological media such as food or gastric juice. Fig. 6 shown some of the equilibrium reactions of the nitrite ion in aqueous solution. Nitrite ion is in equilibrium with nitrous acid (pK_a=3.35).

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Table 1 Optima pH and rate constants for the nitrosation of some secondary amines

Amine	pK_a	Optimum pH	Rate Constant[a,b]
Piperidine	11.2	3.0	0.027
Dimethylamine	10.72	3.4	0.10
Morpholine	8.7	3.0	14.8
Mononitrosopiperazine	6.8	3.0	400.0
Piperazine	5.57	3.0	5000.0

a: Values at the optimum pH, in $mole^{-2}l^2min^{-1}$.

b: Rate constant for equation 1 where concentration referes to total concentrations of amine and nitrite.

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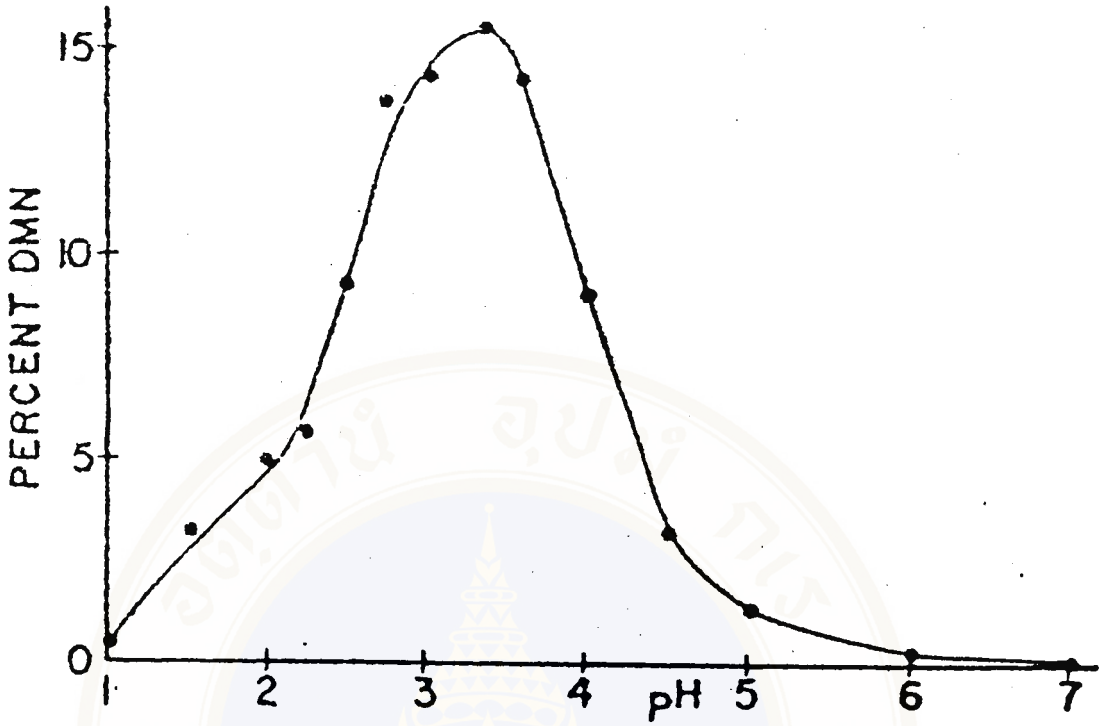

Figure 5 The curve shown pH optimum for the nitrosation of dimethylamine.

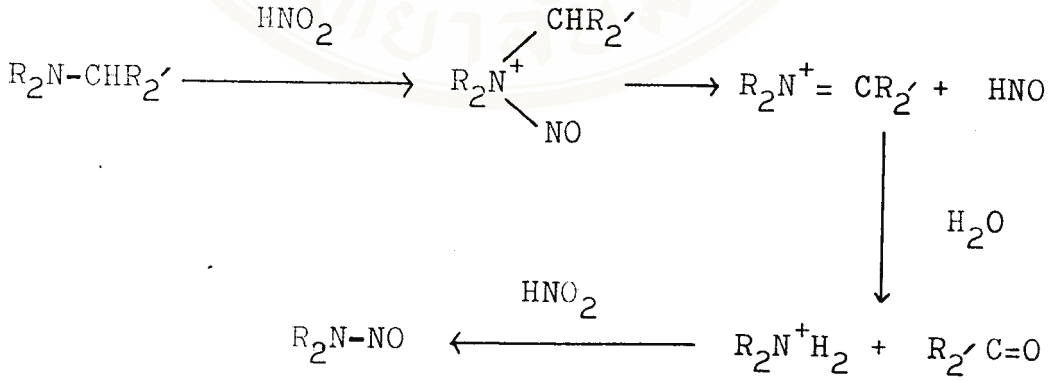

Figure 7 Proposed mechanism for the conversion of tertiary amines to nitrosamines.

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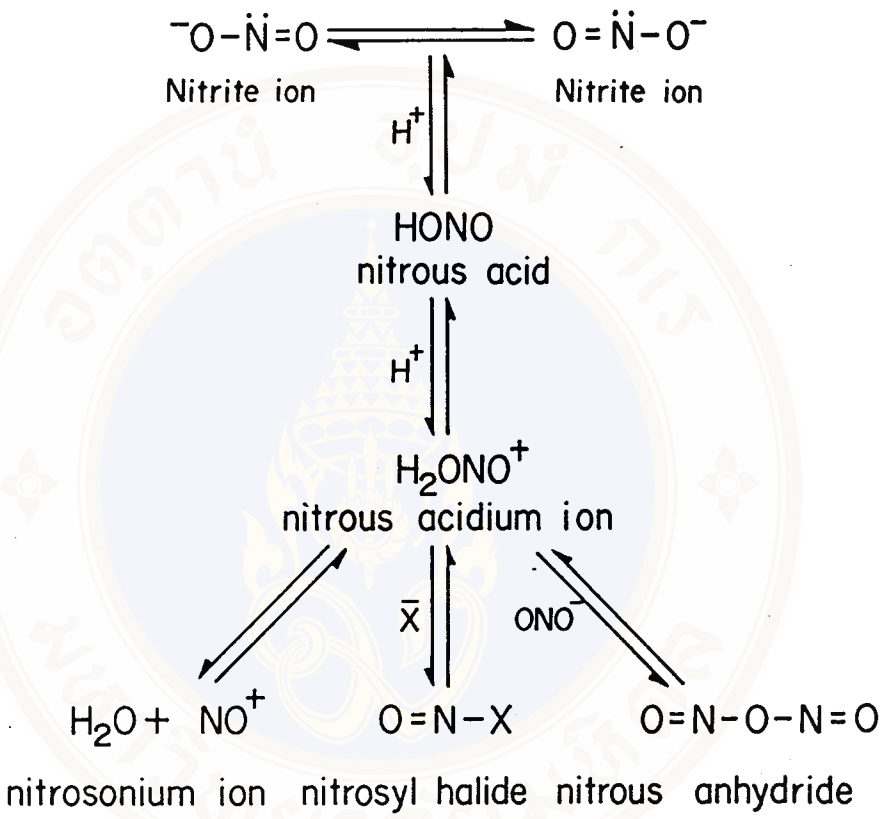

Figure 6 Some equilibria of nitrite ion in aqueous solution.

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Although the most common method for the preparation of nitrosamines is by the reaction of secondary amines with nitrite, nitrosamines can also be formed from tertiary amines, quaternary ammonium compounds, and primary amines. The controversial literature regarding the reaction of tertiary amines with nitrous acid spans back more than 100 years(6). The main products of the nitrosative cleavage of tertiary amines by nitrous acid include a carbonyl compound, a nitrosamine, and nitrous acid. Smith and Loeppky(10) after considering several possible mechanisms for the tertiary amines with nitrous acid, proposed the scheme shown in Fig. 7. The first step for the nitrosation of tertiary amines is similar to the reaction path for primary or secondary amines in which the unshared electron pair on the unprotonated amine reacts with a nitrosating species (i.e., nitrous anhydride). The nitrosoammonium ion first formed undergoes cis elimination of nitroxyl to form and imonium ion which is hydrolyzed to a carbonyl and a secondary amine. The secondary amine can then be nitrosated to the coressponding nitrosamine.

In general the formation of nitrosamines for tertiary amines is less efficient than from secondary amines when working with approximately equel concentrations of amine and nitrite.

Many investigators have used elevated temperatures (37 to 100 ^{o}C) for the formation of

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nitrosamines from tertiary amines. It has been demonstrated(11), however, that trimethylamine will form dimethylnitrosamine in low yields at room temperature.

Scanlan(11) working with trimethylamine demonstrated that the pH optimum for the conversion to the corresponding nitrosamines was approximately pH 3.3. At present it is still unconclusive whether tertiary amines in foods serve as precursors to nitrosamines. *In vivo* experiments have failed to demonstrate toxic effects from feeding nitrite with tertiary amines. The addition of 0.5% trimethylamine and 0.5% nitrite to diet of rats did not cause any toxic effects(12). These results, of course, do not preclude the possibility of nitrosamine formation in foods from tertiary amines.

The reaction of primary aliphatic amines with nitrous acid principally produces compounds other than nitrosamines. Nitrosamines, in low yields, are produced from the nitrosation of primary aliphatic amines. Dipropylnitrosamine was reported as a product of propylamine and nitrous acid. The yield of nitrosamine from primary aliphatic amines is low, particulary in solution. The yield of nitrosamines from some of the compounds investigated are shown in Table 2 . The formation of a stable (secondary) nitrosamines from a primary amine apparently requires conversion of the primary amine to a secondary amine which can then be nitrosated to the nitrosamine. A pathway proposing to essential steps involved for aliphatic primary monoamines

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Table 2 Nitrosamine produced from the reaction of nitrite with primary amines, diamine and diamino acids in aqueous, buffer solution.

Amine	$NaNO_2$	Reaction condition	Nitrosamine	Percent Yield
Butylamine[a]	1.00M	pH 3.4 100°C,1hr	Dimethylnitrosamine	0.01
Putrescine[b]	0.05M	pH 3.8 100°C,1hr	Nitrosopyrrolidine	2.8
Putrescine[b]	0.25M	pH3.4 22°C,6days	Nitrosopyrrolidine	9.2
Lysine[b]	0.05M	pH3.8 100°C,1hr	Nitrosopipecolic acid	0.5
Lysine[b]	0.25M	pH3.8 22°C,6days	Nitrosopipecolic acid	0.3

a: Concentration = 0.5M.

b: Concentration = 0.025M.

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in Fig. 8. Formation of secondary amines from aliphatic primary monoamines involves dimerization in which the unshared electrons on an unreacted amine nitrogen undergo electrophilic attack by the carbonium ion(13). The necessity for the reaction of these two species to form the secondary amine accounts for the low yields of nitrosamines from aliphatic primary monoamines in solution.

The formation of secondary amines from four or five carbon primary diamines or diamino acids involves cyclization to five or six membered hetrocyclic compound as shown in Fig. 9 .

Based on the low yield of nitrosamines from aliphatic primary monoamines, formation of significant amounts of nitrosamines in foods from this source seems very unlikely. On the other hand primary diamines and possibly diamino acids should be considered potential precursors to nitrosamines, particulary in foods exposed to high temperature. Lijinsky and Epstein(14), called attention to the possibility of pustrescine and cadavarine forming cyclic nitrosatable secondary amines during the cooking of meat and fish.

In biological materials, the polyamines, spermine and spermidine, are widely occurring and contain both primary and secondary amino functions. Hildrum's study(6) demonstrated the potential complexity of several aspects of the nitrosamine formation in food problems. From a single compound plus nitrite at least five

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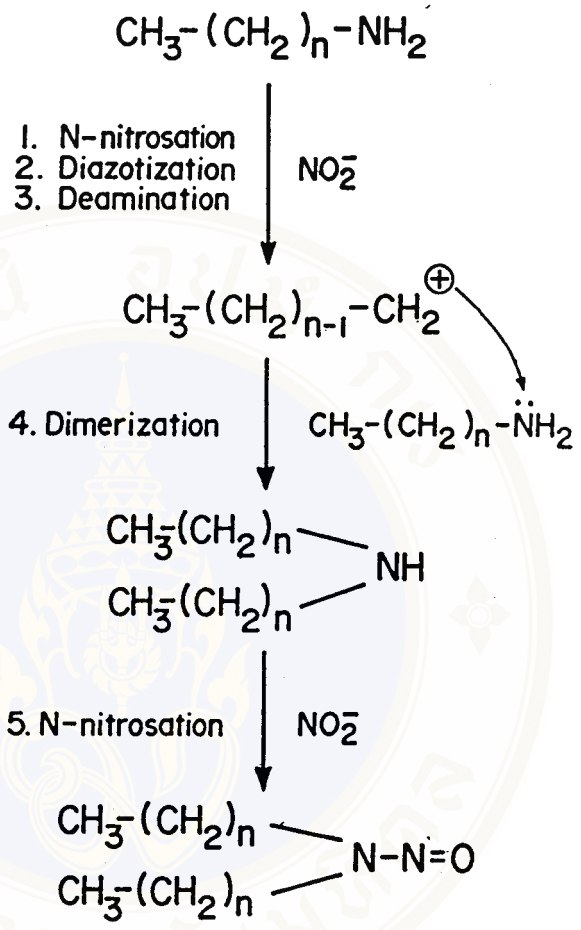

Figure 8 Proposed pathway for nitrosamine formation from primary monoamines.

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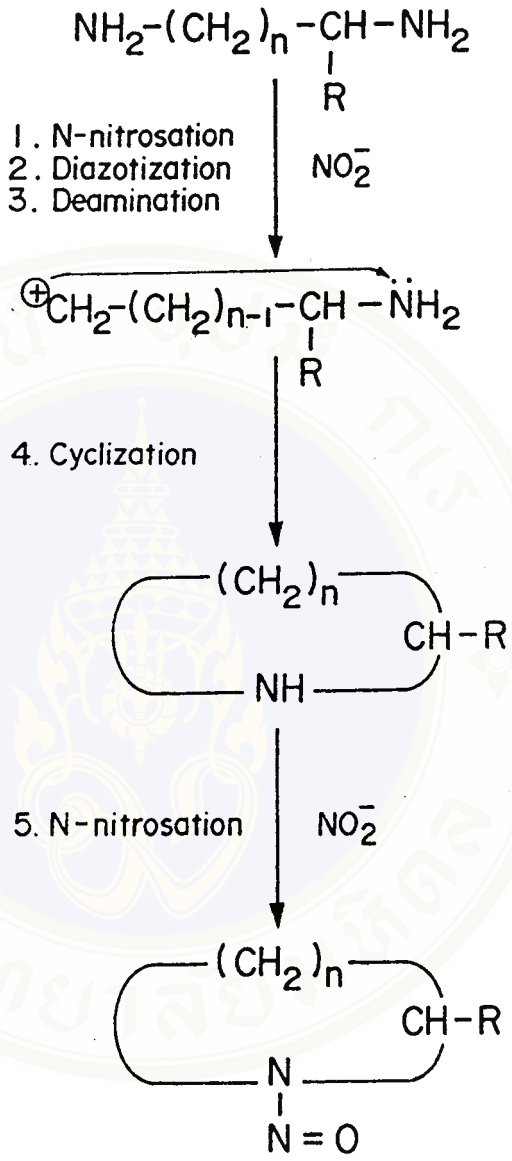

igure 9 Proposed pathway for heterocyclic nitrosamine formation from primary diamines and diaminoacids.

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different nitrosamines were formed. This was probably a minimal number. In fact, assuming the likely possibility of rearrangment to a secondary carbonium ion, 36 combinations of unsaturated , chlorinated and / or hydroxylated nitrosamines are theoretically possible from spermidine. Obviously, establishing the toxicological properties and analytical procedures for this number of nitrosamines would be a formidable task(6).

In addition to three classes of amines discussed thus for, nitrosamines can be formed from the reaction of quaternary ammonium compounds though in rather low yield. Fiddler *et al.*(15) , demonstrated the formation of dimethylnitrosamine from the nitrosation of several quaternary ammonium compounds, in pH 5.6 buffer for 4 hours at 78°C. Tetramethylammonium chloride, the quaternary ammonium compound that formed the most nitrosamine, produced approximately 6% as much dimethylnitrosamine as was formed an equal amount of dimethylamine. Dimethylnitrosamine, diethylnitrosamine, and dipropylnitrosamine (predominately dimethylnitrosamine) were reportedly formed by boiling lecitin with nitrite in buffer solutions (pH 3.5 to 7.0). The highest yields were obtained at pH 3.5. It is not surprising that dimethylnitrosamine would be formed under the condition described; however considering the structure of lecithin, it was not clear how either diethylnitrosamine or dipropylnitrosamine would be formed(16).

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The formation of N-nitrosamides is slightly different as the principal nitrosating agent appears to be the nitrous acidinium ion ($H_2NO_2^+$); this ion also forms in acid solution of nitrite.

$$NO_2^- + H^+ \longrightarrow HNO_2$$

$$HNO_2 + H^+ \longrightarrow H_2NO_2^+$$

$$RNH.CO.R' + H_2NO_2^+ \longrightarrow RN(NO).CO.R' + H_2O + H^+$$

The nitrosation reaction rate does not show a pH maximum and is proportional to the hydrogen ion concentration and the nitrite concentration (not the square of the nitrite concentration)(4).

$$Rate = k_1(amides)(nitrite)(H^+)$$

3. Accelerators and Inhibitors.

Nitrosation can be influenced by a wide range of accelelators and inhibitors(17). A number of anionic or nucleophilic species form effective nitrosating agents when they are present with nitrite in aqueous, acidic solution. Examples are nitrosyl thiocyanate (SCO-NO) and nitrosyl iodine (I-NO). Undoubtedly, food constituents and the physical make up of foods influence nitrosation. A relatively large number of phenolic compounds are known to exist in foods. Initially it was proposed, that

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phenols would reduce the nitrosation of amines in their present by acting as nitrite scavengers(18). Since many foodstuffs contain phenolic bodies such as tyrosine, it was considered likely that their present would reduce the nitrosation of secondary amines in the stomach. This view was rainforced by the difficultly experienced in the determination of nitrite by diazotisation in the present of tannins(19). More recently however, it has been reported that oxidisable phenols such as chorogenic acid can stimulate markedly the formation of nitrosopiperidine from its parent amines(20),although lately some doubt has been cast on the validity of the observation. Many foods contain comparatively hight concentrations of this and related phenols. Gallic acid has also been found to catalyze nitrosation but only around pH 4 (21). However, the situation within the human stomach so far as nitrosation is concerned is far more complex than the model systems in which the actions of phenol have been evaluated, thus the outcome of their present is very difficult of predict. Some carbonyl compounds catalyze nitrosamine formation, particularly under neutral and basic conditions(22). Enhanced nitrosation of lipophilic secondary amines has been demonstrated in aqueous systems containing micelles(23).

Important in the inhibition of nitrosamine formation in foods are ascorbic acid, α- tocopherol, and sulfer dioxide. It is now required to add ascorbic acid (550 mg/kg) in the manufacture of all bacon in the

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United States. Presumably, ascorbic acid reduces nitrous anhydride to nitric oxide, a non-nitrosating species in the absence of catalysts (Equation 1).

$$\text{Ascorbic acid} + N_2O_3 \rightarrow \text{dehydroascorbicacid} + 2NO + H_2O \quad (1)$$

It has been known for sometime that nitroso-pyrrolidine formation in cooked bacon is associated with the lipid portion of bacon. Presumably, the insolubility of ascorbic acid in the lipid phase has limited the effectiveness of ascorbic acid as a nitrosation inhibitor in that product. More recently, α-tocopherol, presumably due to its lipid solubility, has been proposed for use in combination with ascorbic acid for nitrosamine inhibition in bacon (24,25). α- Tocopherol acts like ascorbic acid in that the α- tocopherol is oxidized to α- tocoquinone while nitrous anhydride is reduced to nitric oxide (26). For ascorbic acid or α- tocopherol to be effective, they must be added in excess since nitric oxide can be oxidized to nitrogen dioxide, and these 2 species can combine to reform nitrous anhydride (Equation 2).

$$NO + NO_2 \rightleftharpoons N_2O_3 \quad (2)$$

It is important to consider the effect of sodium chloride on nitrosation, since sodium chloride is added with sodium nitrite to most cured meat products.

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Hildrum *et al.*(27). demonstrated that whether an accelerating or an inhibitory effect by chloride will be observed depends on acidity. Working with the nitrosation of proline, those investigators found a definite accelerating effect by chloride at pH 0.5, a slightly inhibitory effect at pH 2.5, and definte inhibitory effects at pH 4.0 and 5.5. Since the pH of cured meat products to which nitrite and chloride are added is above pH 4.0, one would expect chloride to inhibit nitrosamine formation in these products.

4. Toxicity and Carcinogenicity of N-Nitroso Compounds.

N-nitroso compounds have been shown to be toxic, carcinogenic, teratogenic, and mutagenic. The biologocal effect which concerns humans the most is their carcinogenicity. Toxicity of N-nitroso compounds vary considerably according to their structures. Some of them can be considered highly toxic compounds showing low LD_{50}(Table 3), for example, N-nitrosodimethylamine. While many others may be many folds less in acute toxicity. Their carcinogenic activities have been studied quite extensively and shown to be carcinogenic in variety of animals(28).

Since practically all the data concerning the biological effects of N-nitroso compounds have been gathered using experimental animals one wonders whether nitrosamines are indeed carcinogenic to man. This is not an easy question to answer. Indirect evidence has been

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Table 3. Acute toxicity of some N-ntroso compounds(29).

Coumpound	LD_{50}[a] (mg/kg)
Dimethylnitrosamine	27-41
Diethylnitrosamine	216
Di-n-propylnitrosamine	>400[b]
Di-n-buthylnitrosamine	1200
Methylbenzylnitrosamine	18
Nitrosomorpholine	282
Nitrosopyrrolidine	900
Nitrosopiperidine	200
Nitrosodipropylamine	216

a: single oral dose,adult male rats.

b: Male and female Syrian golden hamsters.

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reported which suggests these compounds would also be active to man. Many studies have shown that N-nitroso compounds are a versatile class of carcinogens. Varions N-nitroso compounds have induced tumors in practically all the organ of various experimental animals including the liver, lung, esophagus, urinary bladder, stomach, small intestine, brain and nervous systems. The organ specificity depends on chemical structure of the N-nitroso compounds, dose, route of application, and animal species.

A number of reports indicate that nitrosamines, as such, are not actively carcinogenic, but are metabolized to an active carcinogen *in vivo*. Similarities exist in the metabolism of nitrosamines by human and animal livers. The mechanism of carcinogenic activity is believed to cause by the formation of strong alkylating agent called alkyl cation(RCH_2^+). The schematic reaction that may occur are shown in Fig.10 (30). For nitrosamines it is activated by microsomal mixed function oxidase enzymes to form α-C-hydroxyl compounds which behave like nitrosamide. The hydrolysis occurs with some further reaction to form alkyl cation that alkylates biological macromolecules, DNA and RNA as examples. It is quite possible with some evidences that site of tumor formation depends upon the rate of the formation of alkyl cation in the body. Some amide as nitrosomethylcyanamide produced methyl cation quickly in stomach. While some other compounds may have half life in miniute, so it can

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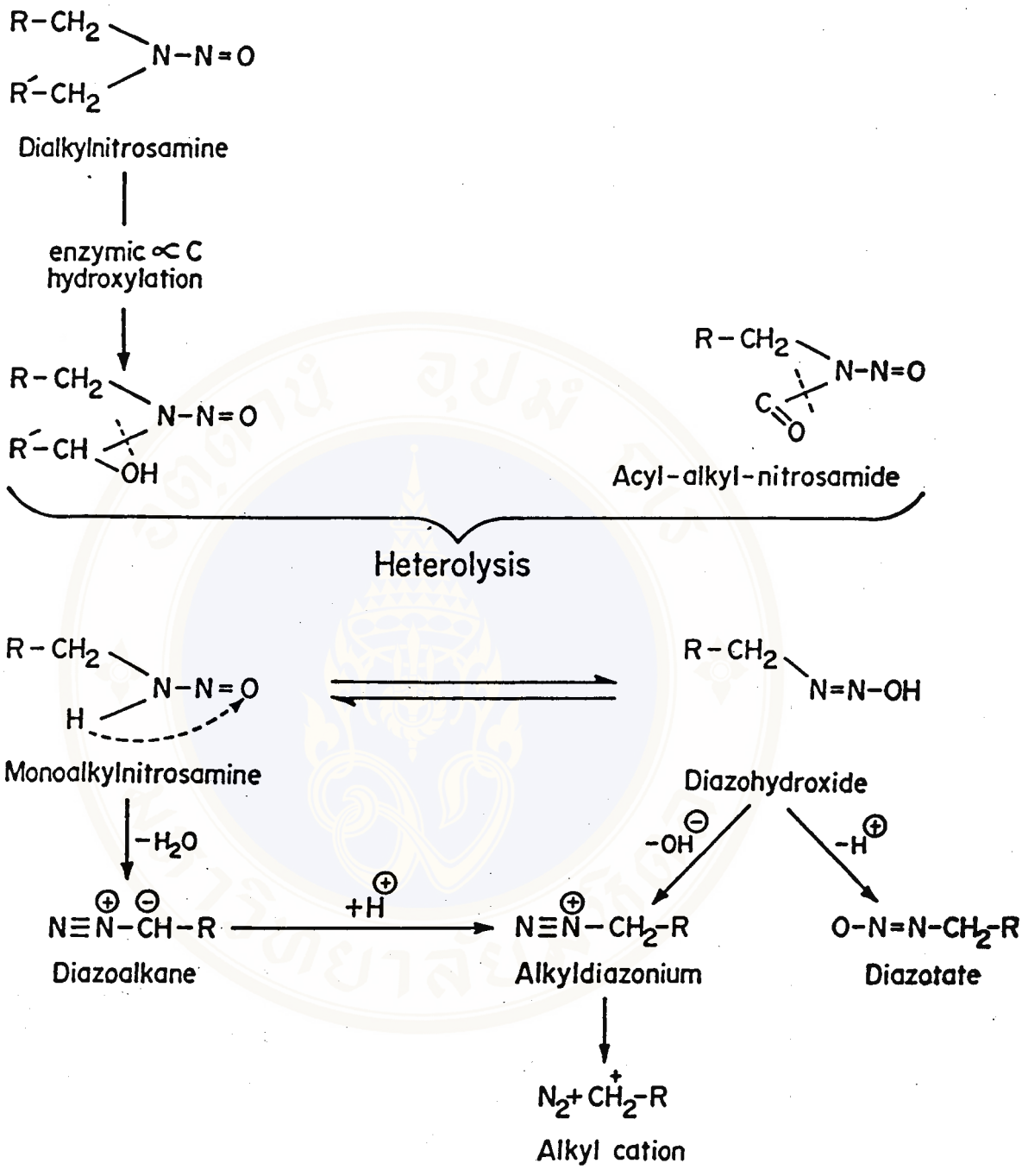

Figue 10 Proposed mechanism for the formation of alkylation intermediates from dialkylnitrosamines and acylalkylnitrosamide.

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traval away from the site of absorption, even to produce tumor in the brain as methylnitrosourea. Some examples of carcinogenic N-nitroso compounds and key organ affected in rodent are shown in Table 4. Montesano and Magee(31) studied the *in vitro* metabolism of dimethylnitrosamine by human and rat livers. They observed comparable rate of metabolism and similar levels of nucleic acid methylation in the two species. This provides one more piece of indirect evidence which suggests that man would respond in the same way as other species to nitrosamines. Clearly, man would have to be the exception to the rule to be exempt from the carcinogenic effect of nitrosamines.

5. Occurrence of N-Nitroso Compounds in Foods and Foodstuffs.

The occurrence of nitrosamines in a wide variety of food products was reported. The review by Shank(32) cites many of the earlier reports. A summary of some of the more recent investigations and the amounts of dimethylnitrosamine, diethylnitrosamine, nitrosopyrrolidine, and nitrosopiperidine found invarious foods and foodstuffs are summerized in Table 5 (32).

5.1 Cured Meats.

Cured meats have received considerable attention since sodium nitrite, and sometimes sodium nitrate is added in the manufacture of these products. Of

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Table 4. Tumor sites of some N-nitroso compounds(29).

Site	Compounds
Skin	Methylnitrosourea
Nose	Diethylnitrosamine
Nasal sinus	Nitrosopiperidine
Tongue	Nitrosopiperidine
Esophagus	Nitrosopiperidine
Stomach	Nitrosopiperidine
Duodenum	Methylnitrosourea
Colon	Cycasin
Lung	Diethylnitrosamine
Bronchi	Diethylnitrosamine
Liver	Dimethylnitrosamine
Pancreas	Nitrosomethylurethane
Kidney	Dimethylnitrosamine
Urinary bladder	Dibuthylnitrosamine
Brain	Methylnitrosourea
Spinal cord	Nitrosotrimethylurea
Thymus	Nitrosobutylurea
Lymph nodes	Ethylnitrosourea
Blood vassels	Nitrosomorpholine

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Table 5. Carcinogenic N-nitroso compounds in human foods and foodstuffs.

	nitrosamine concentration (μg/kg[a])			
	NDMA	NPYR	NDEA	NPIP
Food product				
Bacon	1-4	up to 40	1	
Uncooked	(1/8) 30	10-108		
Fried	(6/8) 2-5	(7/8) 4-25		
Fried			1.5	
Fried	(50/50)up to 5	(50/50)1-200		(50/50)0.08-0.25
Ham	5			
Frankfurter	(3/34) 11-84			
Salami	20-80			
Dry sausage	10-20			
Souse	(6/7) 3-63	(1/7) 19		
Cured meats	(29/80) 2-35	(17/80)13-105	(9/80)2-25	
Salami Hungarian	1-4			
Mettwurst sausage		105		20-60
Luncheon meat	1-4			
Pork,Danish	1-4			
Meat curing mix	850	2500-6000		7000-25000
Fish,fried	up to 1	(1/70) 0.01		
Haddock,fried	1-9			

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	nitrosamine concentration (μg/kg[a])			
	NDMA	NPYR	NDEA	NPIP
Food product				
Cod	1	6	1.5	
Yellow croaker	10-60			
White herring	40-100			
Anchovies	20			
Croaker	20			
Salmon				
Smoked	(1/2) 5			
Smoked, NO_3^-, treated	16,17			
Smoked, NO_2^-, treated	4,6			
Cheese, Cheshire			1.5	
Fruit, canned	(5/12)<0.1	(1/12) 0.09		
Soup[b]	(3/30)<0.09	(1/30)0.06		(1/30) 0.06
Sanwich or beer	1.27			

a: No.of positive samples/No. of samples analyzed in brackets

b: Also contained di(n)Buthylnitrosamine, 2/30 samples 0.2 and 0.5 μg/kg.

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the cured meats, cooked bacon has received the most attention because nitrosopyrrolidine, and usually nitrosodimethylamine, occur consistently in this product. In addition to nitrosopyrrolidine and dimethylnitrosamine in cooked bacon and cooked-out fat, considerable quantities of these volatile nitrosamines are lost in the fumes when bacon is fried. Gough *et al.*(33) reported that 60 to 95% of the total nitrosopyrrolidine produced and 75 to 100% of the total dimethylnitrosamine produced escape in the fumes.

Data presented by Havery *et al.*(34), clearly show that nitrosopyrrolidine levels in United States cooked bacon decreased from 1971 to 1977. Lower levels of nitrite and increased amounts of ascorbate in bacon-cured mixtures have contributed to the reduced levels of nitrosopyrrolidine. Cured meats other than bacon sporadically contain nitrosopyrrolidine, nitroso-dimethylamine, nitrosodiethylamine, nitrosopiperidine, nitrosomorpholine, and nitrosoproline. However, when these nitrosamines are present, the levels usually do not exceed 1 g/kg.

5.2 Dairy Products.

A number of cheeses and dried dairy products have been shown to contain low level of dimethyl-nitrosamine. There is no apperent explanation why some cheese samples contain dimethylnitrosamine and others do not. Gough *et al.*(35) analyzed 58 samples of cheese

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including some cheeses to which nitrate had been added. There was no correlation between addition of nitrate and dimethylnitrosamine in the cheeses. Approximately 17% of the cheeses examined contained dimethynitrosamine; most of the positive samples were in the 1-5 g/kg range. Certain dried products such as nonfat dry milk and dried buttermilk have been shown to contain dimethylnitrosamine. Most of the samples analyzed contain slightly less than 1.0 g/kg. Recent research in Scanlan laboratory has shown that the source of dimethylnitrosamine in nonfat dried milk is the direct-fire drying process(36).

5.3 Fish.

Since many salt-water species of fish contain considerable levels of dimethyl- and trimethylamine, fish has been examined for volatile nitrosamines. Gough *et al.* (37) reported dimethylnitrosamine levels of 1-10 g/kg in 38% of 61 samples of uncooked fish. Nine samples of cooked fish did not contain dimethylnitrosamine. Since dimethylnitrosamine is volatile, it may be removed from the fish during cooking, but Matsui *et al.*(38) found that the dimethylnitrosamine content of cooked fish depended on the method of cooking. This investigators examined 11 species of fish and found that the mean concentration of dimethylnitrosamine increased from 2.4 to 6.4 g/kg upon broiling on a city gas range. Presumably, oxide of nitrogen produced from the gas range reacted with amines in the fish during the cooking to

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produce dimethylnitrosamine.

5.4 Beer and Malt.

Mangino *et al.* (39) summarized dimethylnitrosamine levels found in beer by investigators thoughout the world. The source of dimethylnitrosamine in beer is barley malt; dimethylnitrosamine is produced from reaction of oxide of nitrogen and barley malt alkaloids during the direct-fire drying of this product. Efforts by the melting and brewing industries during the past several years to reduce dimethylnitrosamine levels in beer have been reasonably successful. Conversion to indirect-fire kilns and use of sulfer dioxide during drying are principally responsible for the reductions(36).

6. Precursors of N-Nitroso Compounds in Foods and Foodstuffs.

Factors which can influence the formation of nitrosamines in foods include nitrite concentration, type and basicity of amines, concentration of amines, reaction time and temperature, pH and presence of accelerators and/or inhibitors(6).The reason for listing these factors is because they are known to influence formation in simpler systems. However, since foods are complex chemical media, it is probable that additional factors may also be important.

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6.1 Nitrite.

Since the rate of nitrosation of secondary amines is proportional to the square of the nitrite concentration, the amount of nitrite in foods and in the environment has received considerable attention in recent years. Nitrite and nitrate are used as food additives in the manufacture of cured meat and fish(14). It has been demonstrated that nitrite is the active ingredient and, therefore, some manufactures have abandoned the practice of using nitrate in their cures. Apparently nitrate serves only as a potential source of nitrite which is produced by bacterial reduction of nitrate in the cured meat. It is claimed that nitrite, in combination with sodium chloride and mild heat treatment, prevents outgrowth and toxin production by *Clostridium botulinum*. The reasons for the addition of nitrite to cured meats include its role as a color fixation, a preservative, and its beneficial effect on the flavor and color of cured meat products(40).

Nitrite is also formed by reduction of the nitrate which is a common constituent of vegetables and is present in some (spinach, beets) at levels of several thousand parts per million. Nitrate is also present in water, sometimes at concentrations exceeding the recommended maximum of 45 ppm (41). This reduction is carried out by bacteria in the mouth and is the source of the nitrite found in saliva in varying amounts(42), usually between 1 and 10 ppm but sometimes considerably

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higher. Because saliva is secreted continuously (approximately 50 ml/hr),the amount of nitrite present in the stomach from this source at any time is less than the amount of nitrite introduced in cured meat and fish. Therefore, salivary nitrite, although significant, is usually a less important source of nitrosamines than is residual nitrite in cured foods. This is because the laws of mass action relating the rate of nitrosamine formation to the concentration of amine and of nitrite in the reaction medium show a dependence on the square of the nitrite concentration. Since, during digestion of a meal, the stomach contents are heterogenous, there must be locations of relatively high nitrite concentration at which formation of nitrosamines from amines will be especially high. This factor, together with the probability that mixtures of amines, rather than single amines, will be present and therefore will convert relatively more nitrite to nitrosamine, makes it very difficult to model the system and predict the quantity of nitrosamine to which an individual might be exposed from this source(41).

6.2 Amines.

The limiting factor, then, in the formation of nitrosamines seems to be the availability of nitrosatable secondary amines. Because secondary amines have seldom been sought in the environment. Table 6 can demonstrate that amines have been found to occur in a wide variety of

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Table 6 Food systems from which amines have been identified.

Bakery products

Bleached bread

Biscuites

Janpanese crackers

Beverages

Beer, Coffee, Tea, Wine,

Cocoa and Chocolate

Fishery products

fruits

Apple, Avocado, Banana,

Currants, Grapefruit,

Grape, Lemon, Mandarin,

Orange, Plantain, Plum,

Strawberry, Tangerine

Grains

Barley, Corn, Oats,

Rice, Sorghum, Wheat

Meat products

Bacon, Beef, Frankfurters,

Ham, Mutton, Pork, Sausage

Milk products

Butter, Casine, Cheese,

Milk, Dried milk,

Evaporated milk,

Whey, Yogurt

Poultry products

Chicken, Eggs

Vegetables

Bamboo shoots,

Baked beams, Eggplant,

Potato, Sauerkraut,

Spinach, Sweet potato,

Tomato

Miscellaneous

Almonds, Bacteria,

Mushrooms,

Soybean product,

Vinegar, Yeast

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foods(43). In such sources, secondary amines are usually found together with primary and tertiary amines.

Man is only species which cooks and preserves foods, and cooking possibly affords a source of secondary amines. Pyrolysis of protein and cooking of protein food might produce free amino acid such as proline, arginine and hydroxyproline, and nitrosatable secondary amines such as pyrrolidine and piperidine. The diamines cadaverine and pustrecine present in partially decayed meat and fish are converted by heating into piperidine and pyrrolidine, respectively; these two amine could therefore be formed when meat or fish is cooked(14).

The number and types of amines in each foods in Table 6 are reported in "Amines In Foods" (43). These indicate that amines are naturally present and cannot be avoided in foods. However, the question arises as to whether the amounts found in foods are of much importance with respect to N-nitrosamine formation.

6.3 Gaseous Oxides of Nitrogen.

Challis *et al* (39) have shown that nitrosamines are rapidly formed, even under alkaline conditions, when gaseous nitrous anhydride or dinitrogen tetroxide (N_2O_4) were used as nitrosating agents. Oxide of nitrogen, formed as a result of conbustion in direct-fire drying operations, have recently been shown to lead to the formation of nitrosating agents and subsequently to the formation of dimethylnitrosamine in barley malt and in

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nonfat dried milk (44). During a direct-fire drying process, the products of combustion are incoporated into the drying air and hence come in direct contact with the product being dried. A series of reactions which could lead to the formation of nitrosating agents in direct-fire drying air are:

$$N_2 + O_2 \xrightarrow{\Delta} 2NO \qquad (1)$$

$$2NO + O_2 \longrightarrow 2NO_2 \qquad (2)$$

$$NO_2 + NO \rightleftharpoons N_2O_3 \qquad (3)$$

$$NO_2 + NO_2 \rightleftharpoons N_2O_4 \qquad (4)$$

Nitrogen and oxygen in the air react in the energy-rich vicinity of the flame to form nitric oxide (Equation 1), some of which is air oxidized to form nitrogen dioxide (Equation 2). Nitrous anhydride, formed by Equation 3, and dinitrogen tetroxide, formed by Equation 4, are both effective nitrosating agents. Reaction between nitrosating agents in the drying air and nitrosatable amines in the product being dried can lead to the formation of nitrosamines (36).

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Chapter IV

Materials and Methods.

1.Materials.

1.1 Surveyed samples.

Five interested brands of sausages were randomly bought from ten supermarkets in Bangkok. All brands of samples were produced in Thailand which were:

SS-1	10 samples
SS-2	10 samples
SS-3	10 samples
SS-4	10 samples
SS-5	10 samples

All samples were kept in freezer at -20 °C immediately after arrival at laboratory. They were analysed in the next day.

1.2 Processed samples.

Sausages were processed by the Department of Food Science and Technology from Faculty of Agricultural Industry, Kasetsart University. All ingredients were the same batch bought from local retail markets in Bangkok. Normal formula for processing of sausages used by the Department of Food Science and Technology were:

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Sausage

Ingredients:

beef	1	kg
pork	1	kg
pork fat	400	g
salt	40	g
prek powder	2	g
ground ice	400	g
sugar	20	g
acort	6	g
pepper powder	10,(25)	g
nut meg powder	1.5	g
coriander seed powder	12	g
fresh garlic	30	g

Method:

1.Pork and beef were cut into 1x1x1 inch. Mixed with salt and prek powder then took to closed bowl. And kept in cold room at 4 °C or below at least 24 hours.

2.Pork and beef from(1) were grinded to pass small grill and grinded pork fat seperately from pork and beef.

3.Pork and beef that have been grinded were taken to grinder mixer, added all spices and mixed together. Ground ice was added to made it cool all the time (temperature must not be above 18 °C). Grinded them to fine and sticky paste.

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4.Gound pork fat were added to (3) and grinded

together.

5.The mixture from (4) were filled and press to pork intestine and tied it to form sausage pieces.

6.Sasages were boiled at 70-80 °C for 15 minutes and then immediantely soak in cool water. And dried sausages, pack in the bag. Two kilograms of susages were obtained.

To study the origin of NPIP in sausages, all ingredients of these sausages were the same as normal formula except the amount of nitrite and pepper which were varried.

Processing I Varying the amount of pepper (Table 7)

Table 7 Varying the amount of pepper.

	pepper(g/kg)
P0	0
P5	5
P12.5	12.5

After processing these sausages were stored in refrigerator at 2 °C and the level of residual nitrite, nitrate, NPIP, piprine and piperidine were determined at day 1, day 7 and day 12

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Processing II Varying the amount of nitrite and pepper (Table 8)

Table 8 Varying the amount of nitrite and ppepper.

	added nitrite[a](ppm)	pepper(g/kg)
P25-1	0	25
P25-2	300	25
P50-1	0	50
P50-2	300	50

a: added nitrite was in the form of $NaNO_2$

After processing these sausages were stored in refrigerator at 2°C and the level of residual nitrite, nitrate, NPIP, piperine and piperidine were determined at day 1 and day 7.

2.Chemical and apparatus.

2.1 Chemical

All chemicals were analytical grade or laboratory reagent grade.

Sulfamic acid ($NH_2.SO_3H$), N-1-napthylethylene diamine dihydrochloride ($C_{10}H_7.NH.CH_2.CH_2.NH_2.2HCl$) Ammonia solution(p20=0.91, 25%NH_3), di-Sodium tetraborate ($Na_2B_4O_7.10H_2O$), Glacial acetic acid (CH_3COOH), Sodium nitrite ($NaNO_2$), Sodium hydroxide (NaOH), Sulfuric acid (H_2SO_4,p20=1.84), Heavy liquid paraffin, carborandum (Coarse), Sulphanilic acid were of BDH Chemicals

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Ltd.,England.

Activated charcoal, Morpholine, Isopropanol (C_3H_8O), were of Fluka,Switzerland.

Sodium chlorinde(NaCl), Hydrochloric acid(HCl), Cadmium Sulfate($3CdSO_4.8H_2O$), Zinc acetate dihydrate[$(CH_3COO)_2Zn.2H_2O$], Potassium ferrocyanide trihydrate[$K_4Fe(CN)_6.3H_2O$], Potassium nitrate(KNO_3), Zinc rod(length about 15 cm and diameter of 5-7mm), Dichloromethan(CH_2Cl_2) (using K-D flask,70 ml of each bottle was evaporated to 1 ml and checked for interfering peaks on GC-TEA system), Boric acid cryst.(H_3BO_3), Sodium sulfate anhydrous(Na_2SO_4) were of Merck,Germany.

Perchloric acid($HClO_4$) (p20=1.68,70%$HClO_4$) was of Farmitalia Carlo Erba,Italy.

Dinitrofluorobenzene(DNFB), N-nitroso compounds reference standard(ie. NDMA, NDEA, NDPA, NPIP, NPYR and NMOR) Amine reference standard (ie. piperine and piperidine) were of Sigma Chemical Co.,U.S.A.

2.2 Apparatus

2.2.1 Gas chromatograph - Varian model 37000, stanless steel column, 2.8m × 2.0mm id (3.0mm od) pecked with 10% carbowex 20M and 5% KOH, condition as follows: injector 200°C; column 145°C to 185°C, isotermal 3 min at 145°C and then increasing 30°C/min. Direct interface was made from gas chromatograph to pyrolyzer furnace of TEA, Nitrogen was used as carrier gas with flow rate at 30 ml/min.

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2.2.2 Thermal energy analyzer (TEA) - model 502 A, operated according to manufacturer's instructions, with ethanol and liquid nitrogen slush bath was used as trap coolant.

2.2.3 High pressure liquid chromatograph - Du Pont series 8800 Gradient Controller was used to determine piperine. Column of Zorbax ODS-C_{18} with 25.0cm 4.6mm dimension was used. Mobile phase was methanol:water(75:25) with a flow rate of 1 ml/min. For determination of piperidine, column of Zorbax C_8 golden series with dimension of 8cm × 6.2mm, 3 micron was used. Mobile phase was methanol:water(62:38) with a flow rate of 1.5 ml/min. Variable wavelength UV spectrophotometer was used as detector.

2.2.4 UV spectrophotometer - Model No. 852001.902. Wavelength 340nm was used for piperine and of 368nm was used for piperidine.

2.2.5 Spectrophotometer - The Bausch & Lomb Spectronic 2000, with cells of 1 cm length of ligt path was used.

3.Methods.

This study was divided into 2 experiments:

3.1 Experiment I (Servey of market sausage samples)

All 5 brands of sausage which produced by 5 commercial meat processing companies in Thailand were analysed for nitrite, nitrate contents on the next day of

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buying. On another day, they were analysed for 6 VNAs:NDMA, NDEA, NDPA, NPIP, NPYR and NMOR by using NDPA as internal standard. Then they were analysed for piperine and piperidine.

3.2 Experiment II. (Processing experiment)

This experiment is divided into 2 processings.

3.2.1 Processing I.

Processing I was done by varying the amount of pepper in the formula; P0, P5 and P12.5 while the amount of nitrite was constant (from prek powder in this formula). These sausages were stored in refrigerator at 2 °C after processed. Then thay were analysed for nitrite, nitrate, NPIP, piperine and piperidine at day 1, day 7 and day 12 after processing.

3.2.2 Processing II.

Processing II was done by varying the amount of pepper and nitrite in the formula;P25-1, P25-2, P50-1 and P50-2. After processing they were stored in refrigerator at 2 °C. Then they were analysed for nitrite, nitrate, NPIP, piperine and piperidine at day 1 and day 7.

4.Methods of analysis

4.1 Sample preparation.

Frozen sample was homogenized in a blender and kept cool thoughout the blending time to avoid artifical formation of VNAs during blending. It was kept in a compleatly filled, air tight, closed container and stored in such a way that deterioration and changed in

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composition were not occurred until analysis.

4.2 Determination of nitrite and nitrate

The nitrite and nitrate contents were determined according to the procedure described by International Standard Organization (ISO/DIS 2918 and 3091)(46,47) They were done with in 24 hrs and expressed as milligrams of sodium nitrite and sodium nitrate per kilogram of food. The recovery of sodium nitrite was 98-100% and sodium nitrate was 93-95%.

Principle: The food samples were extracted with hot water, precipitated of protein and filtered. The filtrate contained nitrate and nitrite from food. Nitrate extracted was reduced to nitrite by metallic cadmium. Sulphanilic acid and N-1-naphthylethylene diamine were used to form color with the filtrate before and after conversion of nitrate to nitrite. The intensity of pink color developed was measured sprectrophotometrically. The total nitrite content was caculated by comparing the optical density against those of a series of sodium nitrite standard solution, treated in the same manner. Calculation of the nitrate contents were done by multiplying with a correction factor, by using nitrite content after reduction of original nitrite content of product.

The procedure of nitrite and nitrate determination are decribed in appendix I.

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4.3 Determination of volatile N-nitrosamines.

Volatile N-nitrosamines were analysed according to Mineral Oil Vacuum Distillation Method with GC-TEA described by Greenfield *et al.*(47) with some modification.

Principle: Volatile N-nitrosamines were distilled under vacuum from a mixture of a test portion of the sample, paraffin, water, inhibitor and sodium hydroxide. Cotton wool could be added to prevent foaming or bumping. The distillate was extracted after acidification in a separatory funnel with dichlorometane. The concentrate extract was analysed by the combination gas-liquid chromatograph / chemiluminescence detector (GC-TEA).

The detail of analysis procedure are discribed in appendix II.

4.4 Determination of piperine.

Piperine contents were determined according to the procedure described by Verzele *et al.*(48) with some modifications.

Principle: The food samples were extracted 3 times with methanol and the combined extracts were filtered. The filtrate was extracted 3 times with dichloromethane and the combined extracts were evaporated to dryness under vacuum. The residue was redissolved with methanol. The solution was analysed by

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the high pressure liquid chromatography.

The detail of analysis procedure are described in appendix III.

4.5 Determination of piperidine.

Piperidine contents were determined according to the procedure described by Bellatti *et al.*(49) with some modifications.

Principle: The food samples were rapidly extracted 3 times with perchloric acid and the supernatant was filtered. The combined clear extracts were neutarized with borate buffer and adjusted pH to 10 with sodium hydroxide and the derivertization with dinitrofluorobenzene. The derivertives were extracted 3 times with dichloromethane, the combined extracts were evaporated to dryness under vacuum. The residue was redissolved with methanol. The solution was analysed by the high pressure liquid chromatography.

The detial of analysis procedure are described in appendix IV.

5.Statistical Analysis.

Kolmogorov - Smiranov was used for statistical normal distribution test, Unpaired student's"t" test and simple linear correlation was employed for statistical comparison of data.

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Chapter V

Results

1.Experiment I: Survey of Market Food Samples

Residual nitrite, nitrate, VNAs, piperine and piperidine occurred in 5 popular brands of suasages which produced in Thailand are shown in Table 9, 11 and 13.

Residual nitrite and nitrate were expressed as $NaNO_2$ and $NaNO_3$ in ppm or mg per kg. The residual nitrite (mean ± SD) in samples SS-1, SS-2, SS-3, SS-4 and SS-5 were 108.75 ± 18.16(range 66.9-128.56), 94.61 ± 11.92(range 84.92-116.82), 118.78 ± 31.15(range 53.75-156.69), 83.23 ± 19.00(range 51.59-105.49) and 34.47 ± 27.29(range 11.74-97.31) respectively. Sodium nitrite in sample SS-2, SS-4 and SS-5 were significantly lower than in sample SS-1 and SS-3 whereas that in sample SS-1 and SS-3 were not significantly different among each other. The residual nitrate(mean ± SD) in sample SS-1, SS-2, SS-3, SS-4, SS-5 were 187.40 ± 30.55(range 155.33-222.76), 224.41 ± 59.67 (range129.49-309.79), 229.52 ± 51.41(range172.53-308.48), 233.61 ± 86.59(range 139.56-342.09) and 201.10 ± 63.09 (range79.91-292.67) respectively. For residual nitrate, sample SS-1 was significantly different from SS-3 and SS-5 whereas that in SS-2, SS-3, SS-4 and SS-5 were not significantly different among each other.

Occurrence of VNAs in 5 popular blands of sausage which produced in Thailand could be found only two kinds which were NPIP and NPYR. NPIP and NPYR

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Table 9 Residual nitrite and nitrate occurred in 5 popular brands of Sausage (mean ± SD)

Nitrite, nitrate	Sausage sample				
	SS-1	SS-2	SS-3	SS-4	SS-5
$NaNO_2$	108.75 ± 18.16^{abc}	94.61 ± 11.92^{adef}	118.78 ± 31.15^{dgh}	83.23 ± 19.00^{begi}	34.47 ± 27.29^{cfhi}
(ppm)	(66.9-128.56)	(84.92-116.82)	(53.75-156.69)	(51.59-105.49)	(11.74-97.31)
$NaNO_3$	187.40 ± 30.55^{jk}	224.41 ± 59.67	229.52 ± 51.42^{j}	233.61 ± 86.59	201.10 ± 63.09^{k}
(ppm)	(155.33-222.76)	(129.49-309.79)	(172.53-308.48)	(139.56-342.09)	(79.91-292.67)

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Table 9 - continued

Note: 1. The mean values shown are the average of positive samples.

2. For nitrite and nitrate all 10 samples of each brands were detected.

3. Superscripts a, b, c, d, e, f, g, h and i denote statistical significance of nitrite among each match of sample groups with p values less than 0.05, 0.005, 0.0005, 0.025, 0.05, 0.0005, 0.005, 0.0005 and 0.0005 respectively.

4. Superscripts j and k denote statistical significance of nitrate among each macth of sample groups with p values less than 0.01 and 0.05 .

5. Unpaired student's "t" test was employed for statistical compairison of data.

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Table 10 No. of positive sample of $NaNO_2$ and $NaNO_3$ from each brands.

Sausage sample	No.of positive sample/No.of samples analysed	
	$NaNO_2$	$NaNO_3$
SS-1	10/10	10/10
SS-2	10/10	10/10
SS-3	10/10	10/10
SS-4	10/10	10/10
SS-5	10/10	10/10

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Table 11 Residual VNAs occurred in 5 poppular brands of sausages (mean ± SD).

VNAs	Sausage sample				
	SS-1	SS-2	SS-3	SS-4	SS-5
NPIP	27.27 ± 13.29 abc	14.31 ± 12.26	4.36 ± 2.62 ad	1.79 ± 0.77 bde	5.49 ± 5.26 ce
(ppb)	(10.81 - 45.98)	(0.99 - 26.66)	(1.71 - 8.40)	(1.24 - 2.33)	(0.31 - 17.19)
NPYR	3.59 ± 1.63	6.75 ± 5.08	2.87 ± 1.19	0.72 ± 0.19	2.22 ± 2.14
(ppb)	(1.61 - 7.27)	(3.16 - 10.34)	(2.03 - 3.71)	(0.58 - 0.85)	(0.32 - 5.17)

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Table 11 - continued

Note: 1. The mean values shown are the average of positive samples.

2. For NPIP; SS-1 and SS-5, 9 out of 10 samples were detected. SS-2, 4 out of 10 samples; SS-3 6 out of 10 samples; SS-4, 2 out of 10 samples that were detected.

3. For NPYR; SS-1, 9 out of 10 samples were detected. SS-2, SS-3, SS-4, 2 out of 10 samples that were detected. SS-5, 5 out of 10 samples were detected.

4. Superscripts a, b, c, d and e denote statistical significance of NPIP among each match of sample groups with p values less than 0.0005, 0.0005, 0.005, 0.05, 0.05 respectively.

5. Unpaired student's"t" test was employed for statistical comparison of data.

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Table 12 No.of positive sample of NPIP and NPYR from each brands.

Sausage sample	No.of positive sample/No.of samples analysed NPIP	NPYR
SS-1	9/10	9/10
SS-2	4/10	2/10
SS-3	6/10	2/10
SS-4	2/10	2/10
SS-5	9/10	5/10

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Table 13 Residual piperine and piperidine occurred in 5 popular brands of sausages (mean ± SD).

Amine	Sausage sample				
	SS-1	SS-2	SS-3	SS-4	SS-5
PIPR (ppm)	abc 129.80 ± 7.79 (123.39-140.09)	ad 52.10 ± 34.87 (27.50-130.37)	aef 188.21 ± 9.92 (173.71-200.76)	bef 47.33 ± 3.45 (41.84-52.81)	cdf 105.04 ± 34.66 (77.02-200.9)
PIPD (ppm)	ghi 0.30 ± 0.08 (0.19 - 0.38)	gikl 0.1 ± 0.09 (0.04 - 0.27)	jm 0.39 ± 0.16 (0.04 - 0.61)	hkm 0.05 ± 0.03 (0.02 - 0.09)	ilm 0.19 ± 0.09 (0.11 - 0.36)

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Table 13 - continued

Note: 1. The mean values shown are the average of positive samples.

2. For PIPR all 10 samples for each brands were detected. For PIPD SS-1, SS-2, SS-3 and SS-5 all of 10 samples for each brands were detected. SS-4, 8 out of 10 samples that were detected.

3. Superscripts a, b, c, d, e and f denote statistical significance of PIPR among each match of sample groups with p values less than 0.0005, 0.0005, 0.025, 0.0005, 0.0005 and 0.0005 respectively.

4. Superscripts g, h, i, j, k, l and m denote statistical significence of PIPD among each match of sample groups with p values less than 0.0005, 0.0005, 0.005, 0.0005, 0.05, 0.05 and 0.0005 respectively.

5. Unpaired student's "t" test was employed for statistical comparison of data.

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Table 14 No.of positive sample of PIPR and PIPD from each brands.

Sausage sample	No.of positive sample/No.of samples analysed	
	PIPR	PIPD
SS-1	10/10	10/10
SS-2	10/10	10/10
SS-3	10/10	10/10
SS-4	10/10	8/10
SS-5	10/10	10/10

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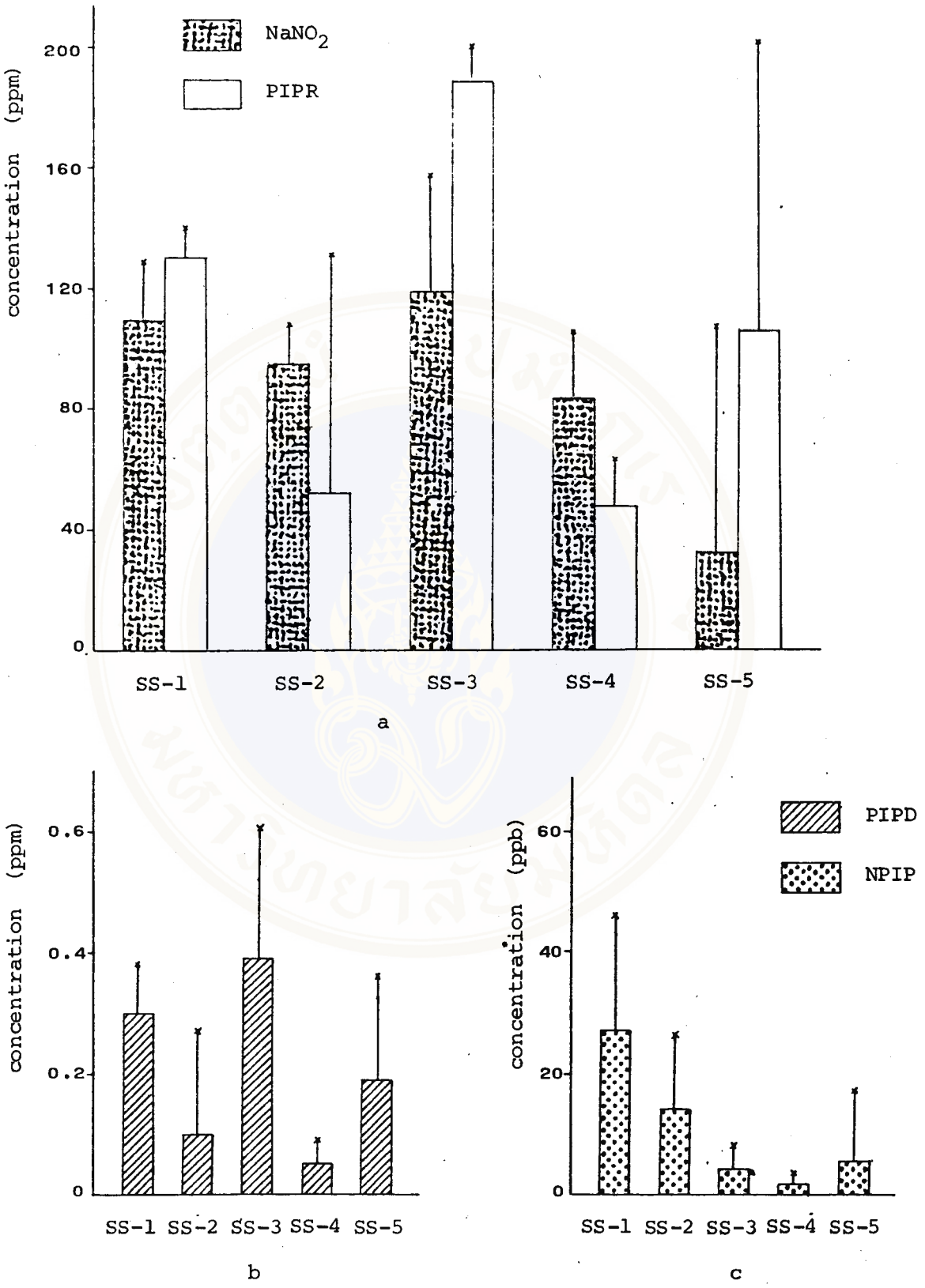

Figure 11 a) The levels of residual $NaNO_2$ and PIPR in samples SS-1, SS-2, SS-3, SS-4 and SS-5.

b) The levels of residual PIPD in samples SS-1, SS-2, SS-3, SS-4 and SS-5.

c) The levels of NPIP occurred in samples SS-1, SS-2, SS-3, SS-4 and SS-5.

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were expressed in ppb or µg/kg. For sample SS-1, nine out of ten were detected for NPIP and NPYR in the range 10.81-45.98(mean± SD = 27.27± 13.29) and 1.61-7.27(mean ± SD =3.59± 1.63) respectively. For SS-2, four out of ten samples were detected for NPIP in the range 0.99-26.66 (mean ± SD = 14.31± 12.26) and two samples were detected for NPYR 3.16 and 10.34(mean± SD = 6.75± 5.08) For SS-3, six out of ten samples were detected for NPIP in the rang 1.17-8.40(mean ± SD = 4.36± 2.62) and two samples were detected for NPYR 2.03 and 3.71(mean± SD = 2.87 ± 1.19) for SS-4, only two out of ten samples were detected NPIP and NPYR, NPIP of 1.24 and 2.33(mean± SD = 1.79 ± 0.77) and NPYR of 0.58 and 0.85(mean± SD = 0.72± 0.19). For SS-5, nine out of ten samples were detected for NPIP in the range 0.31-17.17(mean ± SD = 5.49± 5.26) and five samples were detected for NPYR in the range 0.32-5.17 (mean ± SD = 2.22± 2.14). Levels of NPIP in sample SS-3, SS-4 and SS-5 were significantly lower than in sample SS-1 and in sample SS-4 was significantly lower than in sample SS-3 and SS-5, whereas in sample SS-2 was not significantly different from sample SS-1, SS-3, SS-4 and SS-5.

Residual piperine expressed in ppm or mg/kg. Piperine(mean± SD) in samples SS-1, SS-2, SS-3, SS-4 and SS-5 were 129.8 ± 7.79(range 123.39-140.09), 52.10 ± 34.87(range 27.50-130.37), 188.21 ± 9.92(range 173.71-200.76), 47.33± 3.45(range 41.84-52.81) and 105.04± 34.66 (range 77.02-200.90) respectively. Residual piperine in

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samples SS-2 and SS-4 were not significantly different among each other whereas in samples SS-1, SS-3 and SS-5 were significantly different among each other and among SS-2 and SS-4.

For residual piperidine which were expressed in ppm or mg/kg. Piperidine in samples SS-1, SS-2, SS-3 and and SS-5 were detected in all ten samples but for SS-4 only eight out of ten samples were detected. Residual piperidine(mean ± SD) in samples SS-1, SS-2, SS-3, SS-4 and SS-5 were 0.30± 0.08(range 0.19-0.38), 0.10± 0.09 (range 0.04-0.27), 0.39 ± 0.16(range 0.04-0.61),0.05 ± 0.03 (range 0.02-0.09) and 0.19 ± 0.09(range 0.11-0.36) respectively. Residual piperidine in samples SS-1 and SS-3 were not significantly different among each other whereas in samples SS-2, SS-4 and SS-5 were significantly different among each other and among samples SS-1 and SS-3.

Detail of r values between NPIP and $NaNO_2$, $NaNO_3$, piperine and piperidine are shown in appendix V.

2.Experiment II: Processing experiment

2.1 Processing I

The amount of nitrite, nitrate and piperine in the processing sausage are shown in Table 15. Nitrite and nitrate are from prek powder which is an ingredient in this formula (sodium nitrite and sodium nitrate in prek powder from analysis were 46,000 ppm and 590,000 ppm). Piperine were from pepper(mean of piperine in

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Table 15 The levels of nitrite, nitrate, pepper and piperine in processing I.

	pepper (g/kg)	piperine[a] (ppm)	$NaNO_2$[b] (ppm)	$NaNO_3$[b] (ppm)
P0	0	0	46	590
P5	5	100	46	590
P12.5	12.5	250	46	590

Note:

a:Piperine was from pepper. The mean level of piperine in pepper from analysis was 20,097.5 ppm(mg/kg). In this processing I varying amount of pepper was 0, 5 and 12.5g in sausage 1 kg. So they contained piperine of 0, 100 and 250 ppm (mg/kg) respectively.

b:$NaNO_2$ 46 ppm and $NaNO_3$ 590 ppm were from 2 g of prek powder which was added in this formula ($NaNO_2$ and $NaNO_3$ in prek powder from analysis were 46,000 ppm and 590,000 ppm .

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Table 16 Residual nitrite, nitrate, piperine, piperidine and NPIP occurred in processing I

		P0	P5	P12.5
$NaNO_2$ (ppm)	d1	12.48	12.39	13.03
	d7	24.90	23.66	23.38
	d12	20.74	20.44	21.70
$NaNO_3$ (ppm)	d1	157.36	152.61	175.78
	d7	333.74	353.06	315.47
	d12	313.31	292.29	332.21
PIPR (ppm)	d1	19.36	62.21	88.29
	d7	16.93	59.16	73.88
	d12	10.48	44.38	62.50
PIPD (ppm)	d1	ND	ND	ND
	d7	ND	ND	ND
	d12	ND	ND	ND
NPIP (ppb)	d1	ND	ND	ND
	d7	ND	ND	ND
	d12	ND	ND	ND

Note: ND = non detectable

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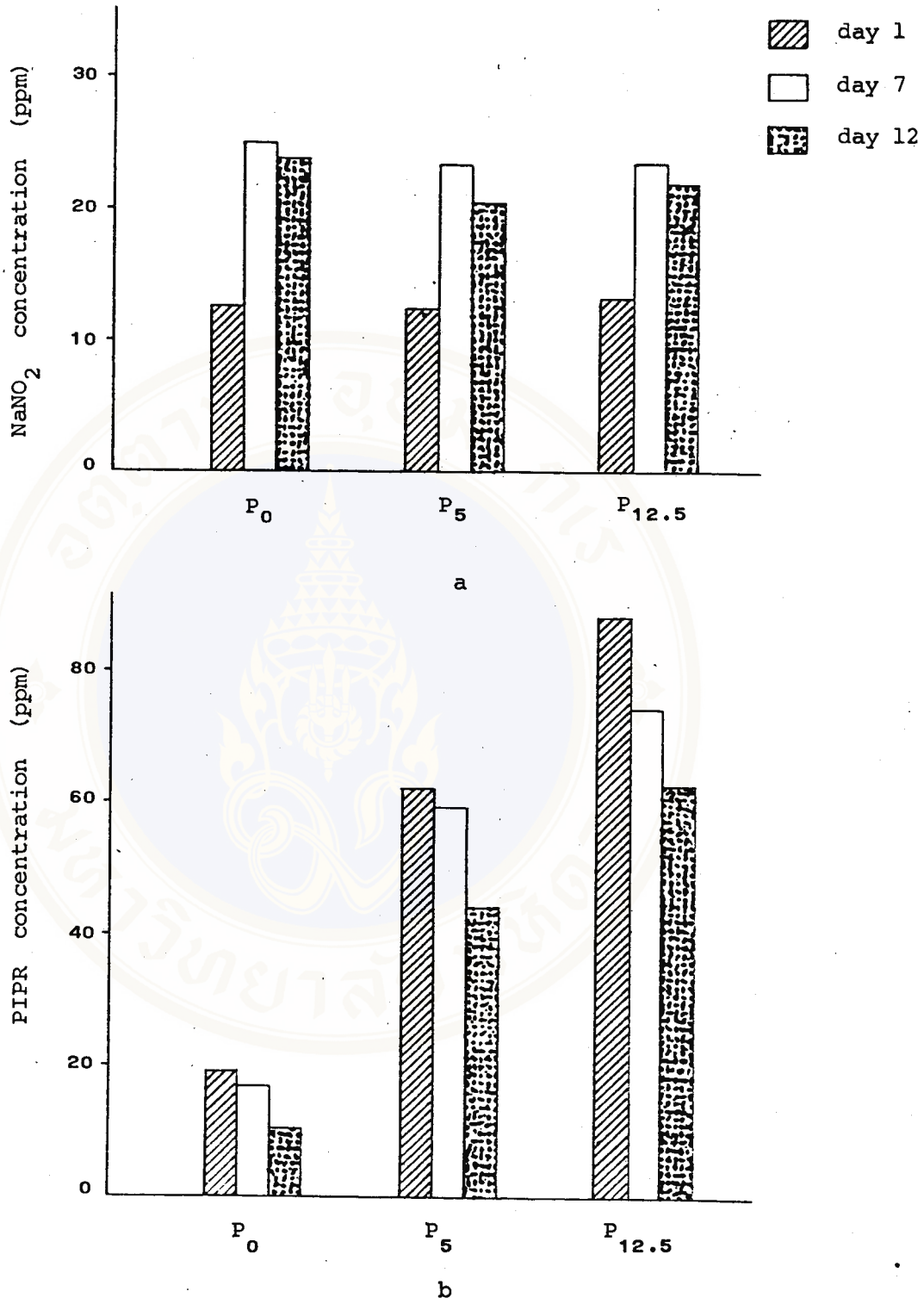

Figure 12 a) The levels of residual $NaNO_2$ in samples PO, P5 and P12.5 at day 1, day 7 and day 12.

b) The levels of residual PIPR in samples PO, P5 and P12.5 at day 1, day 7 and day 12.

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pepper from anlysis were 20,097.5 ppm).

Residual nitrite, nitrate, NPIP, piperine and piperidine in day 1, day 7 and day 12 are shown in Table 16.

For residual nitrite at day 1 in P0, P5 and P12.5 were 12.48, 12.39 and 13.03 ppm respectively. At day 7 the levels were 24.90, 23.66 and 23.38 ppm respectively. And at day 12 they were 20.74, 20.44 and 21.70 ppm respectively. Sodium nitrite levels were highest at day 7 and were lowest at day 1. They were similar for residual nitrate. Residual nitrate levels at day 1 in P0, P5 and P12.5 were 157.36, 152.61 and 175.78 ppm respectively. At day 7 the levels were 333.74, 353.06 and 315.47 ppm respectively. And at day 12 they were 313.31, 292.29 and 332.21 ppm respectively. However for residual piperine the highest levels were at day 1, lower at day 7 and lowest at day12. Residual piperine levels in P0, P5 and P12.5 at day 1 were 19.36, 62.21 and 88.29 ppm respectively. At day 7 the levels were 16.93, 59.16 and 73.88 ppm respectively. And at day 12 they were 10.48, 44.38 and 62.50 ppm respectively. Residual piperidine at day 1, day 7 and day 12 were non detectable.

Only NPYR was detected at day 7 and day 12 from P5 and P12.5, however the levels were less than 0.20 ppb. For all P0, P5 and P12.5 samples at day 1, day 7 and day 12, NPIP was not detected.

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2.2 Processing II

The amount of nitrite, nitrate and piperine (calculated from analysed pepper) in the processing sausages are shown in Table 17. Nitrite of 46 ppm was from prek powder and another 300 ppm was added in the form of sodium nitrite.

Residual nitrite, nitrate, NPIP, piperine and piperidine are shown in Table 18. The levels of residual nitrite in samples P25-1, P25-2, P50-1 and P50-2 at day 1 were 19.78, 91.77, 17.35 and 87.15 ppm respectively and at day 7 the levels were 20.96, 101.08, 20.80 and 91.67 ppm respectively, which were higher than at day1 liked in processing I. For residual nitrate in P25-1, P25-2, P50-1 and P50-2 at day 1 the levels were 230.30, 160.30, 180.10 and 180.10 ppm respectively and at day 7, there were 279.49, 281.86, 315.21 and 226.48 ppm respectively, which, were also higher than at day 1.

Residual piperine levels at day 1 and day 7 were similar to those in processing I. The levels at day 7 were lower than at day 1 in all P25-1, P25-2, P50-1 and P50-2. For residual piperine levels in P25-1, P25-2, P50-1 and P50-2 at day 1 the levels were 377.80, 343.52, 736.80 and 784.80 ppm respectively and at day 7 they were 339.30, 311.79, 684.51 and 709.73 ppm respectively.

For residual piperidine levels in P25-1, P25-2, P50-1 and P50-2 they were 19.45, 13.49, 44.91 and 30.32 ppm at day 1. The levels at day 7 were 25.38, 18.14, 51.72 and 39.45 ppm respectively.

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Table 17 The levels of nitrite, nitrate, pepper and piperine in processing II.

	pepper (g/kg)	piperine[a] (ppm)	$NaNO_2$[b] (ppm)	$NaNO_3$[b] (ppm)
P25-1	25	500	46	590
P25-2	25	500	300 + 46	590
P50-1	50	1000	46	590
P50-2	50	1000	300 + 46	590

Note:

a:Piperine was from pepper. The mean level of piperine in pepper from analysis was 20,0097.5 ppm(mg/kg). In this processing II varying amount of pepper was 25 and 50 g in sausage 1 kg. So in processing II they obtained piperine of 500 and 1000 ppm (mg/kg) respectively.

b:$NaNO_2$ 46 ppm and $NaNO_3$ 590 ppm were from 2 g of prek powder which was added in this formula ($NaNO_2$ and $NaNO_3$ in prek powder from analysis were 46,000 ppm and 590,000 ppm. 300 ppm $NaNO_2$ added was in the form of $NaNO_2$.

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Table 18 Residual nitrite, nitrate, piperine, piperidine and NPIP occurred in processing II.

		$NaNO_2$ 46 ppm		$NaNO_2$ 46 + 300 ppm	
		Pepper 25 ppm	Pepper 50 ppm	Pepper 25 ppm	Pepper 50 ppm
		P25-1	P50-1	P25-2	P50-2
$NaNO_2$	d1	19.78	17.35	91.77	87.15
(ppm)	d7	20.96	20.80	101.08	91.67
$NaNO_3$	d1	230.30	180.10	160.30	180.10
(ppm)	d7	279.49	315.21	281.86	226.48
PIPR	d1	377.80	736.80	343.52	784.80
(ppm)	d7	339.30	684.91	311.77	709.73
PIPD	d1	19.45	44.91	13.49	30.32
(ppm)	d7	25.38	51.72	18.14	39.45
NPIP	d1	ND	1.98	3.91	3.25
(ppb)	d7	ND	2.90	3.96	5.55

Note: ND = non detectable

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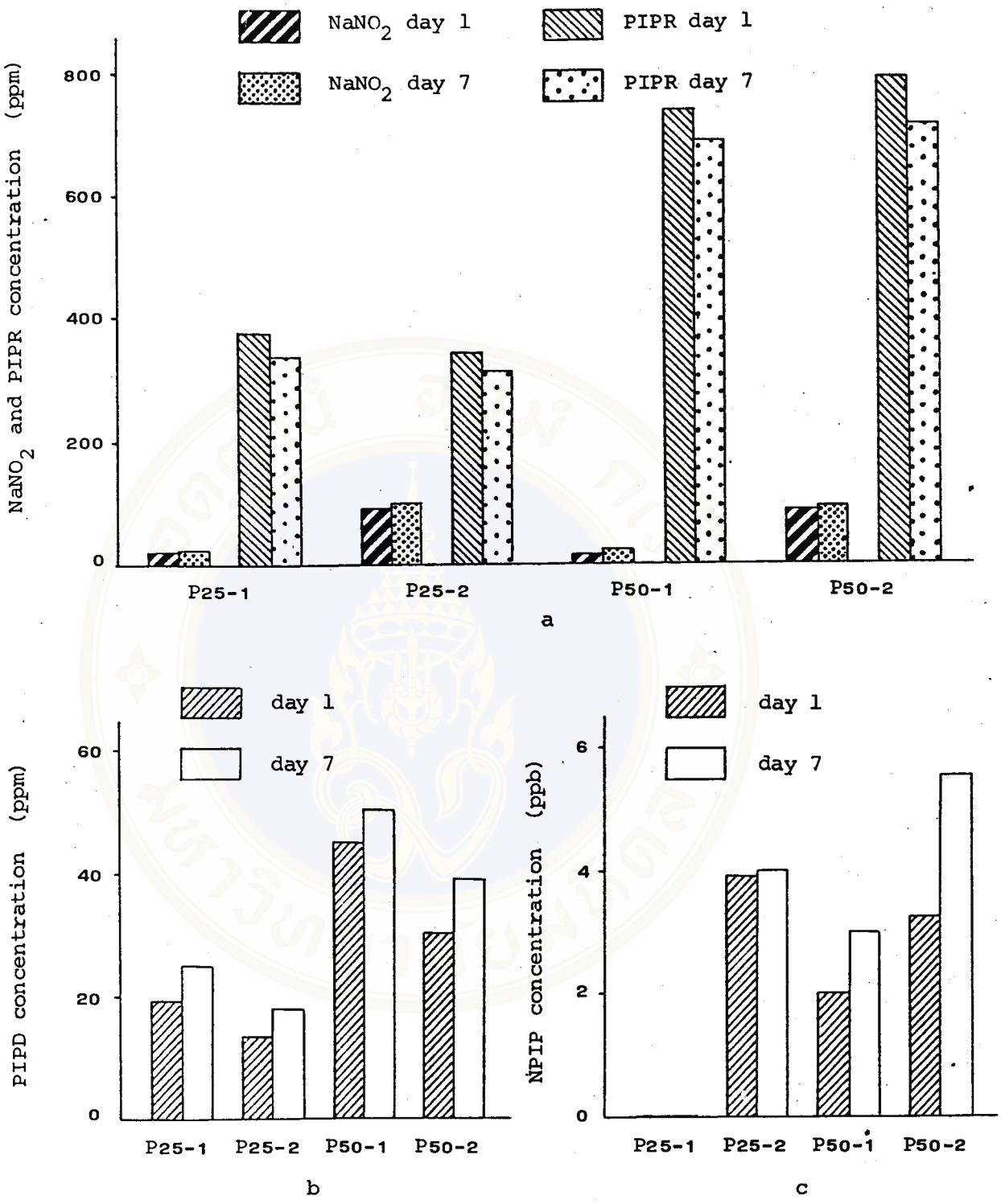

Figure 13 a) The levels of residual $NaNO_2$ and PIPR in samples P25-1, P25-2, P50-1 and P50-2 at day 1 and day 7.

b) The levels of residual PIPD in samples P25-1, P25-2, P50-1 and P50-2 at day 1 and day 7.

c) The levels of NPIP occurred in samples P25-1, P25-2, P50-1 and P50-2 at day 1 and day 7.

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Only NPIP was detected in P25-2, P50-1 and P50-2, it was not detected in P25-1. At day 7 level of NPIP was higher than at day 1. Maximum level of NPIP(5.55 ppb) was from P50-2 at day 7 and lowest level(1.98 ppb) was from P50-1 at day 1.

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Chapter VI

Discussion

1.Experiment I: Survey of Market Food Samples

Sausages are one of the cured meat products which nitrite-spices are used. Nitrite is used as a curing agent as it imparts an attractive red color to the meat. In combination with sodium chloride, it also induces a particular type of flavor. In addition, the combination of salt and nitrite plays a significant role in controlling the outgrowth of *Clostridium botulinum* spores(50). It seemed obvious that some amine components of spices reacted with nitrite to from nitrosamines(51). In this study two VNAs, NPIP and NPYR were found in sausages (surveyed from 5 popular brands of sausages from retial market in Bangkok) ranged 0.31-45.98 ppb and 0.32-10.34 ppb respectively. As the previous study Srianujata(52) reported NPIP and NPYR in some brands of sausages in Bangkok area in the range 1.49-304.37 ppb and 1.13-5.93 ppb respectively. In analysis of 11 meat products (sausage and luncheon meats) in Japan, Kawasaki *et al.*(53) reported 1.5 ppb of NPIP in 1 sample. Sen *et al.*(51) while conducting a survey of nitrosamines in meats, observed the consistent occurrence of NPIP and NPYR in Mettwurst sausages made by certain firms in Canada in the range 50-60 ppb and 10.50-24.00 ppb respectively. The indentity of the nitrosamines was confirmed by mass spectrometry. Upon investigation, it

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was discovered that in practically all cases the occurrence of nitrosamines correlated with the used of curing premixes. The premixes were dry powders consisting of spices (black pepper, paprika and two other spices), salt, sodium nitrate and sodium nitrite. Analyses of serveral commercially available premixes revealed 2.5 to 6 ppm NPYR and 7 to 25 ppm NPIP. It was found that the reaction between nitrite and the black pepper predominantly formed NPIP whereas paprika plus nitrite mainly formed NPYR. Apparently the piperidine and pyrroridine in this spices reacted with nitrite in the premixes to form the corresponding nitrosamines. In 1976, Havery *et al.*(61) reported NPIP in spice-cure mixtures at level range from 50 to 2000 ppb. Neurath *et al.*(54) studied primary and secondary amines in the human environment and found that precursor of pyrrolidine and piperidine were paprika. The main source of piperidine is pepper, Oersted in 1820,(55) isolated from the extract of black pepper a yellow crytalline substance call piperine, which was recorded as pungent. Piperine, $C_{17}H_{29}O_3N$ (mp 128 to 130 C) was shown earlier to be a weak base which is hydrolyzed by aqueous alkali or nitric acid to give a volatile base, $C_5H_{11}N$. This base was later shown to be piperidine. In this study piperine and piperidine were found in the rang 27.50 to 200.90 ppm and 0.02 to 0.61 ppm. The level of piperidine was less than the level of piperine because piperidine is the hydrolytic product of piperine(51). The level of piperine in this study was

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fairly high, so it could form piperidine to react with nitrite in the sausage to form which can be detected in this study. Some brands of sausages in this study, NPIP level was low but some brand was high. From this study NPIP level was found to be lower than that reported by Srianujata in 1985 (52). As mention earlier, the concentration of nitrosamines formed depend on many factors such as the nature of the amines, concentration of the nitrosating agents, pH, temperature of reaction medium and type of accelelator and inhibitor(6,56). It is, therefore, very difficult to compare the results of one sample to the other.

Nitrite concentration is one of the most important precursor on the yields of nitrosamine formation(15,30,57). In this survey study, the level of residual of sodium nitrite were in the range 11.74 to 156.69 ppm. The initial nitrite that was added in the sausages was not known but it seem to be possible to estimate. Since nitrite is a reactive chemical, when it is added to meat for the purpose of curing, it was found that less than 50% of added will be left for chemically detectable after the completion of processing. Many cured meat items contained from only a fill ppm to 50 ppm of nitrite, eventhough up to 156 ppm may had been added. An enormous amount of work has demonstrated that only a minuscule fraction of the added nitrite is, on occassion, available to form volatile nitrosamines in cured meat. Therefore, it must be assumed that the nitrite has been

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lost from the meat to the atmosphere or remains in the meat as reaction products undetectable by current analytical methods. The factors considered as influencing the level of detectable nitrite were : (1) time and temperature employed during processing; (2) amount of protein, fat and carbohydrate; (3) concentration of salt; (4) concentration of nitrate; (5) number and kind of microorganisms; (6) acidity (40). From the study of Cassens *et al.* (40), it may be minimize estimated that initial nitrite added in the sample of survey sausages were ranged from 23.48-313.38 ppm (residual nitrite ranged from 11.74-156.69 ppm)

From estimated initial nitrite added in the sample, some samples had high level of $NaNO_2$ added such as 313.38 ppm which is higher than Regulation of Food and Drug Administration of Thailand (125 ppm). In this study, there was no correlation between residual nitrite or nitrate and the level of NPIP (r values were shown in Appendix V). This agree with several resent surveyed of the amount of nitrosamines in variety of cured meat. Panalak *et al.*(58), observed no clear-cut correlation between the levels of nitrosamine and the residual levels of nitrite or nitrate. Sen *et al.*(30) found that no correlation between the final level of sodium nitrite (analysed before frying) and that of nitrosamines in the fried products. Since the initial nitrite concentration in the commercial products was not known therefore no statistical analysis was carried out.

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2.Experiment II: Processing experiment

From the result of the survey of market sausage samples in experiment I, experiment II was desiged. First, processing I, sausages were prepared with varying the amount of pepper in the normal level that used in processing sausages 5 g/kg and 12.5 g/kg, and sample without pepper added were used as control, to observed the formation of NPIP. The levels of residual nitrite, nitrate, piperine and piperidine during the storage time of 1 day, 7 days and 12 days after processing were observed. In this study the level of residual nitrite (contained in prek powder) was very low at day 1, higher at day 7 and become lower at day 12 but still higher than day 1 in all of samples (P0, P5, P12.5). The levels of residual nitrite detected were less than 50% of initial nitrite which was similar to the study of Cassens *et al.*(40). The levels of residual piperine were highest at day 1 and lowest at day 12 in all samples. In the control sample small amount of piperine was also detected, this may due to some contamination of pepper from other spices in the formula (such as nut meg, coriander seed powder and garlic). The level of piperidine was not detected, and NPIP was not found. It seemed to be because the amount of nitrite was too lower to form nitrosamine. The result in this processing I was used as the guide line to desiged processing II.

In processing II, sausages were prepared by varying the amount of nitrite and also varying the amount

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of pepper both in excess than in processing I. Since it was expected that residual nitrite found in sausages collected from market and in processing I could reflect a much higher initial levels of nitrite, and also for piperine and piperidine. In sample P25-1 which nitrite was not added in the form of sodium nitrite (except in prek powder), and 25 g/kg of pepper was added (approximately 2 times the amount of normally used in normal formula), the level of NPIP was not detected. While the sample P25-2 which was processed in the same way as P25-1 but a higher level of nitrite used. It was 300 ppm initially, but the detected nitrite levels at day 1 and day 7 were 91.77 and 101.08 ppm respectively. This level of nitrite caused the formation of NPIP at level of 3.91 and 3.96 ppb at day 1 and day 7 respectively. At the same time when higher level of pepper(50 g/kg) was used which could contribute to higher level of piperine and interm of piperidine precursor, even no extra nitrite was added (sample P50-1), NPIP was also detected at 1.98 and 2.90 ppb at day 1 and day 7. It was seen even clearer when both nitrite and pepper were added in excess initially, 300 ppm and 50 g/kg respectively. The nitrosamine, NPIP, was found 3.25 and 5.55 ppb at day 1 and day 7. Therefore it indicated quite clear that both nitrite and pepper played a very important role in the formation of NPIP in sausage.

It might be also conceivable that NPIP found in the market sausages both in previous and this study

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reflect the excessive initial used of nitrite or pepper or both. However from data in this study , it is quite possible to speculate that product in the market could be produced by excess initial used of nitrite other than pepper. Since the level of piperine or piperidine did not change very much before and after processing. It was observed also that the product was not so strong in the hot taste which could be if the pepper was used in excess. Fiddler *et al.*(15) studied the effect of sodium nitrite concentration on NDMA formation in frankfurters. Level of nitrite ranged from 150 to 2500 ppm. The formation of NDMA appeared to be directly related to the amount of nitrite added to the product and it took, 1500 ppm of nitrite (approximately 10 times the amount normally used) to produce 10 to 14 ppm NDMA. Frankfurter produced with 150 ppm added nitrite did not contain measurable amounts of NDMA. In the study on the effect of sodium nitrite concentration on the formation of NPYR and NDMA in fried bacon by Sen *et al.*(30) it was reported that statistical analysis of the data by Kendall's rank correlation test indicated high correlation between the level of NPYR and that of sodium nitrite initially used to prepare the bacon ($p<0.001$).

As mention earlier, the amount of pepper also affected the formation of NPIP (compare the level of NPIP between P25-1 and P50-1 or between P25-2 and P50-2), it is shown that precursor of NPIP was from the pepper used. Piperidine could formed from piperine in the

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pepper(51),which could be detected in sausage from this study. Nakamura *et al.*(59), also reported that precursors of NPIP were present in pepper which was the origin of NPIP in salami. Yamamoto *et al.*(60), reported that NPIP was detected in most of salami sample but was rarely found in any other food because of the presence of large amounts of piperine in pepper which is an ingredient of salami.

In processing II, time of storage was studied at day 1 and day 7, it was found that at day 1 the level of NPIP was low than at day 7 in all samples. Havery *et al.*(61), reported that time could effect the level of NPIP formation by studying NPIP from spice-cure mixtures at day 30, 150 ppb NPIP was found and at day 60, 420 ppb NPIP was found. The storage temperature was also found effect to the formation. Sen *et al.*(30) also found that the nature of spices, temperature and duration of storage could affected the formation of nitrosamines. It was observed in general that the premixes containing only ground black pepper and/or paprika, with no other addition or filler, could found the highest level of nitrosamines. There was also some differences in results between different brands (or origin) of spices used. For example, the sample of Spanish paprika formed nitrosamines very rapidly, the concentration of NPYR reached 4 ppm with 15 days of storage at 38°C. It is also interesting that certain samples did not formed detectable level of nitrosamines when stored at 4°C, even

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for long time. However, when the same samples were brought out to store at 23 °C for additional 50-56 days, three out of four samples gave positive results for nitrosamines.

From these evidences , it may be possibly concluded by present study that excessive used of nitrite and pepper could lead to the formations of NPIP in sausages. The levels found in processing samples seemed to be quite lower than in samples collected from the market. This could be due to the differences in the processing, origin of pepper used, time and temperature of storage.

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Chapter VII

Conclusion

It was shown earlier by Srianujata that some brands of sausages sold in market contained considerably high level of NPIP. Therefore, it is the main objective of this study to investigate more extensive on the occurrence of Nitrosopiperidine in sausages and the factors that influence the formation of this nitrosamine in sausages. The study was divided into two parts, firstly the survey of NPIP content in sausages sold in market, and secondly the processing experiment to find the main factors of the formation of NPIP.

From the survey samples, it has been shown that sausages in Bangkok area were contaminated with NPIP and NPYR ranged from 0.31 - 45.98 ppb and 0.32 - 10.34 ppb respectively. Some brands of sausages were often found NPIP (SS-1 and SS-5, 9 out of 10 samples were found) some were found not so often (SS-4, 2 out of 10 samples were found). In most of the samples, nitrite contents (residual nitrite) were quite high which could estimated that nitrite added were higher than that allow by the regulation of FDA (125 ppm). The NPIP contents of several brands of sausages were varried between very low (traces) to fairly high level (up to 45.98 ppb).

From processing experiment, in the normal formula with low levels of nitrite and pepper, the level of NPIP was very low and mostly nondetectable. When

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higher initial levels of nitrite used, considerable NPIP formed, as well as when higher initial level of pepper used. It was found that level of nitrite added must be as high as 300 ppm in order to cause the formation of NPIP considerably when normal amount of pepper used. Some NPIP was also found when very high level of pepper, as high as 50 g/kg, and normal nitrite used. All these, however, the levels of NPIP found in experimental samples were still lower than those found in market samples.

From these results it could be possible to speculate that excess initial level of nitrite might have been used in market samples. The levels found as residual nitrite were still under the limit allow by FDA. But it was found that residual nitrite could be as low as 50% of initial used. Furthermore other factors could effect the formation, such as brand or source of pepper, temperature and duration of storage, and also some inhibitors and accelerators present in the formula.

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Appendix I

Procedure for Nitrite and Nitrate Determination.(45,46)

Briefly, quantitatively transfer 10.0 g of homogenized food sample into a 300 ml Erlenmeyer flask. Add 5 ml of saturated borax solution, and disperse in 100 ml hot water of temperature not below 70 °C. Heat the fask for 20 minutes on a boiling water-bath and shake repeatedly. Then chill the mixture to room temperature, deproteinize with successive addition of 2 ml of potassium ferrocyanide(10.6%w/v) and 2 ml of Zinc sulfate(22% w/v in 3% glacial acetic acid v/v), dilute to 200 ml with distilled water and leave for 30 minutes. After adding activate charcoal powder to remove pigment, finally filter the mixture through fluted filter paper (240mm diamiter and nitrite free). The filtrate obtained is analysed for nitrite, nitrate. Reduction of the nitrate extracted to nitrite is done by passing 20 ml aliquot of the filtrate through a column of metallic cadmium(Fig.14). Take an aliqout of the filtrate and of the effluent for nitrite and nitrate estimation by the Griess reagent [N-(1-naphthyl)-ethylenediamine(0.45g/l) and sulfanilamide(6g/l) in glacial acetic acid(20%v/v) and 200ml sodium chloride solution(100g/l)]. Add 5 ml of Griess reagent to and 5 ml aliquot of filtrate/effluent required for azo dye formation, mix the solution with vertex and leave for 20 minutes, protect from light.

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Measure photometric the resulting pink color from this reaction at 540 nm, using a reagent blank as the reference.

Culculate the nitrite content of this extract by comparing with the optical density obtained with that of the standard sodium nitrite solution treated in the same manner. Culculate the nitrite content by multiplication with a conversion factor of the nitrite content after deduction of the original nitrite content of the product. The nitrite and nitrate contents are expressed in mg of their sodium salt per kg.

Preparation of the calibration curve: prepare 6 concentrations of sodium nitrite standard solutions: 0.10, 0.25, 0.50, 1.00, and 2.00 ug/ml on the day of use. React with the color reagent as described in the determination. Draw the calibration curve by plotting the optical densities measured against the concentrations, in ug/ml of the sodium nitrite standard solution(fig. 15).

Repeatability: the difference between the results of two determinations carried out simultaneously or in rapid succession shall not be greater than 10% of the nitrate content.

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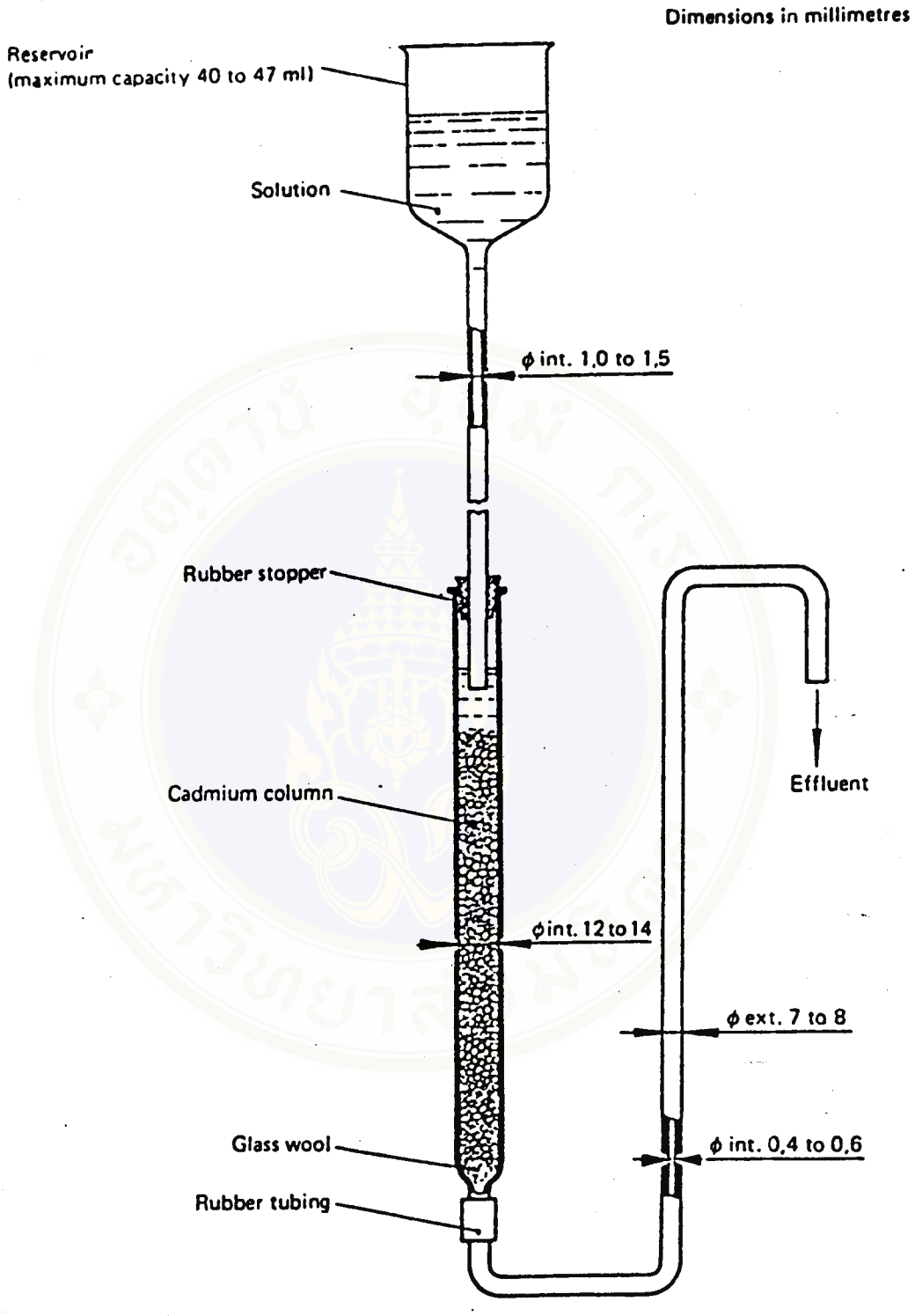

Figure 14 Apparatus for nitrate reduction

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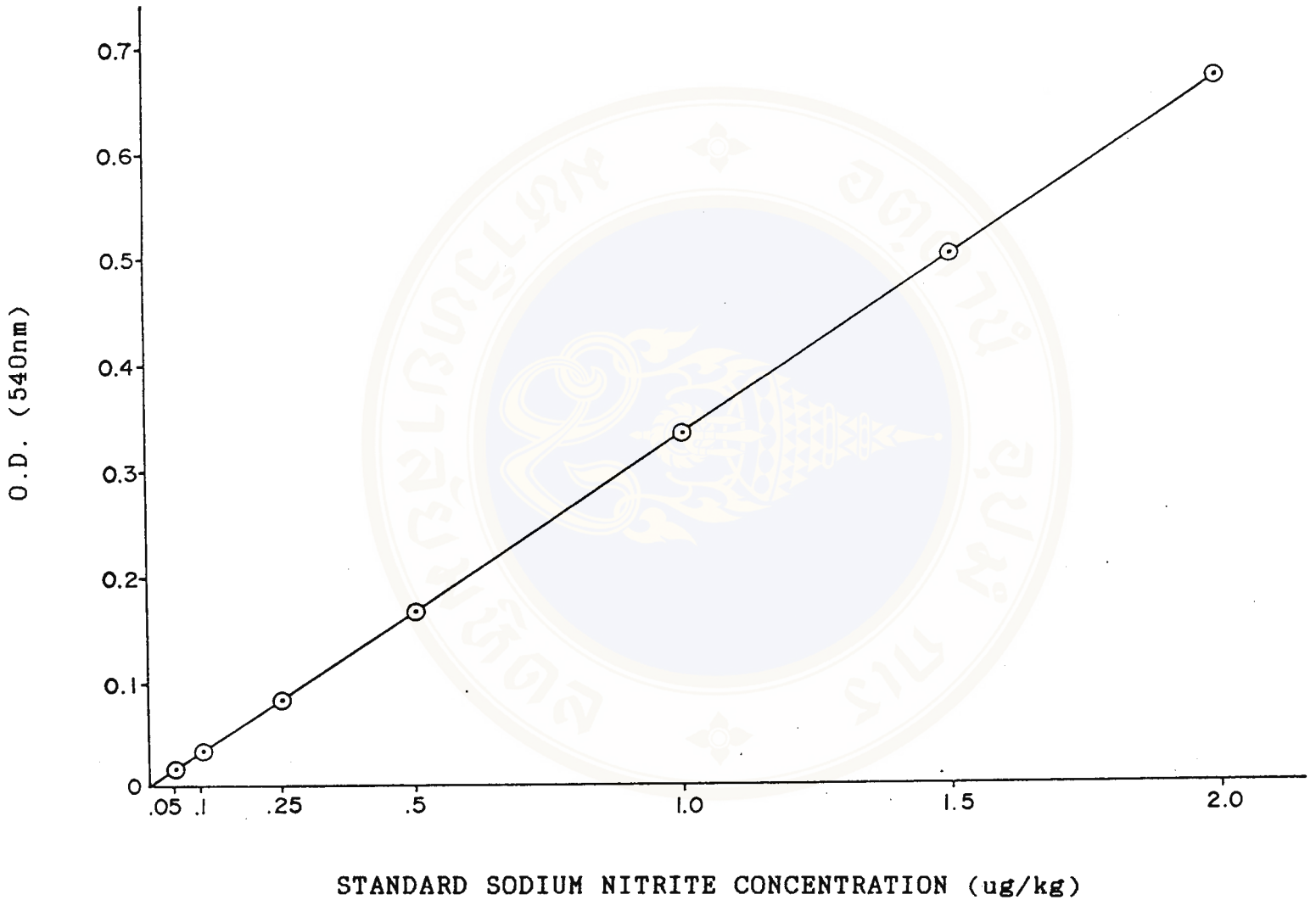

Figure 15 Calibration curve of standard sodium nitrite.

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Appdendix II

Procedure for Determination of Volatile N-nitrosamines(46).

1.Sample preparation:

a.Weigh 22.0 g of homogenized sample, add some water;

b.Add 5.5 ml of inhibitor(1.8 g of sulfamic acid and 10 ml of 10N H_2SO_4 in 50 ml of water) and leave for 5 minutes;

c.Adjust pH to about 7.5 with 10N NaOH; and

d.Weigh sample equivalent to 20 g of initial sample into 500 ml boiling flask with thermometer well.

2.Distillation:

Add paraffin oil 30 ml, follow by sufficient cotton wool. Avoid to contaminate the joint with sample, solvent or cotton wool.

Assemble the apparatus as shown in Fig. 16 Place flask in heating mantle. Use adapter to connect boiling flask to trap which was equilibrated in liquid nitrogen.

Connect the trap to vacuum and check for leaks as system comes to operate with pressure of less than 2 torr. Maintain vacuum for 5 minutes before applying heat.

Increase temperature in boiling flask, as indicated by termometer in oil filled well, from ambient

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to 120 °C. At the end of heating period, turn off heat and let the system stand for 10 minutes with vacuum maintained. Carefully release vacuum and move boiling flask away from heating mantle. Disconnect flask. Place trap with adapter attacked in hood. Remove adapter and rinse with 10 ml dichloromethan(CH_2Cl_2), collecting the rinse in trap.

3.Extraction:

Add 2 ml of 2N H_2SO_4 and 5 g of NaCl to 250 ml separator. Transfer distillate and CH_2Cl_2 to separator. Wash trap with 15 ml CH_2Cl_2 3 times. Each time of washing, shake for 1 minute and transfer CH_2Cl_2 to seperator and extract for 1 minute. Let the separator stand until the phases separated.

Drain each lower CH_2Cl_2 layer through 12 g of anhydrous Na_2SO_4 (held on filter paper in 60 ml gas funnel prewetted with 5 ml of CH_2Cl_2) into 125 ml modified K-D flask with 2 ml bottom tube. Add 0.5 ml isopropanal as keeper.

4.Concentration.:

Place the carborandum grains in K-D flask, attach 3 ball Synder column to it, and carefully concentrate solvent to about 1 ml in 55 °C water bath. Remove concentrator, let dry and air cool to ambient temperature, let remaining solvent in Synder column drain into K-D flask. Measure the final volumn of concentrated

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sample and transfer into 1.5 ml screw-cap vial with teflon seal, and store in a refrigerator.

5.Determination:

Inject a 5 µl aliquot of concentrate into GC-TEA by mean of 10 µl syring and quantify the observed peaks by compering chomatographic response to those of known reference standard. Feed the TEA signal into an electronic integrator (Spectra Physics,SP4270) which was programmed to calculate the result in ug/kg sample (ppb) for each compound(Fig. 17). And then correct the detected values according to the recoveries data of the coressponding VNAs and samples.

Recoveries of the method for volatile N-nitrosamines analysis were estimated by addition of 0.2 ml of standard mixture of six VNAs (NDMA, NDEA, NDPA, NPIP, NPYR and NMOR) to 20 g of food samples, at a concentration equivalent to 5.0 µg/kg (ppb). The result of percent recoveries were shown in Table 19.

The method of analysis was regularly checked by addition of 0.2 ml internal standard NDPA to all 20 g of samples analysed (equivalent to 5.0 µg/kg,ppb). And samples blank was also done to absence of NDPA in these samples.

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Table 19 Percent recovery of spiked external standard volatile N-nitrosamines (0.5 ppm) at 0.2 ml from 20 g food sample.

	Recovery (%)					
	NDMA	NDEA	NDPA	NPIP	NPYR	NMOR
range	75-98	78-85	76-91	86-97	84-99	75-93
mean	86	82	83	92	90	86
SD	8.76	2.64	6.12	4.66	6.7	7.22

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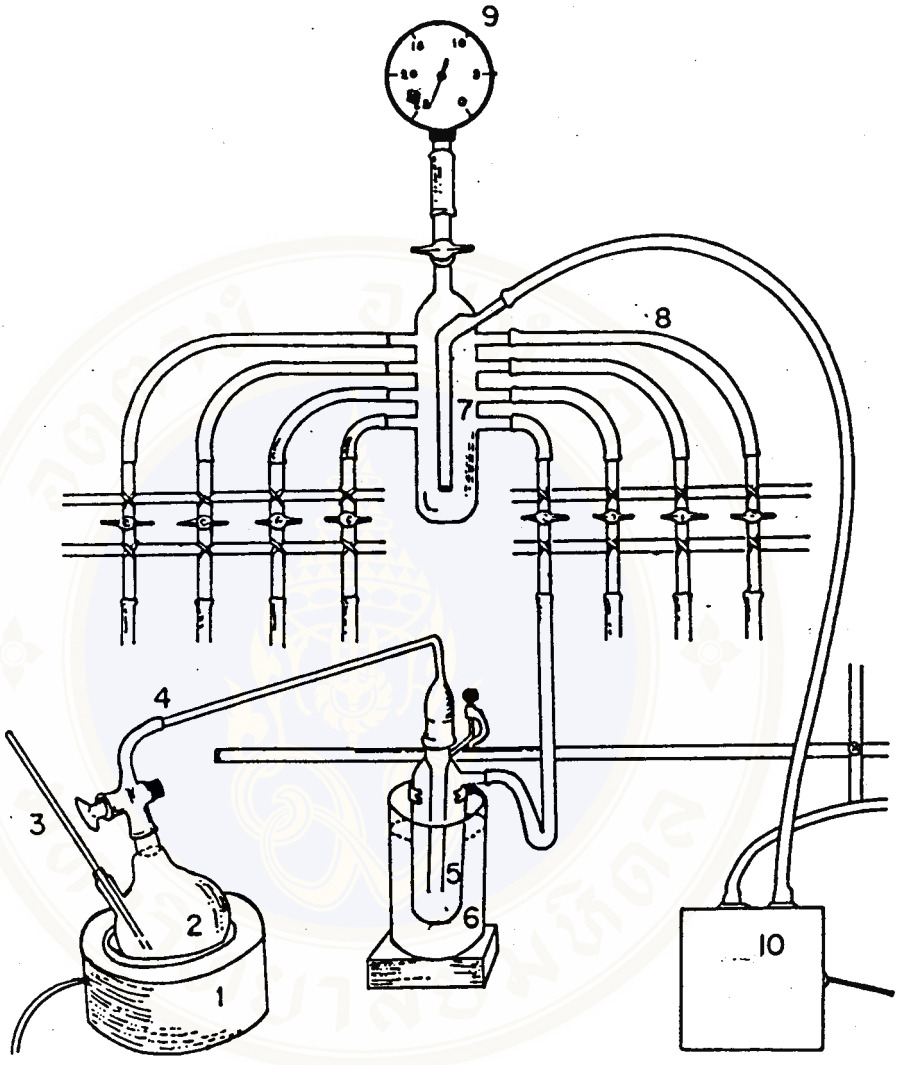

Figure 16 Modified Mineral Oil Vacuum Distillation Assembly Single system (Eight distillation set up may be used) :

(1) Electromantle, hemispherical of 500 ml capacity. (2) Round bottommed flask, 500 ml of 29/32 $ neck with 14/23 $ thermometer well; (3) Thermometer,100mm immersion, -10 to 250°C; (4) Adapter with stopcock ; (5) Vapor trap; (6) Thermos jar with liquid nitrogen;(7)Vacuum controller; (8) Vacuum tubing; (9) Barometer, expressed in mbar; (10) Hight vacuum pump.

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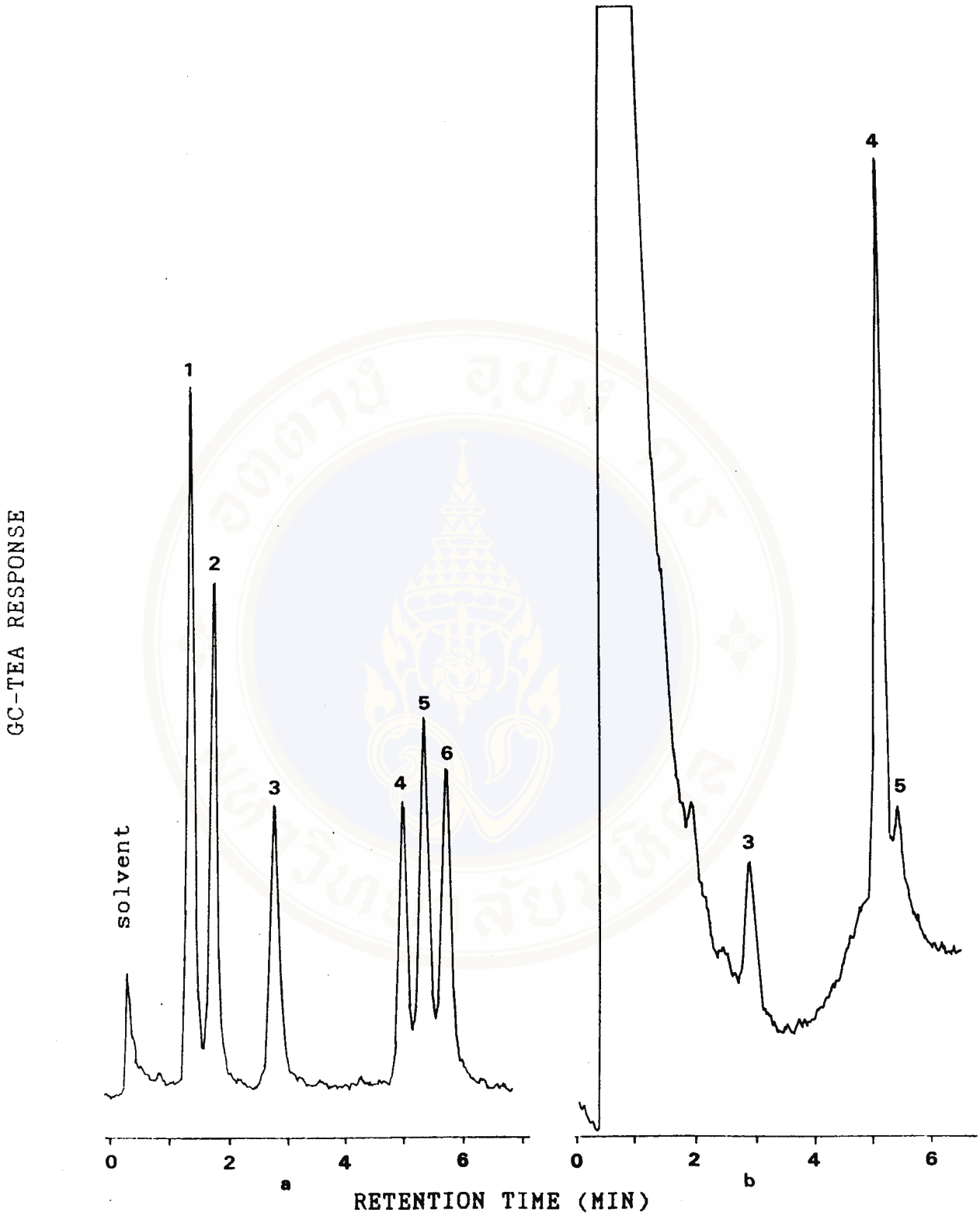

Figure 17

a) The chromatogram (GC-TEA) of 6 standard containing mixture 5 ppb each of the following volatile N-nitrosamines : (1) NDMA (2) NDEA (3) NDPA (4) NPIP (5) NPYR (6) NMOR.

b) The chromatogram showing GC-TEA response of sample spiked with internal standard NDPA.

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Appendix III

Procedure for Piperine Determination (48).

Briefly, weigh 5 g of homogenized sample into 50 ml centrifuge tube and extract sample 3 times with 20 ml $CH_3OH/H_2O(1/1)$ by shaking for 1 minute. After shaking, centrifuge each slurry and filter the supernatant through filter paper into a 250 ml Erlenmeyer flask. Transfer the supernatant to a 250 ml separator and extract 3 times with 30 ml CH_2Cl_2 by shaking for 1 minute. Let the separator stands until the phases separated. Drain each lower CH_2Cl_2 layer through 12 g anhydrous Na_2SO_4 (held on filter paper in 60 ml glass funnel prewetted with 5 ml of CH_2Cl_2) into 200 ml round bottommed flask. Evaporate the combined extracts to dryness under vacuum. Redissolve the residue with 5 ml of CH_3OH.

Inject 10 µl to HPLC. Isocratically elute all solution on a reverse phase Zorbax ODS C_{18} column (25.0cm 4.6mm inner diameter), with methanol:water(75:25) as the mobile phase at a flow rate of 1 ml/min. Operate the UV detector at 340nm. Construct a standard calibration curve for piperine tested and calculate the natural concentration from the calibration curve (Fig.18)

Recovery of 100 ppm piperine added to 5 g of food sample ranged from 63-87% with an overall average of

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73 ± 9.13% (mean ± SD). The sample results were corrected according to the recovery data.

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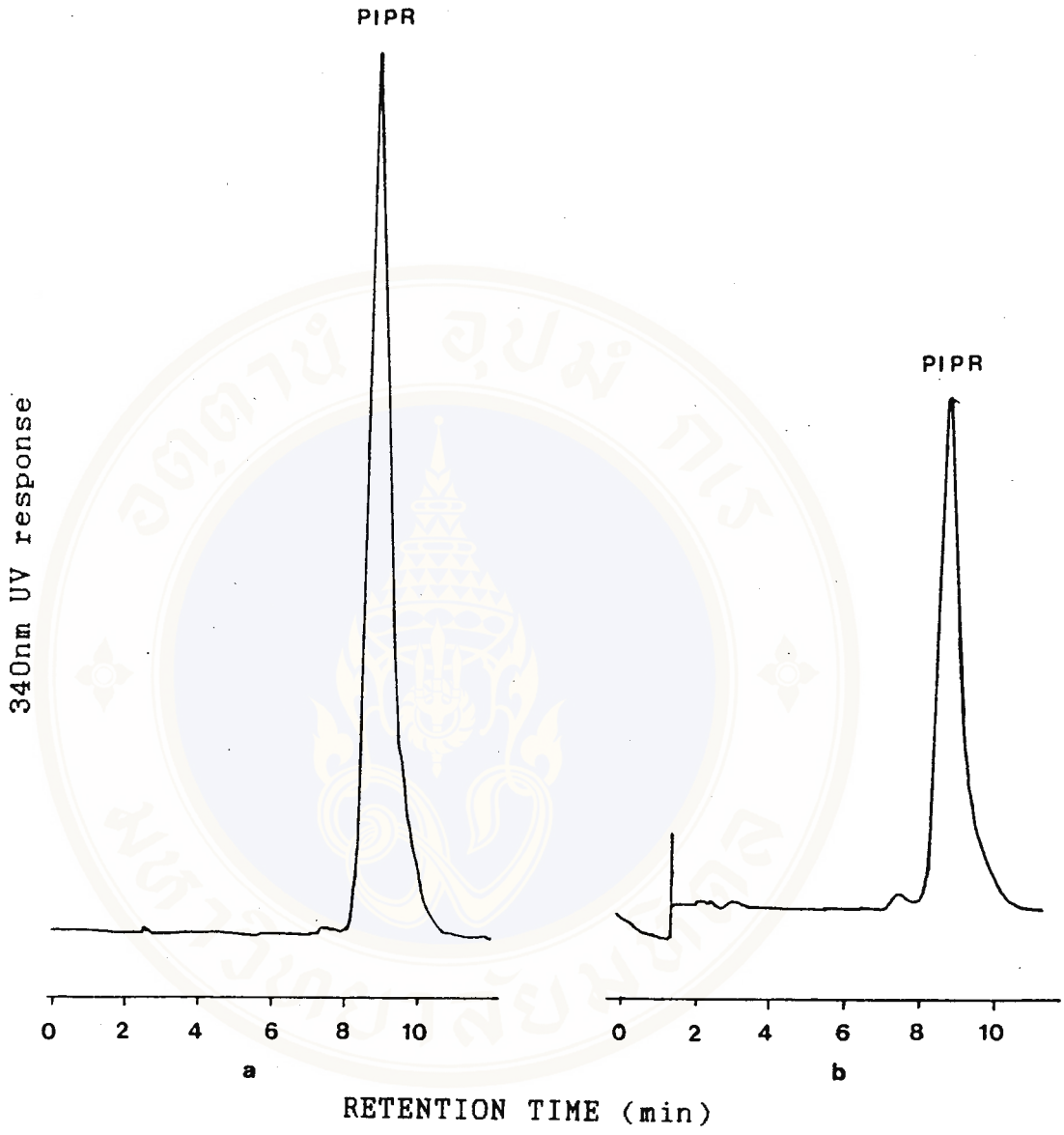

Figure 18 (a) The chromatogram (340nm UV) of standard PIPR 100 ppm.
(b) The chromatogram showing 340nm UV response of sample.

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Appendix IV

Procedure for Piperidine Determination (49).

Briefly, weigh 5 g of homogenized sample into 50 ml centrifuge tube and extract sample 3 times with 0.6N $HClO_4$ (pH 1.1) by shaking for 1 minute. After shaking, centrifuge each slurry and filter the supernatant though filter paper into a 250 ml Erlenmayer flask. Add 10 ml borate buffer to the combined clear extracts and adjust to pH 10 with 1N NaOH. Add a ten fold excess of 10% DNFB in acetone (relative to the largest amount of expected piperidine in the sample) and allow the solution to react for 40 min at 65°C in water-bath. Transfer the cool solution to a 250 ml separator and extract 3 times with 30 ml CH_2Cl_2 by shaking for 1 min. Let the separator stand till the phases separated. Drain lower organic layer through 12 g anhydrous Na_2SO_4 (held on filter paper in 60 ml glass funnel prewetted with 5 ml of CH_2Cl_2) into 250 ml round bottommed flask. Evaporate the combined extracts to dryness under vacuum. Redissolve the residue with 5 ml of CH_3OH.

Inject 50 µl to HPLC. Isocratically elute on a reverse phase 3µ Zorbax C_8 column (8.0cm × 6.2mm inner diameter), with methanol:water(62:38) as the mobile phase at a flow rate of 1.5 ml/min. Operate the UV detector at 368 nm Construct a standard calibration curve for piperidine tested and calculate the natural concentration

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from the calibration curve. (Fig.19).

Note: The study of recovery of piperidine: Added standard piperidine in high concentration recovery values were better than in added low concentration. For example, recovery of piperidine added at 50 ppm to 5 g of food samples ranged 72-76% with an overall average of 74%, but recovery of piperidine added at 5 ppm in the same way ranged 48-59% with and overall average of 55% , obtained the difference may be dued to the problem in extraction step, because when extracted 3 times with CH_2Cl_2 the emalsion formed and the phases separated not so well. Then piperidine could be loss in this step which made recovery decrease if the concentration of piperidine in the sample was very low. In the survey samples study all piperidine detected values were not above 1 ppm, so in this study the samples were corrected by using recovery value of 55%.

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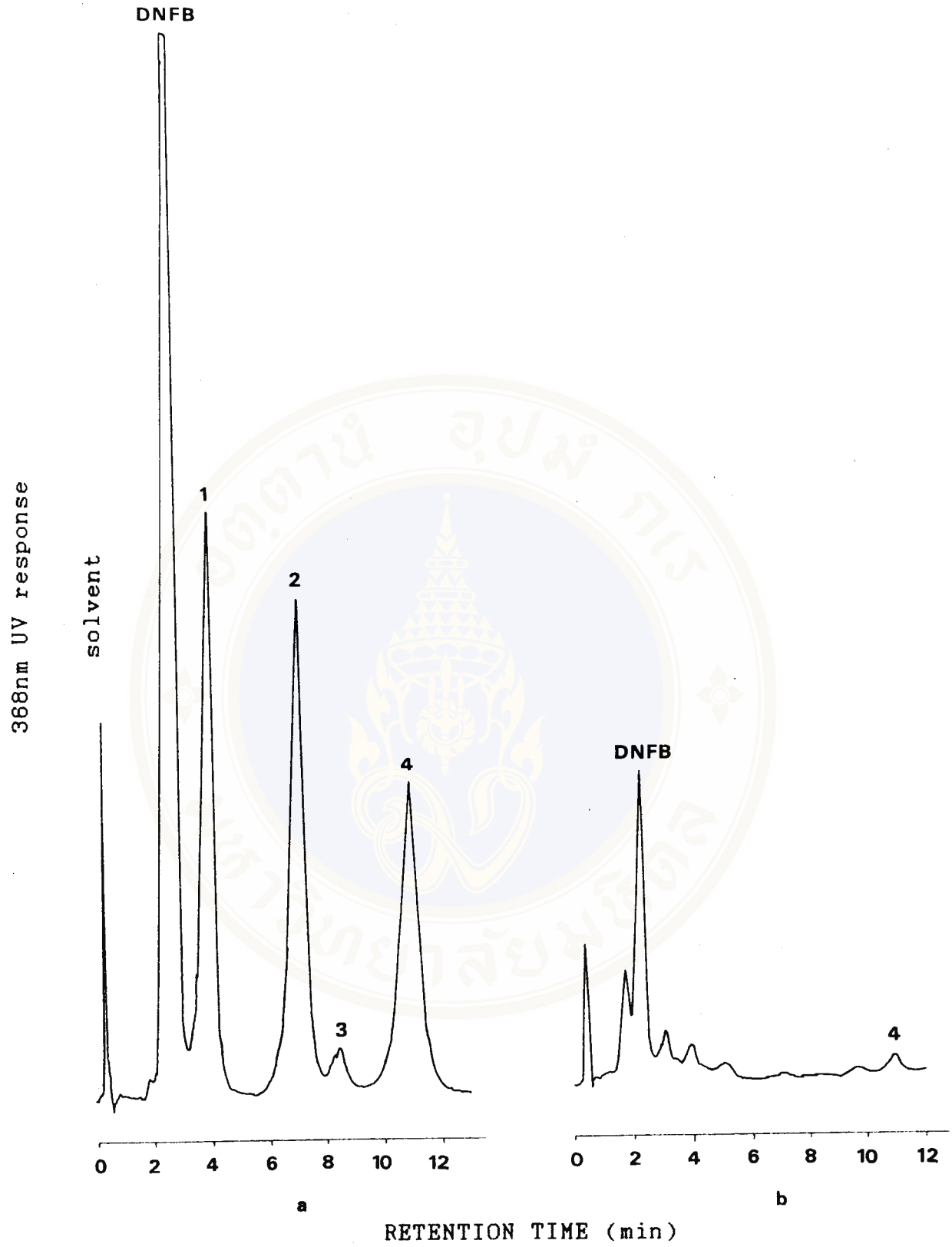

Figure 19 (a) The chromatogram (368nm UV) of 4 standard containing mixture 5 ppm each of the following amines : (1) DMA, (2) PYR, (3) DEA (4) PIPD.

(b) The chromatogram showing 368nm UV response of sample.

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Appendix V

r Values between NPIP and Residual Nitrite, Nitrate, Piperine and Piperidine of SS-1, SS-2, SS-3, SS-4 and SS-5 are :

SS-1 r values between NPIP and Nitrite = -0.5326
Nitrate = -0.1927
Piperine = -0.1586
Piperidine = 0.1431

SS-2 r values between NPIP and Nitrite = -0.1588
Nitrate = -0.5654
Piperine = 0.9592
Piperidine = 0.9771

SS-3 r values between NPIP and Nitrite = 0.3278
Nitrate = 0.5763
Piperine = -0.6068
Piperidine = -0.0463

SS-4 r values between NPIP and Nitrite = 0.1958
Nitrate = 0.0946
Piperine = 0.3093
Piperidine = -0.3188

SS-5 r values between NPIP and Nitrite = 0.2337
Nitrate = -0.1265
Piperine = 0.0604
Piperidine = 0.8305

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