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Supercritical fluid extraction of N-nitrosamines

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SUPERCRITICAL FLUID EXTRACTION OF N-NITROSAMINES

by

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ABSTRACT

SUPERCRITICAL FLUID EXTRACTION OF N-NITROSAMINES

by

Suresh Tewani

Advisor: Professor David C. Locke

A detailed review of N-nitrosamines is presented in the first chapter of this thesis. The detailed chemistry of N-nitrosamines including the mechanism of carcinogenicity, modes of formation, inhibition and destruction are discussed in detail. The occurrence and risk assessment of human exposure of these suspect cancer agents is described along with practical considerations for minimizing their effects. The methods of isolation and analysis of N-nitrosamines are critically discussed with special emphasis on Gas Chromatography (GC) - Electrochemical Detectors, GC-High Resolution Mass Spectrometry (GC-HRMS), GC - Thermal Energy Analyser (GC-TEA).

In the second chapter, the theoretical concepts of supercritical fluid extraction (SFE) are discussed in general. A comparison of physical and chemical properties of supercritical fluids (SF) with other phases is given. A comparison of static, dynamic, recirculating SFEs shows the versatility of this technique. The advantages over conventional methods such as Soxhlet extraction are also discussed. The instrumentation and operational technique of a laboratory built supercritical fluid extractor is explained. A brief review of applications (industrial and analytical) of SFE is given at the end of second chapter.

The evaluation of SFE for analytical sample preparation and analyses of volatile N-nitrosamines (VNAs), tobacco specific N-nitrosamines (TSNAs), and N-nitrosodiethanolamine (NDELA) from spiked matrices, tobacco and cutting fluids is presented in the third chapter. Experimental parameters such as pressure, temperature, equilibration time, collector materials, modifier are evaluated to optimize the recoveries. The extracts are analysed by GC-TEA which is already proven to be highly selective and sensitive to for quantitation of N-nitrosamines. The extraction of VNAs in pure SF-CO_2 is quantitative at moderate pressures and low temperatures. However, for the extraction of TSNAs and NDELA, it is imperative to have high pressures and presence of an additive (~ 10% methanol) to achieve satisfactory results.

Overall, this study offers a simple, rapid, accurate and environmentally advantageous sample preparation technique for the estimation of N-nitrosamines at nanogram levels as an interim alternative to existing analytical methodology which offers only complex, time-consuming procedures.

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CHAPTER 1

1.1 Importance

One of the most potent classes of chemical carcinogens are collectively called the N-nitroso compounds. The problem of chemical carcinogenesis is an area of intense research activity, and there is a general agreement within this community of investigators that a very large percentage of human cancers may be the result of accidental, continued exposure to various chemicals in man's enviroment (1,2,3,4,5). The World Health Organization (WHO), through the International Agency of Cancer (IARC), estimates that over 80% of all cancers in man may be due to environmental factors (6). The general area of environmental chemical carcinogenesis has been discussed by many active researchers in recent years (7,8,9,10). Cairns has stated that : "Almost all cancers appear to be caused by exposure to factors in the environment. The most promising approach to the control of disease is to identify those factors and eliminate them (11)". Because chemical carcinogens are present in minute levels, normally ppb levels, the reliable detection is heavily dependent upon the methods and instrumentation found in modern analytical chemistry.

Since existing analytical methodology offers only complex time-consuming procedures, the present project is focused to develop a simple, rapid, accurate, reproducible and environmentally advantageous sample preparation technique for the estimation of N-nitrosamines at nanogram levels. The use of carbon dioxide as a solvent eliminates all the complications associated with routinely used organic solvents.

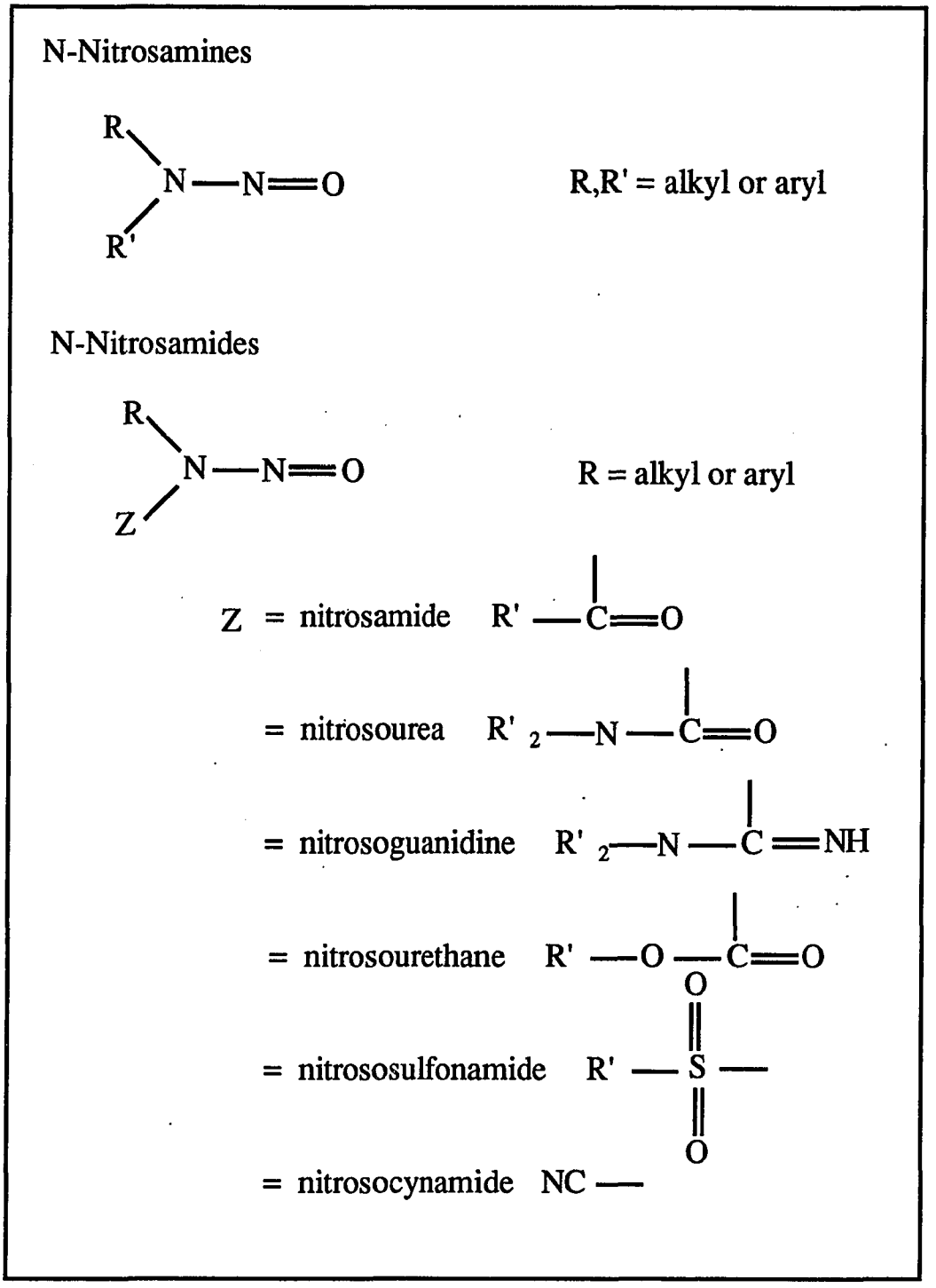
1.2 Type of N-Nitroso Compounds

N-Nitroso compounds are formed by the interaction of a nitrogen-containing organic compound, such as an amine, amide, urea, guanidine, urathane or cyanamide, and a nitrosating agent , such as nitrogen oxide. These compounds can be divided into two categories (Figure 1.1) namely nitrosamines and nitrosamides which differ in their chemical stability, and in the mechanism of their carcinogenicity and mutagenicity (12,13,14). The nitrosamines are very stable once they are formed. They require chemical modification in an enzyme-catalysed in vivo reaction before they exhibit carcinogenic and mutagenic activity. By comparison the nitrosamides can be hydrolysed , especially in neutral and alkaline solution. They exhibit carcinogenic and mutagenic activities without modification and malignant tumors are produced at the site of their application (15).

1.3 Carcinogenicity of N-Nitrosamines

The first report that nitrosamines cause cancer in the laboratory animals was that rats fed low levels of dimethylnitrosamine in their diet developed liver cancer (16,17). Since then more than 130 N-nitroso compounds have been tested thus far, over 100 of these produced tumors in one or more organs and were found to be carcinogenic in wide variety of animals. Although there is no direct evidence that N-nitroso compounds tested cause cancer in man , their carcinogenicity has been demonstrated in many other animal species including mice, rats, hamsters, fish, rabbits, guinea pigs, dogs and monkeys (14,15,18). Only about 20, mostly volatile, N-nitrosamines have been studied extensively. The type of tumor depends on one or more of the following:

Figure 1.1 Types of N-Nitroso Compounds



a. Structure of compound to be tested

b. Species in which it is tested

c. Dose at which it is administered

d. Route of administration.

Comprehensive reviews of results have been published (12,14,15).

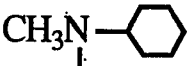
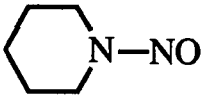
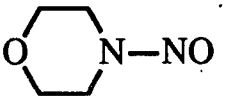
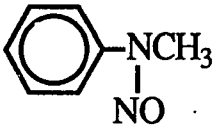
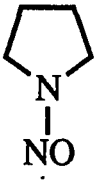
To illustrate the range of activity, representative nitrosamines are classified in Table 1.1 according to carcinogenic potency (2). The carcinogenic dose is expressed in the way suggested by Wishnok et al. so that larger numbers indicate higher carcinogenicity (19).

Wishnok and coworkers demonstrated that the carcinogenic potency of many nitrosamines correlates quantitatively with a combination of their hexane-water partition coefficients and the electronic inductive effects of substituents on the α-carbon (20). Earlier Wishnok and Archer showed that carcinogenicity is inversely related to the number of carbon atoms of acyclic dialkyl nitrosamines (20). Lijinsky found that the reverse is true for cyclic nitrosamines, where larger molecules are more potent, and that there are major changes in target organs with a change in ring size (21). Recently Klopman (22) extended the use of Computer Automated Structure Evaluation program to predict carcinogenicity of 69 N-nitrosamines. The agreement of results with experiment is satisfactory (22) and this method appears to be useful to correlate and predict carcinogenicity.

1.4 Mechanism of Carcinogenicity

The frequently proposed action shown in Figure 1.2 accounts for the enzymatic activation required by nitrosmines, but not by nitrosamides, and indicates that only nitrosamines containing an α-

Table 1.1 Carcinogenic Activity of Nitrosamines

Range of log $(1/D_{50})^a$	Structure	D_{50}^a	$\log(1/D_{50})$
Highly Potent,>3.0	$(CH_3CH_2)_2NNO$	0.00063	3.2
	$CH_3N(NO)CH_2CH_2Cl$	0.00061	3.2
	$CH_3N(NO)CH_2C_6H_5$	0.00080	3.1
Potent, 2.1-3.0		0.00010	3.0
	$CH_3N(NO)CH_2CH_2N(NO)CH_3$	0.0039	2.4
	$(CH_3)_2NNO$	0.0054	2.3
	$(n\text{-}C_3H_7)_2NNO$	0.0088	2.1
Intermediate Activity, 1.1-2.0	$(NCCH_2)_2NNO$	0.012	1.9
		0.012	1.9
		0.011	1.9
		0.025	1.6
		0.039	1.4
Minimal Activity,<1.0	$(i\text{-}C_3H_7)_2NNO$	0.11	1.0
	$(CH_3COOCH_2CH_2)_2NNO$	0.18	0.7
	$(i\text{-}C_5H_{11})_2NNO$	0.26	0.6
	$(HOCH_2CH_2)_2NNO$	0.89	0.05

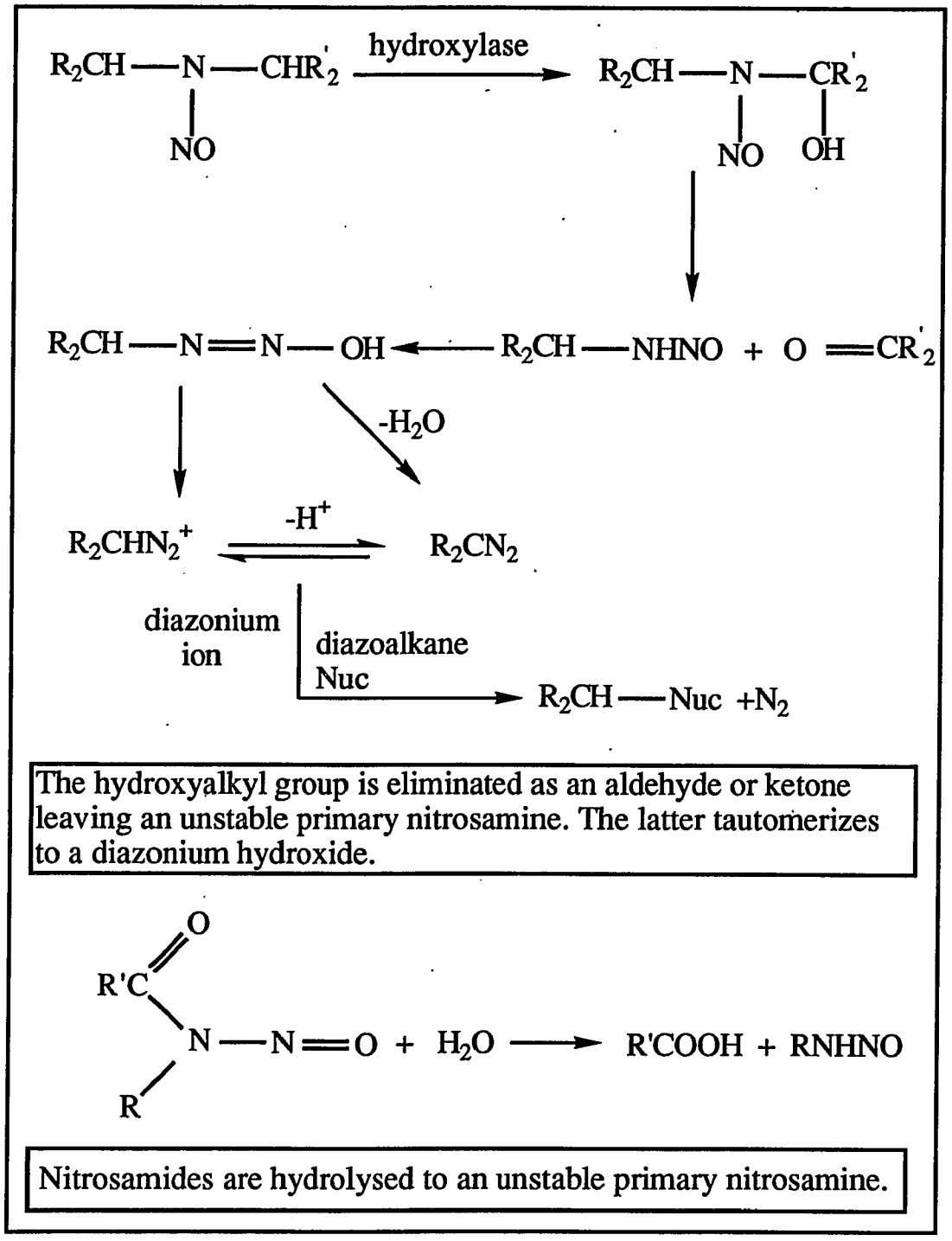
D_{50}^a = *mean total carcinogenic dose, expressed in mol/kg body wt, for production of tumors in 50% of the animals.*

hydrogen are carcinogenic (12,13,23). The requirement for activation of nitrosamines is defined as an enzyme-catalysed hydroxylation of an α-carbon. This step is supported by correlations of the degree of carcinogenicity with α-carbon substituents (20) and by another report showing that preformed α-acetoxy nitrosamines are direct-acting carcinogens not requiring enzymatic modification for activity (24).

It has been demonstrated that like most other chemical carcinogens nitrosamines cause structural alterations in target cell DNA, RNA and proteins by alkylation of nucleophilic sites (14,25). Under in vivo conditions nitrosamines give rise to highly reactive electrophiles which cause the covalent binding of alkyl residues to electron-rich O, N, and S atoms in cellular macromolecules. About a dozen different nucleophilic O and N atoms in DNA are well documented sites of alkylation by nitrosamines (26). The DNA alkylation products O^6-alkyl-2'-deoxythymidine are of particular interest, because the persistence of these DNA lesions through DNA replication can lead to transition mutations due to anomalous base-pairing (27). These and other genetic consequences of structural modifications in genomic DNA is of critical importance in relation to carcinogenesis. In nucleic acids the principle site of alkylation is N(7) of guanine. Alkylation of nucleic acid oxygens has also been demonstrated (28).

Nitrosamides do not require metabolic activation because they can be hydrolysed in vivo to an unstable primary nitrosamine (Figure1.1), the proposed precursor of the alkylating agent (12,13).

Figure 1.2 Mechanism of Carcinogenicity



1.5 Chemistry of N-Nitrosamines

The chemistry of N-nitroso compounds in aqueous solution may be summarized by a set of equations as illustrated in Figure 1.3 . Nitrosation of secondary amines is described by equation 1. The effectiveness of nitrosating agent Y-NO depends on the nature of Y. Catalysis of nitrosation by Y' species results from its prior reaction with Y-NO (eq. 3), which produces the more active nitrosating agent Y'-NO. The irreversible inhibition of nitrosation occurs by reaction of inhibitor Z with nitrosating agent Y-NO , as shown in equation 4, which is much faster than 1 and produces unreactive products. Destruction of N-nitroso compounds by denitrosation is described by equation 2. Addition of Z, in this case termed a scavenger or trap, is necessary to prevent via 4 the reversal of denitrosation, equation 1. The details of these reactions are described below in brief.

1.5.1 Formation

1.5.1.1 Nitrosating Agents

1.5.1.1.A Inorganic species : Several nitrogen oxide species are nitrosating agents, but nitrous acid (HONO) and the nitrite ion (ONO^-) are themselves inactive (29). The nitrosating species are shown in Table 1.2. The interrelationship between active nitrosating agents (bold) and inactive species is summarized in Figure 1.4. In moderately acidic aqueous nitrite solutions the nitrosating agent is nitrous anhydride, N_2O_3 formed from nitrous acid, pK_a = 3.138 at 25°C, after protonation of nitrite ion according to equation 5 and 6. At lower pH, more rapid nitrosation by nitrous acidium ion (equation 7) becomes more important (30,31,32,34). Certain anions, Y^-, catalyse the reaction in water by forming nitrosating species Y-NO (equation 8) which are

Figure 1.3 Chemistry of N-nitroso Compounds

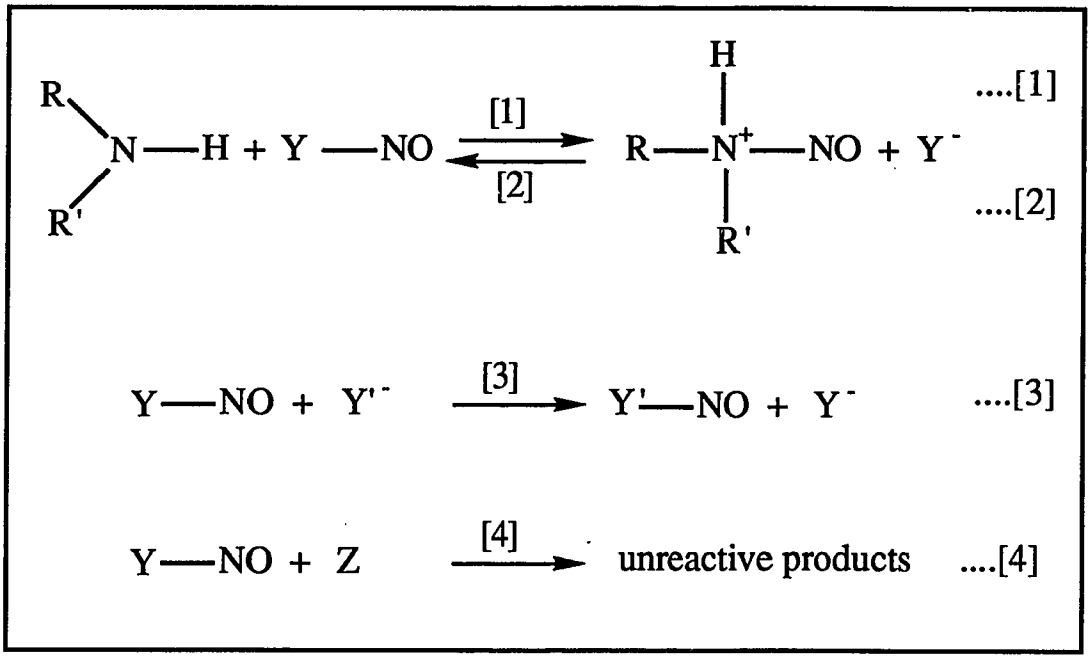
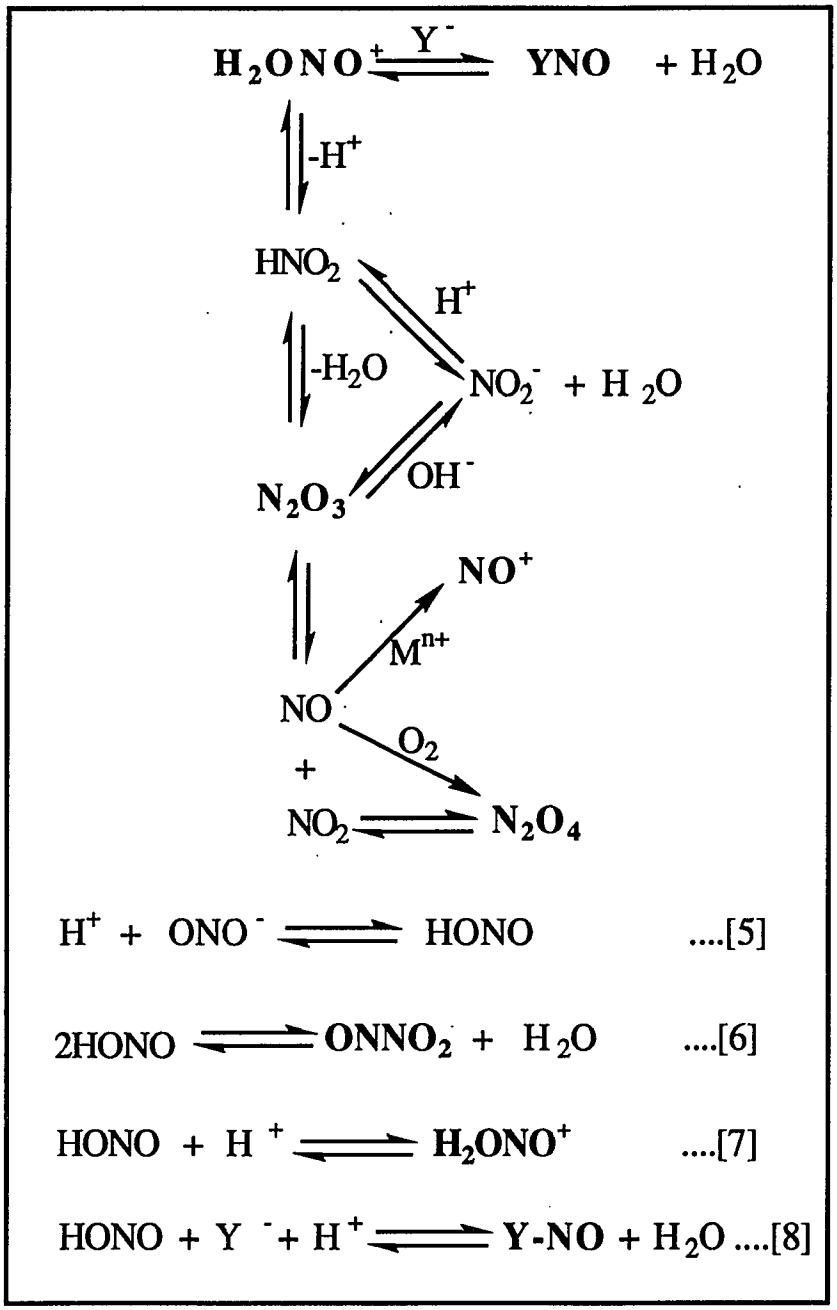


Table 1.2 Inorganic Nitrosating Agents

Substance	Medium
N_2O_3	Gas (30)
	Water (30,)
	Organic solvent (12)
NO_2 / N_2O_3	Gas (31)
	Water (32)
	Organic solvent (32)
YNO	Water (30,33,34)
H_2ONO^+	Water (30,34,35)
NO	With O_2 (32)
	Anaerobic, M^{n+} (30,32)

Figure 1.4 Interrelationship between Nitrosating Agents



more reactive than N_2O_3. Of the anionic catalysts studied thiocynate has the greatest effect. The halide ions are also catalytic in the order SCN^-,I^- >> Br^- > Cl^- (30,33,34,35,36). Nitric oxide (NO) alone is inactive but is oxidised by oxygen to NO_2 and thus to the reactive nitrosating agents N_2O_3 and N_2O_4 (32,37). Rapid nitrosation by NO under anaerobic conditions occurs in the presence of iodine or Ag(I), Cu(I), Cu(II), Zn(II), Fe(III), or Co(II) salts (30,32).

1.5.1.1.B Organic species : N-nitrosamines themselves act as nitrosating agents. Aromatic nitrosamines, such as nitroso-diphenylamine, transnitrosate secondary amines under neutral conditions in organic solvents via free radical mechanism. The process is more rapid in acidic aqueous solution and occurs by a heterolytic mechanism (38,39). The slower transnitrosation between aliphatic secondary amines requires more extreme conditions or catalysis by nucleophilic agents, such as thiocyanate and halide ions (40).

Nitrosation of morpholine by aromatic and aliphatic C-nitro compounds in tetrahydrofuran at 70°C has been reported (41). However it is difficult to ascertain whether nitrosation occurs by direct reaction of amine with the C-nitro function or is caused by agents derived from inorganic nitrite present as a synthetic contaminant or decomposition product. Primary and secondary nitroalkanes decompose to nitrite in dilute alkaline solutions (42).

1.5.1.2 Nitrogen Compounds

1.5.1.2.A Primary amine : The well-known deamination of primary aliphatic amines with nitrite in cold aqueous acid yields a variety of products (34). The rapid reaction proceeds through unstable primary

nitrosamine and diazonium ion intermediates as shown by equation 9 (Figure 1.5). The latter reacts with nucleophiles present to form substitution, elimination and rearrangement products. Secondary amines and subsequent nitrosamines formed by reaction of diazonium ion with the primary amine starting material have been isolated (equation 10). This reaction occurs in low yield because the amine is largely protonated and unreactive under the strongly acidic and low temperature conditions commonly used.

Diamines with a second primary amine function appropriately located for intramolecular reaction with diazonium ion form secondary nitrosamines at high temperatures or long reaction times as illustrated by equation 11 in Figure 1.5 (43).

Higher levels of stable α-alkoxynitrosoamines (equation 12) are produced from the reaction of primary amines with aldehydes in the presence of alcohols and nitrite under mildly acidic conditions (44).

1.5.1.2.B Secondary amines : Nitrosamines produced directly from secondary amines are stable. In moderately acidic aqueous nitrite solutions, N_2O_3 formed from two molecules of HNO_2 (equation 6), is the nitrosating agent. The rate determining step in the reaction is electrophilic attack by N_2O_3 on the free electron pair of the unprotonated amine (equation 13). The rate equation describes the kinetics :

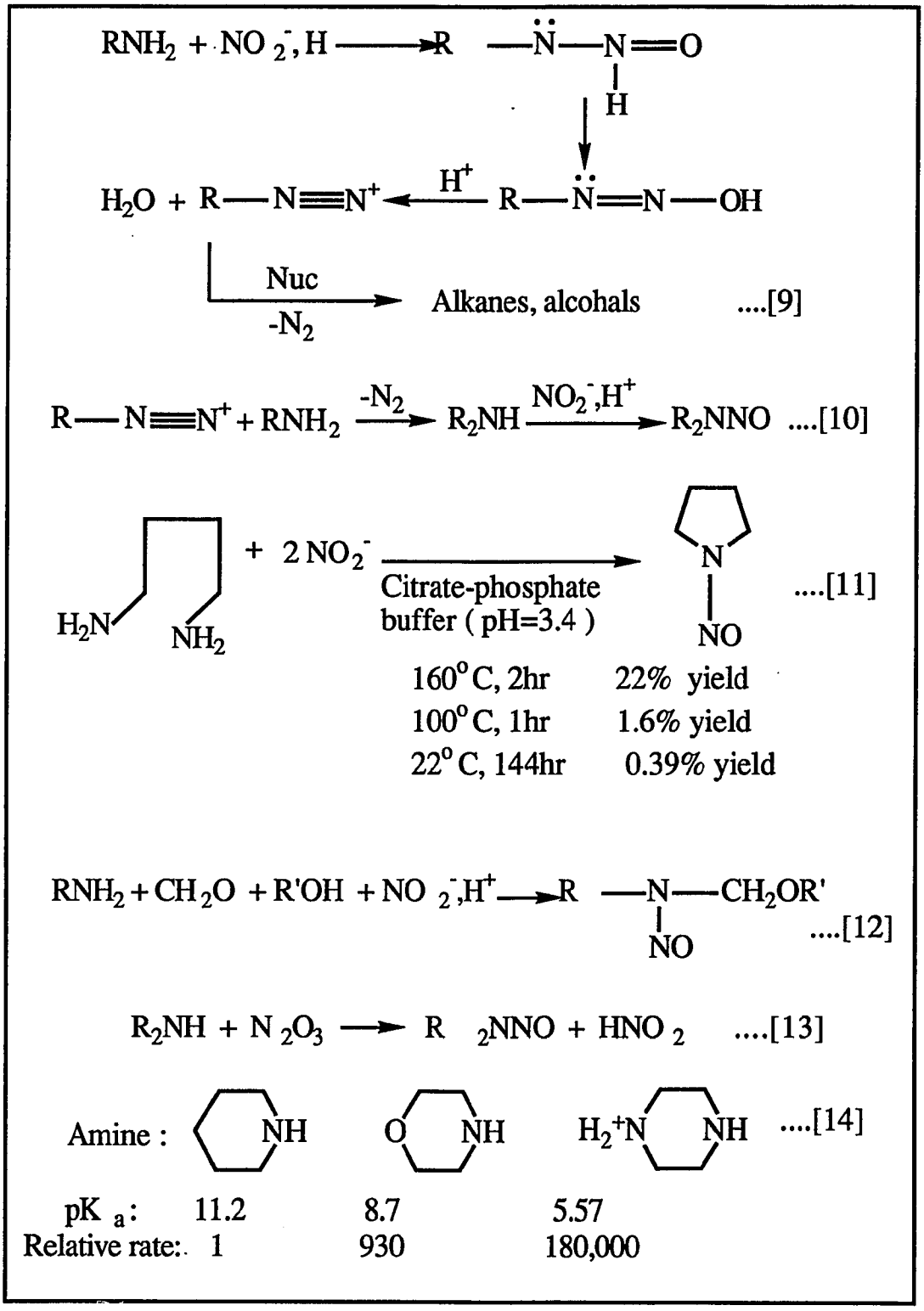
$$\text{rate} = k\,[R_2NH]\,[HNO_2]^2$$

Thus, two factors determine the effect of pH on the rate of nitrosation:

(i) extent of conversion of NO_2^- to HNO_2 and thus to N_2O_3

(favored by lower pH values)

Figure 1.5 Reactions of Nitrogen Compounds



(ii) concentration of unprotonated amine

(favored by higher pH values)

As predicted by the rate law for simple basic amines (pK_a>5), the nitrosation rate in water is maximum at pH_{max} = 3 to 3.4 near the pK_a of HNO_2 (45). For a given amine, as the pH increases above pH_{max} the rate decreases, because the concentration of HNO_2 decreases. As the pH decreases below pH_{max} the rate decreases, because the concentration of unprotonated amine decreases. At a given pH the rate of nitrosation increases as the basicity of amine decreases, e.g. equation 14, because of the higher relative concentration of unprotonated amine present.

Nitrosation of secondary amino acids occurs at an optimum pH_{max} = 2.25 to 2.5 (36,46). The reaction follows the rate law mentioned above but the pH-rate profile is changed by the fact that two amine species react - $RNHCHR'COO^-$ and RNHCHR'COOH.

1.5.1.2.C Tertiary amines : Tertiary amines have generally been regarded as inert to nitrosation, even though their conversion to secondary nitrosamines was reported over 100 years ago (47). Because dealkylation occurs to a significant extent only at elevated temperature in weakly acidic media (48,49). At 25°C and pH 3.4 nitrosation of tertiary amines is about 10,000 times slower than that of related secondary amines (50).

1.5.1.2.D Quaternary amines and amine oxides : Quaternary ammonium compounds apparently react slowly with nitrite in acidic media. The initial dealkylation required accounts for their lower activity compared to tertiary amines and may not involve nitrosating agents. The relative reactivity of secondary, tertiary and quaternary amines is

indicated by the following data gathered for reaction of a ratio of 5 mol $NaNO_2$/mol amine at 78°C and pH 5.6 for 4 hr :

Amine	Yield of $(CH_3)_2NNO$
$(CH_3)_2NH$	9.6
$(CH_3)_2N$	0.9
$(CH_3)_2N^+$	0.6
$(CH_3)_2NCH_2CH_2OH$	1.6
$(CH_3)_2N^+CH_2CH_2OH$	0.0002

Several naturally occurring quaternary ammonium compounds were found be be much less reactive than the tetramethylammonium ion (51).

Tertiary amine oxides in the presence of excess nitrite at pH 1 to 3 and temperatures from 25 to 75°C are converted to secondary nitrosamines to a greater extent than are tertiary amines. However, at 90 to 100°C and pH 4 to 5 both classes show similar reactivity (50, 52).

1.5.2 Inhibition of Nitrosation

Studies of nitrosamine inhibition have consisted of the use of substances which compete with the amine for nitrosating species. The reduction potentials of various nitrogen oxides listed below can aid in selecting appropriate oxidizing and reducing agents for destruction of nitrite.

In acid solution:

Reaction	E° (volts)
$HNO_2 + H_2O = NO_3^- + 3\,H^+ + 2\,e^-$	-0.94
$NO + H_2O = HNO_2 + H^+ + e^-$	-0.99
$N_2O + 3\,H_2O = 2\,HNO_2 + 4\,H^+ + 4\,e^-$	-1.29

In basic solution:

$$NO_2^- + 2\,OH^- = NO_3^- + H_2O + 2\,e^- \qquad -0.01$$

$$NO + 2\,OH^- = NO_2^- + H_2O + e^- \qquad +0.46$$

$$N_2O + 6\,OH^- = 2\,NO_2^- + 3\,H_2O + 4\,e^- \qquad -0.15$$

In Table 1.3 are summarizied few important examples of effect of presence of inhibitor on the formation of nitrosamines in amine-nitrite systems. A brief discussion of most commonly used inhibitors is given below.

1.5.2.1 Inhibition by ascorbic acid : Ascorbic acid inhibits nitrosamine formation by rapid reduction of the nitrosating agent (53), (equation 15; Figure 1.6). Since the product NO can be air-oxidized to the nitrosating agent N_2O_4, excess ascorbic acid must be added to inhibit nitrosation in systems exposed to air. It inhibited the toxic and carcinogenic effects attributable to in vivo nitrosamine formation with two exceptions. In one case adenoma induction by N-nitrosomorpholine and mononitrosopiperazine increased with added ascorbic acid (54). In another it inhibited in vivo synthesis of N-nitrosomorpholine in rats and consequent liver tumors, but enhanced forestomach papillomas and carcinoma (55).

1.5.2.2 Effect of phenol : In most cases phenols inhibited nitrosamine formation, but sometimes their presence intensified nitrosamine production. In systems containing nitrite, phenols and secondary amines several reactions compete:

— formation of quinones (equation 16)

— formation of C-nitrosophenols (equation 17)

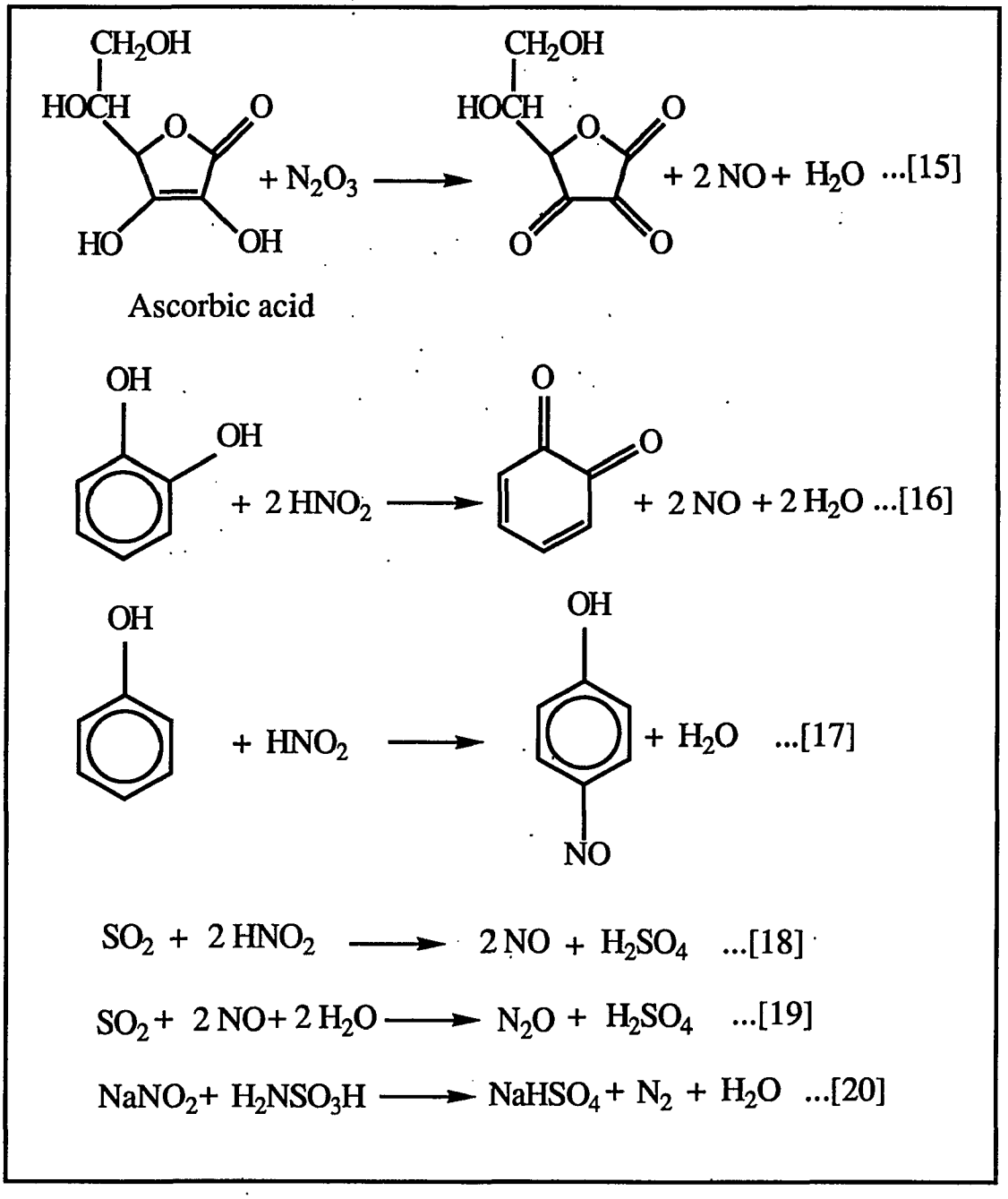
— direct formation of N-nitrosamines

— phenol-catalysed formation of N- nitrosamines

Table 1.3
Inhibition of Nitrosamine Formation in Amine-Nitrite System

Amine	System Studied *[Inhibitor]*	Effect of Inhibitor
Proline	Fried,nitrite cured bacon *[Ascorbic Acid]*	Inhibits formation of nitrosopyrrolidine
Dimethylamine Pyrrolidine Piperidine	Meat-curing mixtures. *[Ascorbic Acid]*	Inhibits nitrosamine formation
Dimethylamine	Acute tox., Rats *[Ascorbic Acid]*	Hepatic necrosis inhibited Got elevation inhibited
Dimethylamine	Cigarettes *[α-Tocopherol]*	Nitrosodimethylamine formation inhibited
Dimethylamine	In vitro *[Gallic Acid]*	Inhibition or catalysis depends on pH & conc.
Morpholine	Adenoma induct.,Mice *[Gallic Acid]*	Adenoma strongly inhibited
Dimethylamine Morpholine	In vitro *[Ammonium Sulfamate]*	Inhibits nitrosamine formation
Piperazine	Human Gastric Juice. *[Sulfamic Acid]*	Inhibits nitrosamine formation
Piperazine Morpholine	In vitro *[Urea]*	Inhibitory effect decreases with time
Morpholine	Lung adenoma,Mice *[Caffeine]*	Moderately inhibited
Dimethylamine	In vitro *[Vanillin]*	Inhibits nitrosamine
Dimethylamine	In vitro *[Cysteine]*	Inhibits nitrosamine

Figure 1.6 Inhibition of N-Nitrosamine Formation



Inhibition of nitrosamine formation by phenols occurs by reduction of nitrite to unreactive nitric oxide (56) or by removal of nitrite via C-nitrosation (57) as shown in Figure 1.5. Whether a phenol inhibits or catalyses nitrosamine formation largely depends on the relative concentration of nitrite and phenol. Excess nitrite C-nitrosates the phenol and subsequently forms the catalytic species. A large excess of phenol removes nitrite so that it is unavailable for reaction with amine, either directly or catalytically.

1.5.2.3 Inhibition by sulphur compounds : Bisulfite reduces nitrite in two steps - first to nitric oxide (equation 18) and then to nitrous oxide (equation 19) (58). Sulfamate reduces nitrite to molecular nitrogen (59). Sodium bisulfate, ammonium sulfamate, cysteine, glutathione, methionine are few more examples of compounds which inhibit in vitro nitrosamine synthesis (60).

1.5.2.4 Miscellanous inhibition : The ammonium ion reacts with nitrite to form molecular nitrogen and hydroxylamine reduces nitrite to nitrous oxide (59). Vitamin A reacts with nitrite in acid solution but not under neutral conditions (61). It has been found that urea, caffeine and ethanol are relatively weak inhibiters, but reduced nicotinamide adenine dinucleotide (NAD) is effective (55,61,62).

1.5.3. Destruction of N-Nitrosamines

N-Nitrosamines are stable compounds once they are formed. They are stable in neutral and in strong alkaline solutions in the absence of light (12,15). Denitrosation (equation 2) occurs slowly in acid solution (1 to 5 M) and is catalysed by nucleophiles in the order of effectiveness $Y = I^- > SC(NH_2)_2 > SCN^- > Br^- > Cl^-$ (38). To prevent reversal of the

reaction, a substance which reacts irreversibly with Y-NO (equation 4) and more rapidly than with the amine, must be added. The relative efficiency of various nitrite traps in 5 M H_2SO_4 was found to be hydrazoic acid and hydrazine > sulfamic acid > aniline > hydroxylamine > urea (63). Ease of denitrosation varies in the order R,R' = aryl > R = aryl, R' = alkyl > R,R' = alkyl (64).

Quantitative denitrosation of nitrosamines can also be achieved at room temperature using a solution of HBr (5 to 10 %) in glacial acetic acid if water is excluded. Analysis of the nitrite released provides a measure of the original nitrosamine concentration (65).

When exposed to ultraviolet light nitrosamines decompose either to aldehydes, nitrogen and nitrous oxide or quantitatively to amine and nitrous acid depending on the wavelength used. The reaction is fastest in acid and faster in neutral than basic solutions (66).

1.6 Occurrence of N-Nitrosamines and Risk Assessment of Human Exposure

1.6.1 Occurrence

N-nitrosamines, suspect cancer agents, are present both in the natural and manmade environments. EPA is considering listing N-nitrosamines as hazardous pollutants under section 112 of the Clean Air Act of 1990. In 1977, the U. S. EPA ruled that all pesticides and herbicides must be screened for N-nitrosamines prior to release for sale and use by public. The USDA in 1979 established a N-nitrosamine limit of 17 ppb in bacon and in 1980, the FDA limited N-nitrosamines to 5 ppb in all malt beers.

Man breathes in nitrosamines from tobacco smoke, frying bacon, spraying aqueous herbicides, etc. These compounds can be dangerous in the human body through ingestion, inhalation, dermal contact, skin absorption, and in-vivo formation. Their presence in products and workplaces may be summarized as follows:

Cigarette and tobacco smoke- NDMA, TSNA (74)

Cosmetics; creams, lotions etc.- NDELA (81)

Beverages made from malted grains ; beer, whisky- NDMA (67)

Preserved meat products; bacon, sausage etc.-NPYR, NDMA (68)

Rubber packings of meat products- NDBA (69)

Pesticides & herbicides- NDMA, NDPhA (70)

Cutting & hydraulic fluids- NDELA (76,71)

Rubber products- NDBA (72)

Air in tire/rubber factory- NDMA, NMOR (73)

Air in iron foundry- NDMA, NDPhA, NDELA (82)

Industrial waste- NDMA, NDPhA (82)

Prescription and nonprescription drugs- NDMA, NDELA (74).

(NDMA = N-Nitrosodimethylamine, NDEA = N-Nitrosodiethylamine, NDBA = N-Nitrosodibutylamine, NDPhA = N-Nitrosodiphenylamine, NDELA = N-Nitrosodiethanolamine, NMOR = N-Nitrosomorpholine, NPYR = N-Nitrosopyrrolidine,TSNA = Tobacco specific Nitrosamines)

The formation & occurrence of NDMA in beer is attributed to a chemical reaction that takes place when sprouted barley malt is dried directly over a hot flame. Barley malt in which no NDMA was detected, was dried by an indirect process in which hot air was piped into the drying klin.

NDELA has been detected in cutting fluids. Its presence results from a reaction of nitrite ion, added to act as a rust inhibitor, with diethylamine/triethylamine, added as lubricants & emulsifying agents .

The preformed nitrosamines, compounds formed prior to ingestion, are present in nitrite-cured meats such as bacon, sausage, some cheeses, and fish. However, the in-vivo formation of nitrosamines in the gastrointestinal tract from the bacterial conversion of nitrites which then nitrosates circulating amines is probably greater then ingestion of preformed nitrosamines. The in-vivo nitrosation of amine-containing drugs is probably also significant.

1.6.2 Exogenous Exposure

Because of improvement in analytical techniques over the past 10-15 years, human exposure to N-nitroso compounds is well documented. It is becoming increasingly apparent that N-nitroso compounds are ubiquitous in the environment, particularly in many chemical, agricultural and consumer products. A typical human exposure to N-nitroso compounds in given in Table 1.4. In order to minimize human exposure, it is essential that the most significant exposure routes be understood first.

1.6.2.1 Ingestion : The cured food products like meat etc. are potential sources of nitrosamines. N-nitrosodimethylamine (NDMA) was found to be present in cooked bacon and some nitrite preserved foods in the concentration range 1-10 μg/kg. N-nitrosopyrrolidine (NPYR) was consistently found to be present in cooked bacon at the 10-50 μg/kg concentration levels (75). Dry premixed cures containing spices and sodium nitrite were found to contain N-nitrosopiperidine (NPIP) up to

Table 1.4 Typical Human Exposure to N-nitroso Compounds

Exposure	NDMA & NDEA,μg	NPYRR μg	NNN μg	NDELA μg
Ingestion				
nitrite preserved foods	1	5		
Inhalation, cutting fluids,				
0.5 to 5 ml concentrate				25-250
Aqueous herbicide sprays	100			
UDMH Factory, Baltimore	40			
Adjacent to UDMH Factory,				
Baltimore	14			
Tobacco smoke, 20 cigarettes	1		3	
Downtown Baltimore	0.3			
Kitchen, from bacon frying	0.01			
Dermal Contact				
cutting fluids, 5 ml concentrate				25,000
Pesticide spill, 1 ml	500			
Facial cosmetics				50
Hair shampoos				3
Hand lotions				2
In vivo	>7			

3000 μg/kg level and NPYR up to the 40 μg/kg level (76). In drinking water, particularly high nitrite well water, the sub 0.01 μg/L levels of NDMA, N-nitrosomorpholine (NMOR) and N-nitrosodiethylamine (NDEA) found so far are too low to be considered significant (77).

1.6.2.2 Inhalation **:** Fine et al. reported (78) that in Baltimore, the prime source was found to be a chemical plant which was manufacturing unsymmetrical dimethyl hydrazine for which NDMA was used as a precursor. Typical NDMA levels were between 6,000 and 36,000 ng/m^3 on the site of the factory, about 1000 ng/m^3 in the residential neighborhoods adjacent to the factory and about 100 ng/m^3 two miles away in downtown Baltimore. If it is assumed that an adult inhales 10 m^3 of air per day, then daily human exposure to airborne NDMA can be estimated. On the factory site, a worker would be exposed to 40 μg NDMA during the course of an 8 hour day. The exposure was 10 μg in the adjacent residential neighborhood and 0.3 μg in downtown Baltimore (Table 1.4). The studies in New York City, Boston and New Jersey revealed that NDMA at the sub 10 ng/m^3 level in 3 out of 40 sites (79).

Gough & Walters (80) and Sen et al. (81) have reported that 70% of the NDMA and 50% of the N-nitrosopyrrolidine was lost from bacon during cooking. In a kitchen, while vaporization of volatile N-nitrosamines from cooked bacon may be detectible, normal ventilation and air leakage would reduce the exposure per person per day to less than 0.1 μg.

The mainstream smoke of a US blended cigarette has been shown by Hoffmann et al. (82) to contain 0.08 μg NDMA and 0.14 μg of N-nitrosonornicotine (NNN). The human intake from smoking 20 cigarettes

per day would therefore be approximately 1 μg of NDMA and 3 μg of NNN (Table 1.3).

Herbicides which are formulated as aqueous amine salts have been found to contain appreciable amounts of volatile N-nitrosamines. Fine et al. (83) reported NDMA levels in dimethylamine formulations of 2,3,6-trichlorobenzoic acid to be as high as 0.06%. Volatile N-nitrosamines were found to be present at the 200 to 600,000 μg/l level in most amine based pesticide formulations which have been tested . These products are applied as a fine aqueous mist and it is assumed that drifting winds could expose herbicide applicators to levels in excess of 100 μg per day.

Synthetic cutting fluids are used in the US by approximately 750,000 machine shop workers. N-nitrosodiethanolamine (NDELA) was shown by Fan et al. (84) to be present in cutting fluids at concentrations as high as 3 percent. All synthetic cutting fluids which have been tested have been shown to contain detectible levels of NDELA (76). During most machine shop operations and particularly during milling and grinding, the dilute cutting fluids are sprayed, splashed and vaporized into the air. The machine operator thereby continuously inhales an aqueous mist which is laden with NDELA. Human exposure could exceed tens of micrograms per person per day.

1.6.2.3 Dermal Contact **:** Agricultural workers who formulate and apply herbicides such as Treflan and Benzac are in contact with volatile nitrosamines, such as NDMA and N-nitrosodipropylamine (75), present at the part per hundred concentration level, some of which may be absorbed. Agricultural workers who handle pesticides containing major N-nitrosamine impurities could be subjected to exposures of the order of 500 μg per day.

Machinists are also exposed to NDELA by dermal contact. Normal procedures require the operators to continuously handle the wet machine parts. Many machinists are in direct contact with milligrams of NDELA per day.

Facial cosmetics, hair shampoos, hand lotions and skin creams all contain significant NDELA impurities. Fan et al. (85) reported NDELA to be present in 27 out of 29 consumer toiletries. Several facial cosmetics contained NDELA at levels in excess of 20,000 to 40,000 μg/kg. Human exposure to NDELA in cosmetics can be estimated (Table 1.3). Assuming 10 to 15 g is used for a shampoo, the human exposure is about 2 μg. For facial cosmetics, where 1 to 2 g is used, the typical daily exposure is about 50 μg of which approximately 1.8% (0.9 μg) would penetrate the skin (86). Persons such as actresses and models may be exposed to even higher levels.

1.6.3 Endogenous Exposure (In Vivo Formation)

Various investigators have demonstrated by direct chemical analysis that if relatively massive amounts of amine plus nitrite are fed to laboratory animals, some of the amine will be nitrosated *in vivo* to form the corresponding N-nitroso compound. Tumours have been induced in laboratory animals by oral administration of precursor amines plus nitrite, presumably via *in vivo* nitrosation. Sander & Schweinsenberg have demonstrated that *in vivo* nitrosation can take place in human subjects fed diphenylamine plus nitrite. The literature pertaining to such studies has been reviewed recently by Mirvish (87). *In vivo* nitrosation, with the precursors at typical environmental

levels, has been demonstrated both in laboratory animals (88) and in man (89).

A volunteer was fed a meal consisting of bacon, spinach, tomato, bread and beer. Blood samples were taken before and after the meal and analysed for volatile N-nitrosamines. Although N-nitrosopyrrolidine was the major N-nitrosamine in the meal, it was not detected in the blood. NDMA was also present in the meal, but more NDMA was found in the blood (assuming a body blood volume of 5750 ml) than was ingested in the food. N-nitrosodiethylamine was not present in the food, but was found to be present in the blood. Surprisingly, fresh blood always contained some NDMA at the 0.03 μg/l (0.03 ppb) level. This background NDMA level was reduced to zero if the volunteer consumed an excess of ascorbate for the previous two or three days. The fact that ascorbate, a nitrite scavenger, reduced the background NDMA level is indicative of continuous in vivo formation from nitrite precursors (81). Human exposure to NDMA from eating cooked bacon has been estimated to be at least 7 μg per meal (Table 1.3).

Using laboratory animals, Rounbehler et al. (80) developed a rapid technique for screening compounds that are likely to be nitrosated in vivo. Mice were fed amine plus nitrite and were then sacrificed at various times after feeding by plunging them live into liquid nitrogen. The frozen whole mouse was ground to a fine powder and analysed for the N-nitroso derivative of the amine to which the animal had been exposed. Control experiments included feeding nitrite alone, amine alone and the N-nitroso compound alone. The technique has been used to demonstrate that, at the typical low amine levels present in normal diets, in vivo nitrosation in

mice occurs more than 100 times faster than would be predicted from the rate expressions of Mirvish (79).

1.6.4 Endogenous vs. Exogenous And Risk Assessment

The estimated average US per capita intake of nitrate and nitrite is shown in Table 1.5. The exposure of humans to both endogenous and exogenous nitrosamines are compared in Table 1.6 (90), are subject to several assumptions: first, that all the dietary intake of amines is 4000 mg per day; second, that all dietary amines behave like proline; third, that the site of endogenous nitrosation is restricted to the stomach; and fourth, apart from tobacco products, the data base for exogenous exposure is limited to volatile nitrosamines. Table 1.7 presents a risk estimate due to both endogenous and exogenous nitrosamines for four different population subgroups, the average non-smoker, the average smoker, a high risk category and a low risk category in the ratio of 14:6:1:1 respectively-based on US population. Then the number of cancer deaths per year attributable to nitrosamines are estimated. The model predicts 108 deaths per year for the average non-smoker group, 291 for the average smoker group, 814 for the high risk group and 4 for the low risk group (91). The high risk group is assumed to have 4 times average cosmetic and dietary exposures, use only new cars, smoke 40 cigarettes per day, and have the highest possible endogenous nitrosamine formation and be exposed to very high nitrosamine levels in the workplace.It must be emphasized that these estimates must be considered speculative.

Table 1.5 Estimates of Average US Per Capita Intake of Nitrate and Nitrite

Food Item	Intake, mg/day	
	Nitrate	Nitrite
cured meats	1.6	0.36
fresh meats	0.7	0.07
vegetables	65.0	0.2
fruit juices	4.3	0.01
cereals, baked goods	1.2	0.24
milk, dairy products	0.2	0.01
water	2.0	0.01
Total estimate	**75.0**	**0.9**

Table 1.6 Human Exposure to N-nitroso Compounds from Endogenous and Exogenous Sources

	All exposures in μg/person/day	
	Average	**High**
Exogenous Exposure		
Cosmetics (volatile)	0.4	
Car interiors (volatile)	0.2	0.5
Dietary (volatile)	1.1	
Tobacco smoke (volatile and non-volatile)	16.0	32.0
Endogenous Exposure (calculated)		
Average U S diet	0.3 - 2 3	
Average U S diet plus 20 cig/day	0.3 - 2.3	
Vegetarian	2.3 - 16	
High nitrate water	3.4 - 24	
High meat diet	0.6 - 3.7	
Worker Exposure to Exogenous Sources		
Leather tanning (volatile)	20-18	440
Tire factory (volatile)	50-130	250
UDMH Factory (volatile)	10-50	260

Table 1.7 Estimated Risk Levels for Various Subgroups

Exposure source	Population subgroups: Average non-smoker	Average smoker	High-risk	Low-risk
	µg	µg	µg	µg
Exogenous				
cosmetics	0.4	0.4	1.6	0.0
car interiors	0.2	0.2	0.5	0.1
dietary	1.1	1.1	4.4	1.1
tobacco smoke	0.0	16.0	32.0	0.0
Endogenous	1.3	1.3	24	0.3
Worker exposure	0.0	0.0	250	0.0
Total exposure	3.0	19.0	312	1.5
Lifetime risk (surface area)	5.4×10^{-5}	3.4×10^{-4}	5.7×10^{-3}	2.7×10^{-5}

1.7 Analysis of N-Nitrosamines

The low levels, a few ppb, at which N-nitrosamines are present in foods and other complex matrices invariably requires a multistep analytical procedure. The first step, sample preparation (extraction or isolation), is followed by separation and quantitative detection steps.

1.7.1 Isolation of N-Nitrosamines

A number of analytical methods sensitive at µg/kg level have been employed to extract N-nitrosamines with varying degrees of success. The routinely used procedures are complex and time consuming. These methods often require multiple steps such as digestion, liquid extraction, steam or vacuum distillation, soxhlet extraction, followed by extensive clean-up. To achieve sensitivity at the µg/kg level in the original sample, concentration of the extract by at least a factor of 1000 is essential.

Fine et al. (92,93) developed a three step method applicable to difficult food matrices such as tuna fish, canned beef *et cetera*. The vacuum distillation from mineral oil followed by extraction with dichloromethane and finally concentration on a Kuderna-Danish (K-D) flask allowed sensitivity at ppb level. Sen et al. (94) carried out direct extraction with methylene chloride of foodstuffs predigested in 3N KOH solution in presence of antifoam'A' (Dow Corning). The methylene chloride extract was dried and concentrated in a K-D flask. If required, the concentrate was purified on a basic alumina column (95). Goodhead and Gough (96) described a steam distillation procedure applicable to food matrices. The sample was slurried with sodium chloride and water, and then steam distilled. The distillate was then extracted with methylene chloride and cleaned-up and concentrated before estimation. Solid phase

extraction using Celite as an adsorbent and as a diluent has been used to analyse beer (97), malt (98,99), non-fat dry milk, frankfurters and surimi (100). Briefly, the sample is mixed with Celite and, in the case of a dry sample, further mixed with ammonium sulfamate solution (1g ammonium sulfamate dissolved in 100 ml of 0.5 M sulfuric acid) to produce a mixture fluffy in consistency. The mixture is packed into a chromatography column (commonly 40 cm x 32 mm i.d.), above a plug of anhydrous sodium sulfate. The column is eluted with methylene chloride. The eluate is collected in a K-D flask and concentrated to 4 ml in a 60 ^{o}C water bath, then to 1 ml under a gentle stream of nitrogen. Fazio and Havery (101) have used vacuum distillation and column elution (described above) to extract volatile N-nitrosamines from direct flame dried processed foods such as soy protein concentrate/isolate, non-fat dry milk, beer and malt beverages (102). The 'Pretube-extraction' from alcoholic beverages has also been done (103). Fine et al.(104) have described extraction of N-nitrosamines from various biological samples using one or more of following : vacuum distillation, Soxhlet extraction, aqueous extraction, steam or mineral oil distillation, pretube extraction, solid phase extraction.

The methods mentioned above, generally lengthy, give acceptable results but incorporate use of large quantities of hazardous organic solvents and chemicals. Direct extraction while simple, requires purified solvents and elaborate precautions to obtain reliable results. Even then, direct extraction is limited to porous matrices. Distillations, especially mineral oil, suffer from artifact formation and excessive foaming leading to misleading results. Solid phase adsorbents, rubber products such as tubing, septa etc., freezing can complicate the analysis. An overview

describing various aspects of artifact formation has been published by Fine (105).

There exists a need for a sample preparation technique which is fast, simple, efficient, environmentally safe, accurate and can be routinely used for extracting N-nitrosamines from a large number of samples. In this present study, N-nitrosamines are isolated by supercritical fluid extraction (SFE) which is a faster and cleaner alternative to conventional analytical extraction methods, and could well come to replace them.

1.7.2 Quantitative Determination of N-Nitrosamines

The quantitative estimation of trace amounts of N-nitrosamines in foodstuffs, biological fluids, vegetation, and other matter has been met using a variety of analytical techniques such as gas chromatography, mass spectrometry, spectrophotometry, thin layer chromatography (106), spectrofluorometery (107) and polarography (108,109). While each of these methods can be highly selective and reliable, they each have certain limitations. For example gas chromatography is more suitable for volatile N-nitrosamines. Mass spectrometry and UV-Vis spectrophotometry, while highly sensitive, can be very difficult to interpret in the case of mixtures.

By and large all the successful analytical methods developed for estimation of N-nitrosamines require a prior separation using Gas Chromatography (GC) whereas Liquid Chromatography (LC) is used to a lesser extent. The degree of sophistication of the GC apparatus varies from single isothermal packed columns to systems incorporating solvent-venting pressure programming (110) and high efficiency narrow bore columns (111,112). The identification of N-nitrosamines is based upon

their retention times. But it is the chromatographic detector which governs the entire scenario. There are three generally recognized 'sensitive and specific' methods of detection, which are described below in brief :

1.7.2.1 G.C.- Electroanalytical Detectors

Electroanalytical methods, being highly sensitive are used to detect the electroactive N-nitrosamines. The voltammetric electrochemical detector (ECD) is chosen over polarographic ECD because it is more selective and less affected by other impurities (113,114,115,116).

The Coulson electrochemical detector (CECD), as described by Coulson in 1965 (117) is a reasonably specific and sensitive detector of compounds containing nitrogen and, by varying the operating conditions, also of compounds containing sulfur and halogens. For the estimation of nitrogen-containing compounds the effluent from a GC column is mixed with hydrogen and passed through a reduction furnace at 800 °C over a nickel catalyst. Nitrogen-containing compounds are reduced to ammonia, which is subsequently dissolved in deionised water and passed through a micro cell in which changes in electrolytic conductivity are measured. Halogens, which, if present, would be reduced to the corresponding acid and hence interfere, are removed by a strontium hydroxide coated plug of glass fibre placed between furnace and the detector cell. Other organic species form hydrogenation products which give no response in the conductivity cell. Several refinements (118) and comparative studies have been reported (119). Bogovski and Walker (120) used this detector to analyse volatile N-nitrosamines.

The alkali flame ionization detector (AFID) is a modification of the FID in which alkali metal ions are introduced into the flames to vary the ionisation processes and to enhance selectivity of response to compounds containing certain hetero-atoms. This phenomenon was first reported by Giuffrida (121), and Karmen and Giuffrida (122), who showed that the response to phosphorus was increased by a factor of 100. The close similarity in electronic configuration between phosphorus and nitrogen prompted Aue et al. (123) to experiment with a variety of alkali salts in order to improve the selective determination of nitrogen, and their results indicated that rubidium sulphate gave the best overall performance. In a comprehensive review of thermionic detectors, Brazhnikov et al. (124) compared and contrasted the performance of the detector with tips from a number of salts, and concluded that rubidium chloride was preferable for nitrogen compounds . Cesium bromide has also been considered suitable in this respect (125). No less conflicting are the reports of the significance of detector geometry (126), electrode spacing (127,128), nature of carrier gas (129) and hydrogen flow (130). The importance of operating under carefully controlled conditions is exemplified by the use of slight adjustment of these parameters to vary the selectivity of the detector and conditions for the following elements have been described: chlorine, iodine and bromine (129, 131,132); silicon, tin and lead (133); and sulphur (134). It seems clear, therefore, that the choice of parameters for thermionic detectors is empirical, and that further development and understanding is essential before they can be considered suitable for anything except specialist or research applications.

Palframan et al. (135) have compared the performance of GC-CECD and GC-AFID. The various parameters influencing the specificity

and sensitivity of these detectors are discussed and experience gained in their use for the analysis of foods for traces of N-nitrosamines is described. Only slight differences in the sensitivities of the two detectors were found but the CECD was more selective and less disturbed by small changes in operating parameters, and hence is considered more suitable for routine use in this field of analysis.

Extracts of biological origin are still complex mixtures even after extensive clean-up and frequently contain nitrogen-containing compounds. Some of these can give rise to false positive results . Thus results needs to be confirmed by another technique, commonly GC-MS.

1.7.2.2 GC-High Resolution Mass Spectrometry (GC-HRMS)

High-resolution mass spectrometry is another technique which can be successfully employed to detect N-nitrosamines (136). The selected ion-monitoring (SIM) mode has two major advantages : (i) it provides detection limits that are 2 or 3 times orders of magnitude lower than possible in the normal scanning mode; (ii) by choosing ions that are characteristic of the compounds of interest , integration of the recorded signal response can be a direct measure of the concentration. Quantitation is normally based on the intensity of selected ions in the spectrum, after calibration using standard N-nitrosamines solutions(137). Using GC-MS several groups have reported presence of N-nitrosamines on food such as cured meat, fish and cheese (138,139,140).

Although GC-MS is proven to be reliable and proven method of confirmation of the identity of N-nitrosamines , there is a need for simpler yet equally 'sensitive and selective' technique that is affordable

and can be used for routine screening purposes without extensive clean-up etc. of sample as in GC-MS.

1.7.2.3 GC - Thermal Energy Analyser (GC-TEA)

GC-Thermal Energy Analyser (141) has proven to be the instrument of choice because of its specificity, speed and accuracy. The details of the TEA detector have been discussed in chapter 3.

Given that comparable results are obtained by GC-TEA on dilute and dirty extracts as compared with GC-CECD and GC-MS obtained on final clean concentrates, it seems that the selectivity and sensitivity claims of TEA are justified. Also, comparative studies of various chromatographic detectors for the estimation of N-nitrosamines (142,143) indicate the same.

1.8 Practical Aspects

The basic problem in minimizing nitrosamine formation is prevention of the reaction between nitrosating species and amines. The nitrosating species are ubiquitous in the environment. Roughly 50 ppb of nitrous oxide and nitrogen dioxide are present in the atmosphere of our cities. In soils, streams and rivers, organisms of the genus nitrosomonas oxidize ammonia to nitrite. Some foods have a high nitrate content. These can be reduced in vivo after ingestion of the food. Nitrites are added to some foods to prevent growth of botulinus organisms. Nitrites are also widely used as metal corrosion inhibitors.

Removal of nitrosating species from our environment is a sociological task not amenable to immediate solution. In certain cases, steps can be taken to minimize such contamination. Already industry is

moving to replace nitrite as a corrosion inhibitor in some applications and to reduce its use as an additive in meat.

A more likely general approach to preventing the reaction of nitrosating species and amines is the inclusion of appropriate scavengers into raw materials and finished products. For example, in the production of organic raw materials, where a nitration step occurs in the synthesis, a small amount of SO_2 can be added before solvent removal in the final step to destroy any traces of nitrite. The excess SO_2 would be eliminated by the drying process. Alternatively, a nontoxic nitrite scavenger, such as ascorbic acid, can be incorporated into the raw material or finished product

Scavengers which reduce nitrosating species can be classified into those which convert nitrite to NO and those which reduce it further. Most inhibitors described reduce nitrite to NO. In the presence of molecular oxygen NO is readily oxidized to N_2O_4 which is a good nitrosating agent. Thus, a sufficient excess of these inhibitors should be incorporated to scavenge oxidized NO. Sulfamates and sulfites reduce the nitrites to N_2 and N_2O respectively, which are not reoxidized by molecular oxygen . These inhibitors are not as innocuous as some of the weaker reducing agents, however.

CHAPTER 2

Supercritical Fluid Extraction

The concept of supercritical fluid extraction was first recognized in 1879 by Hannay and Hogarth (1). They observed the solubility of potassium iodide in supercritical ethanol and demonstrated that the reduction of pressure resulted in the precipitation of the salt from the fluid. They also demonstrated that this concept was applicable to other salts and to low molecular weight hydrocarbons. Later it was proposed that the solvent power of SFs may have been involved in geological processes through the influence of water in rock formation (2). It was also realized that deposits on the blades of steam turbines from water above its critical temperature could be caused by silica dissolving in the steam and then being deposited as the high pressure steam expands (3). During the development of high-pressure hydrogenation it was observed that lubricating oil dissolved in the compressed gas, which led to diminished lubricating efficiency. In petroleum technology it was found that supercritical solutions could exhibit retrograde behavior, such as the condensation of a vapor phase on isobaric cooling (4, 5).

SFE is a technique that exploits the solvent power of supercritical fluids at temperatures and pressures above the critical point. The alternative term "dense-gas extraction" (6) has been used to emphasize the fact that the density of the supercritical phase is the principal factor in the extraction process. From this terminology it is clear that a gas only becomes an efficient solvent at high density. The term "destraction" derived from the Latin words "destillare" and "extrahere", has been used in Germany (7) to acknowledge that the technique is a combination of liquid extraction and distillation. Because SFE blends the application of

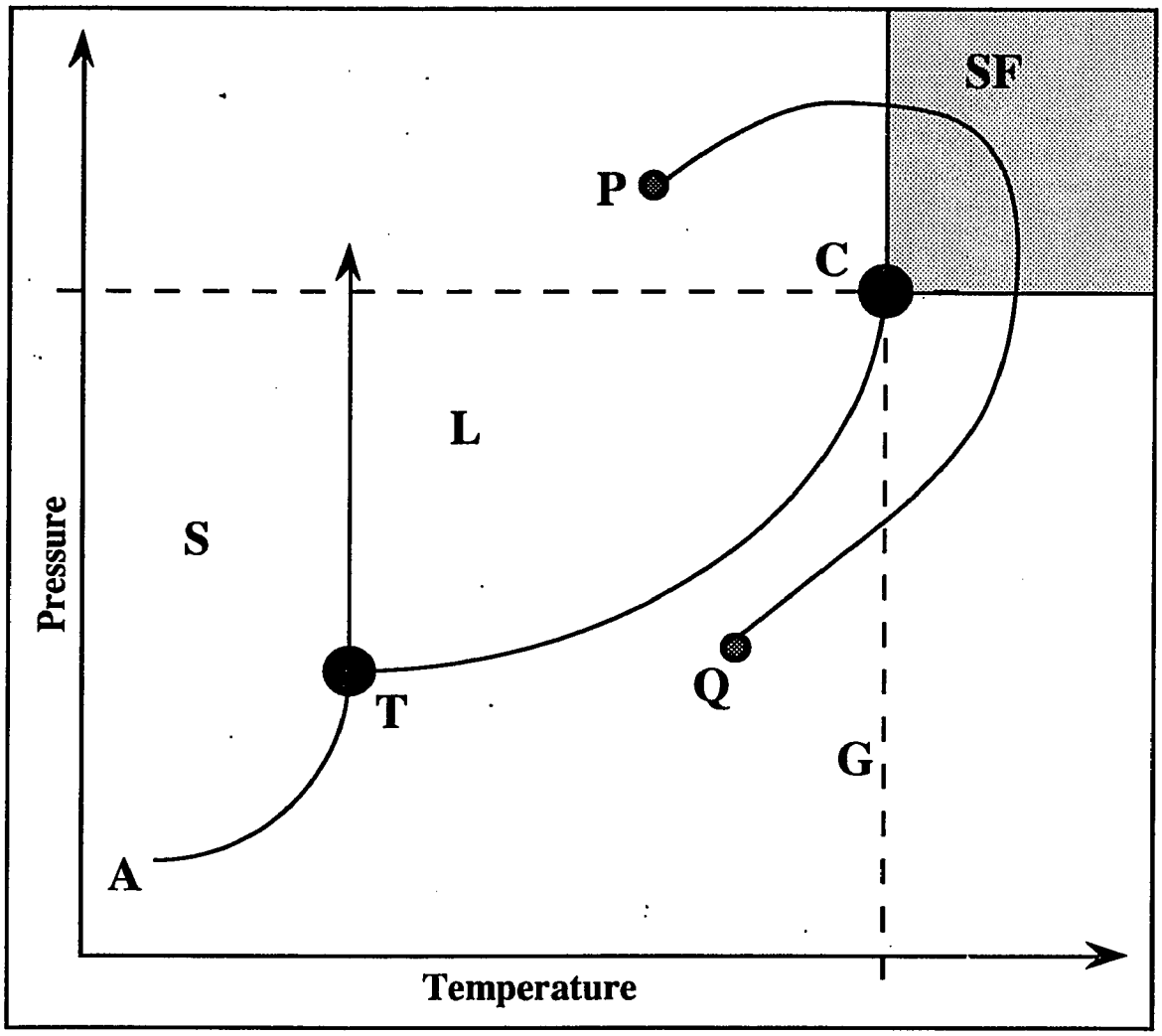
solvent powers and differences in volatility which are the basis of two important separation processes namely distillation and extraction.

A supercritical fluid is a substance heated to temperature higher than its critical temperature and held at a pressure greater than its critical pressure. A supercritical fluid has high diffusivity, high density, and low viscosity. Thus, a supercritical fluid is able better to penetrate porous materials such as tobacco and soil and thus to be a more efficient extractant. For CO_2 the critical temperature is 31.1°C and the critical pressure is 72.8 atm. As the pressure is increased, the density of supercritical fluid increases; supercritical fluid CO_2 has a liquid-like density of 1g/ml at a pressure of 5350 psi. Further, the viscosity of supercritical fluid CO_2 under these conditions is only 1.1×10^{-3} g/cm-sec.

2.1 Supercritical Fluid : The Fourth State of Matter

Figure 2.1 (8) shows the phase diagram of pressure versus temperature for a pure compound. The lines TA, TB and TC divide the diagram into three regions. These regions represent the range of pressures and temperatures where the three different states of matter can be distinguished. These are the gaseous (G), liquid (L) and solid (S) states. On the lines two phases are in equilibrium and at the triple point, T, all the three phases coexist. The line TC, as has been known since the classic experiments of Andrews (9), terminates at the critical point C. Above the critical point, a difference between the gaseous and liquid phase can no longer be observed and result from the formation of the supercritical region.

Figure 2.1 Phase Diagram



S: Solid Phase; L: Liquid Phase; G: Gas Phase;
SF: Supercritical Fluid Regin.
T: Triple Point; C: Critical Point
AT: Sublimation Curve; BT: Fusion Curve;
CT: Vaporization Curve.
PQ: L to G without a phase change.

The border of this region is illustrated by the dashed lines. Crossing one of these dashed lines does not result in a phase change, where crossing a solid line does. A gas can be transformed into a liquid by condensation, which can be achieved by increasing the pressure or decreasing the temperature. The opposite process, evaporation, can be achieved by increasing the temperature or decreasing the pressure. Both condensation and evaporation are phase changes, during which the physical properties (density, viscosity, diffusivity, etc.) change abruptly. A gas can also be transformed into a liquid in a manner indicated by the arrow in Figure 2.1. During this process, a phase change will not take place. It has been stated that physical properties of a pure compound show continuous rather than abrupt variations when passing through one of the dashed lines (10).

The special name "Supercritical Fluid (SF)" signifies the behavior of this fluid which has been heated above and compressed beyond its critical temperature and pressure. The properties of SF's depend on the fluid composition, pressure and temperature. In the supercritical region, it is possible to take advantage of a variety of interesting and useful properties. Table 2.1 (8) compares certain properties of gases, liquids and supercritical fluids and Table 2.2 presents physical properties of some of the SFs (11). The compressibility of a supercritical fluid is largest just above the critical temperature and small changes in pressure result in large changes in density of the fluid. The density of a supercritical fluid is typically two to three orders of magnitude greater than that of a gas and of the same order of magnitude of a liquid. Because of the higher compressibility, shorter intermolecular distances can result in stronger

Table 2.1

Comparison of Physical Properties of SF, Gas, and Liquid (8)

Phase	ρ (g/ml)	η (g/cm-sec)	D(cm^2/s)
Gas¶	$(0.6 - 2)x10^{-3}$	$(1 - 3)x10^{-4}$	0.1-0.4
SF•	0.2 - 0.5	$(1 - 3)x10^{-4}$	$0.7x10^{-3}$
SF*	0.4 - 0.9	$(3 - 9)x10^{-4}$	$0.2x10^{-3}$
Liquid§	0.6 - 1.6	$(0.2 - 3)x10^{-2}$	$(0.2 - 2)x10^{-3}$

where
ρ : density (g/ml)
η : viscosity (g/cm-sec)
D : Diffusivity (cm^2/s)

¶ at 1 atm,15-30 °C
• properties at Tc & Pc
* properties at Tc & 4Pc
§ at 15-30 °C

Table 2.2 Physical Properties of Supercritical Fluids§ (11)

Solvent	T_c,°C	P_c,atm	ρ_c,g/ml	ρ_{SF},g/ml
Ethylene	9.2	49.7	0.217	0.365
Xenon	16.6	57.6	1.113	1.893
Chloro trifluoro methane	28.9	38.7	0.580	1.010
Carbon Dioxide	31.1	72.9	0.466	0.803
Ethane	32.2	48.2	0.203	0.352
Nitrous Oxide	36.4	71.5	0.452	0.764
Sulfur Hexafluoride	45.5	37.1	0.738	1.315
Propane	96.7	41.9	0.217	0.375
Ammonia	32.4	111.3	0.235	0.358
l-Butene	134.9	36.0	0.221	0.401
n-Butane	152.0	37.5	0.228	0.388
Diethyl ether	193.5	35.9	0.265	0.440
n-Pentane	196.5	33.3	0.237	0.393
n-Hexane	231.6	29.3	0.233	0.388

§ : $T_r = 1.02$ & $P_r = 2.0$

r : reduced

c : critical

ρ : density

intermolecular interactions, result in a high capacity of supercritical fluids for solutes.

The viscosity, a measure of the ability of a substance to flow, of a SF is almost as low as that of a gas and two orders of magnitude lesser than that of a liquid. This low viscosity leads to faster mass transport which can be facilitated both by pumping and natural convection. The diffusivity of a SF is three orders of magnitude greater than that of a liquid. The higher diffusivity (or higher diffusion coefficient) of a SF relative to a liquid imparts excellent powers of penetration into a porous matrices structure. Moreover, it can result in shorter interphase equilibration times as compared with a liquid. The low viscosity and the absence of surface tension in supercritical fluids increases the speed of percolation so the passage of the solvent into the interstices of a porous matrix is enhanced. The properties of gas-like diffusivity and viscosity, zero surface tension and liquid-like density combined with the pressure-dependent solvent powers of a SF provides the impetus for applying SF in different separation procedures. Overall it is apparent that a SF can be used as an excellent medium for an extraction process (25,12,13).

2.2 Theoretical Aspects of Supercritical Fluid Extraction

Thermodynamic models describing phase equilibria associated with SCF extraction can be separated into two general classes: those describing SCF-liquid equilibria and those describing SCF-solid equilibria. The main difference between these models are that the solid phase is assumed to dissolve no solvent (14,15) and in multi-component systems, no solid solutions are assumed to be formed (16). These two assumptions simplify

the analysis considerably by eliminating the composition-dependence of the chemical potential of the solid phase. In the case of equilibria between liquids and SFs, multicomponent solutions are common and considerable amounts of SF can dissolve into the liquid phase (17). This leads to a composition-dependence of the chemical potential which must be considered in the analysis. As with all the equilibria, the condition for equilibrium is based on standard thermodynamic principles which equate fugacities for each mixture constituent in all phases. For two phases,', and ",

$$f'_i = f''_i \qquad [1]$$

where i= 1, 2, ...m, and f_i is the fugacity of component i in a mixture of m components. In the case of SFE of solids, solid (i = 2) is brought into contact with the SCF and equilibrium is attained, then at equilibrium

$$f_2^S = f_2^{SCF} \qquad [2]$$

The fugacity of solid component can be determined in each phase from conveniently chosen constitutive equations. Generally, the fugacity of the solid component in the condensed phase is calculated from

$$f_2^S = P_2^{Vap} \{ \phi_2^{Vap} \exp (V_2^s (P - P_2^{Vap}) / RT) \} \qquad [3]$$

where P_2^{Vap} is the saturated vapor pressure of the solid at the temperature T. The molar volume,V_2^s, is a function of temperature and pressure, but at conditions remote from critical, the condensed phase may

often be regarded as incompressible. The fugacity coefficient, ϕ_2^{Vap}, corrects for deviations of the saturated vapor from ideal-gas behavior. Hence, the product, $P_2^{Vap}\phi_2^{Vap}$, accounts for the fugacity of the pure solid at saturated vapor pressure. The exponential term, the Poynting correction, takes into account the fact that the solid is at a pressure P, different from P_2^{Vap}. The fugacity coefficient and the Poynting correction are often small and usually they are negligible. If the temperature T is such that the P_2^{Vap} is low, then ϕ_2^{s} is very close to unity. The Poynting correction, which is an exponential function of the pressure and is small at low pressures, may become large at high pressures or at low temperatures.

The fugacity of the solid component in the fluid is more difficult to determined. Since there is no clear distinction between gas phase and liquid phase at elevated pressures, two conventional approaches are utilized to obtained the fugacities of the components in the SF phase; SF is treated as a highly compressed gas, or as an expanded liquid.

The most common approach is to treat the fluid as a highly compressed gas and to determined the fugacity, f_2^{cg}, from volumetric properties,

$$f_2^{cg} = y_2\phi_2 P \qquad [4]$$

where y_2 is the mole fraction of the solid solute in the SF phase and P is the pressure. The fugacity coefficient ϕ_2 characterizes the non-ideal behavior of the solid component in the gas phase relative to ideal-gas behavior, and can be calculated from an equation of state (EOS) using exact thermodynamic relationships (18). The results are frequently very

sensitive to the interaction energies and size factors used, necessitating the development of improved mixing rules to estimate the mixture size and energy parameters needed in the EOS. Combining equations [2], [3] and (4), solubility of the solid in SF can be represented by

$$y_2 = (P_2^{Vap} \{ \phi_2^{Vap} \exp (V_2^s (P - P_2^{Vap}) / RT) \} / \phi_2 P) \qquad [5]$$

The validity of this equation relies on two reasonable assumptions, i.e. the solid phase is pure and the molar volume of the solid is fixed.

By assuming ideal gas behavior, solubility of the solid component in the gas phase can be calculated from Dalton's Law (19)

$$y_2^{ideal} = P_2^{Vap} / P \qquad [6]$$

The ratio of the non-ideal to ideal solubility of the solid is defined as the enhancement factor, E, which is a dimensionless measure of the SCF solvent power.

$$E = y_2 / y_2^{ideal} \qquad [7]$$

By substituting equations [5] and [6] into equation [7], an expression can be derived to estimate the enhancement factor for the solubility of a solid in the SF phase.

$$E = (\phi_2^{Vap}/\phi_2) \exp (V_2^s (P - P_2^{Vap}) / RT) \qquad [8]$$

High enhancement factors are common in supercritical systems. Since the contribution of ϕ_2^{Vap} and the Poynting correction are

relatively small, the primary contribution to the enhancement factor is through the fugacity coefficient ϕ_2. There are many empirical EOS's and new ones continue to appear which can be used to estimate the fugacity coefficient of different types of solutes in SF mixtures. These equations contain empirically determined constants which are derived from mixture rules which state how these constants are dependent on the mixture composition.

A theoretically significant EOS is the virial equation in which virial coefficients can be related to the intermolecular potentials. The virial equation can readily be extended to mixtures. Whereas for any pure component the virial coefficients depend only on the temperature and on the intermolecular potential for the component, for a mixture they depend also on the interaction potentials between molecules of those different components which comprise the mixture. The fundamental advantage of the virial equation is that it directly relates fugacities in the mixtures to intermolecular forces. The practical disadvantage of the virial equation follows from insufficient understanding of the molecular forces. As a result the virial equation is applicable only to those mixtures whose components are non-polar or weakly polar. At moderate pressures, below approximately one-half the critical density of the fluid component, ϕ_2 can be calculated from a truncated virial equation of state (20,21,22). At higher pressures, i. e. higher densities of the SF, the utility of the virial equation is limited by a lack of knowledge of higher order coefficients (28).

At high densities, an essentially empirical EOS must be used to relate the fugacity coefficient to the pressure, temperature and the fluid phase composition. Most of the cubic EOS's are derived from

perturbation theory with certain assumptions. In perturbation theory, the properties of the fluid mixture are related to those of a simpler reference fluid whose EOS and other properties are accurately known. Pure fluids, treated as a mixture of hard spheres or a mixture of non-spherical hard bodies, have been used as the reference states in these perturbations which is dependent on the type of solute. The van der Waals EOS was derived by assuming that the integral of the perturbing intermolecular potential for a pair of molecules is a constant which is called the mean field approximation (23) . The Soave-Redlich-Kwong EOS (24) and the Peng-Robinson EOS (25) are obtained by introducing a temperature and density into the perturbing intermolecular potential. Use of the Carnahan-Starling repulsive term (26), instead of the van der Waals version, was based on the derivation of the Carnahan-Starling-van der Waals equation (27) and augmented van der Waals equation (28). Another perturbation method that has been applied to SCF is the perturbed hard-chain theory (29) and its variations (30,31). This model addresses the asymmetry of the size of the molecules, taking into consideration the ability of the solvent to interact with only part of the solute.

A different approach has been the use of lattice gas models or those including scale laws (32) which are nonanalytic. The lattice gas models are based on the idea of distribution of molecules over the sites in a three dimensional lattice. These approaches address the fact that behavior in the immediate vicinity of the solution critical point is non-classical and can not be described correctly by a classical EOS or its modifications. Introduction of the so-called universal critical exponent can be used to describe the thermodynamic properties in terms of the distance from the critical point. Hence, lattice gas models can be used to impose the correct

asymptotic behavior at the critical point. Although this approach is useful in the complete understanding of the critical region, there is not much significance in this approach in terms of SFE because nearly all the SFE processes operate away from the critical region.

An alternative approach is to treat the SF as an expanded liquid (33) and obtain the fugacity, f_2^{el}, of the solid component in the fluid phase from solution theories,

$$f_2^{el} = y_2 \gamma_2 f_2^{o} \qquad [9]$$

where f_2^{o} is the fugacity of solid component in a specifled standard state. The activity coefficient γ_2 characterizes the non-ideal solution behavior of the solid component relative to the chosen standard state. Therefore the solubility of a solid in the SF phase can be calculated from combining the equations [3] and [9] into [2] as

$$y_2 = (P_2^{Vap} \{ \phi_2^{Vap} \exp (V_2^{s} (P - P_2^{Vap}) / RT) \} / \gamma_2 f_2^{o}) \qquad [10]$$

When the standard-state fugacity is specified as the fugacity of the pure liquid at the system temperature and pressure, γ_2 characterizes deviations from Raoult's law behavior for the solid component. The activity coefficient can be evaluated at a fixed reference pressure and at constant temperature at which it is a function of composition only. The best reference pressure is the critical pressure P_c. At this pressure, the solubility of the solid component is negligible and the activity coefficient is essentially the activity coefficient at infinite dilution, $\gamma_2^{\infty}(P_c)$, which is constant at a fixed temperature. For the limited solubility range under

consideration, it is a good approximation to use both this infinite dilution activity coefficient and the partial molar volume at infinite dilution. Since the SF mixture is highly compressible in the critical region, the partial molar volume at infinite dilution has to be evaluated from an EOS. The infinite dilution activity coefficient is a characteristic parameter for the binary mixture and therefore is obtained from mixture data.

2.2.1 Enhancement vs Density

Johnston and Eckert (27) reported an empirical correlation between the log enhancement factor and the density. Later, Schmitt and Reid (34) noted that the plots of log enhancement versus the pure solvent density, instead of the mixture density, can also be used to correlate the solubility results. Interestingly, these plots showed fine splitting of the solubility isotherms which formed parallel lines corresponding to the various system temperatures. Furthermore, they suggested that the solubility isotherms can be made to collapse into a single generalized line, by proposing the empirical model

$$\log E = \alpha \rho_r + \beta + \sigma (T\text{-}T_{ref}) \qquad [11]$$

where α and β represents the slope and y-intercept of the reference temperature, σ the isotherm spacing constant, and T and T_{ref} are the system and reference temperature, respectively.

2.2.2 Concentration vs Density

Chrastil (35) derived a relationship relating the solubility of a solute (g/l) to the density (g/l) of the supercritical solvent based on the assumption that a molecule of the solute associates with a fixed number of solvent molecules at a given temperature and this solvated complex is in

equilibrium with its surroundings. From equilibrium considerations and the approximation of the Clausius-Clapeyron equation, the following equation was derived:

$$\ln C = k \ln \rho + \Delta H/RT + q - k \ln M_1 + \ln (M_2 + kM_1) \qquad [12]$$

where k is the association constant, ΔH ($= \Delta H_{sol} + \Delta H_{Vap}$) represents the total heat of the reaction where ΔH_{sol} is the heat of solvation and ΔH_{Vap} is the heat of vaporization, R the universal gas constant, q is a constant and, M_1 and M_2 are the molecular weights of the solvent and solute, respectively. Chrastil suggested that a plot of the log of the solute concentration versus log of the solvent density should result in direct proportionality over a wide range of temperatures and pressures. For a given solute-solvent system equation (12) can be rewritten as

$$C = \rho^k \exp (a/T + b) \qquad [13]$$

This simple three-parameter equation was modified by Adachi and Lu (36) since k is density-dependent. Introduction of two more additional parameters produced a better correlation for a total of 37 different systems. Generally the unmodified equation successfully fits data for non-polar solutes. However, it was found that the slopes of the solubility isotherms decrease with increasing temperature when the solutes are polar (37,38). This indicates that at high temperatures fewer molecules are involved in the solvato complex.

Recently Kumar and Johnston (39) showed that the solute solubility varies linearly with the solvent density when graphed in either log-log or

log-linear co-ordinates. The relationship between the concentration of the solute in the SF and the fluid density was derived by expressing the fugacity coefficient of the solute in terms of solvent density instead of pressure as is done in the conventional thermodynamic treatment. Interestingly, they were able to relate the slopes of these plots to the isothermal compressibility of the solvent. Furthermore, the partial molar volumes generated from this model were in good agreement with the available experimental data.

2.2.3 Enhancement vs Pressure

A semi-empirical correlation can be derived from equation [8], using the assumption that the fugacity coefficient and the solid molar volume do not depend on the pressure of the system. Under these conditions equation (8) can be rewritten as

$$\ln E = \ln (\phi_2^{Vap}/\phi_2) + (V_2^s (P - P_2^{Vap}) / RT) \qquad [14]$$

Hence, the plot of log enhancement versus $(P - P_2^{Vap})$ should fit a straight line where the slope of the line is a measure of the solid molar volume and the intercept is a measure of the fugacity coefficient.

Zieger and Eckert (40) developed this correlation using regular solution theory and the van der Waals EOS. Their proposed semi-empirical correlation uses the van der Waals EOS and mixing rule to determine the fugacity coefficient of the solute in the fluid phase in terms of the solubility parameters of the solute and solvent as

$$\ln\phi_2 = \ln (1+\delta_1^2 / P) - \varepsilon_2^* \Delta\{2-\Delta) + V_2^L P/ 2.3RT \qquad [15]$$

where ε_2^* is the dimensionless energy parameter given as

$$\varepsilon_2^* = \delta_2^2 V_2^L / 2.3RT \qquad [16]$$

δ_1 and δ_2 are the solvent and solute solubility parameters, $\Delta=\delta_1/\delta_2$ represents the ratio of the solubility parameter of the solvent to that of the solute, and V_2^L is the molar volume of the solute which has been evaluated by treating the solid as a subcooled liquid and thus extrapolating liquid properties below the melting point. Alternatively such properties can be more readily estimated by atomic and group contribution methods (41).

The Hildebrand solubility parameter is introduced to relate the enhancement of volatility with pressure, temperature and the size and the nature of the solutes. An appreciation of the meaning of the solubility parameter in physical terms is well described by Fedors (41). In the above treatment, Zieger and Eckert defined the solubility parameter as originally defined by Giddings (42) according to van der Waals theory

$$\delta_1 = a_1^{1/2} \rho_1 \qquad [17]$$

The constant a is the energy parameter in the van der Waals equation. The solubility parameters of the solutes are calculated from knowledge of their thermal properties.

Substituting equation [15] into equation [14] and introducing two empirical temperature-independent parameters η and ν, the final correlation can be derived as

$$\log E = \eta\,(\varepsilon_2\,\Delta/y_1\{2-\Delta/y_1\} - \log\{1+\delta_1{}^2/P) + \nu \qquad [18]$$

where y_1 is the equilibrium mole fraction of the solvent. Zieger and Eckert claimed that the above semi-empirical correlation will produce linear behavior with the collapsing of the solubility isotherms over a wide range of SF conditions, thereby resulting in a single generalized line. The parameters η and ν represent constants that are characteristic of each solvent and solute respectively.

Correlations based on this equation indicates that solubility data do collapse onto a straight line (42). Moreover, it was found that the binary systems involving the same SF solvent produced lines of similar slopes implying that η is a function of the SF only.

2.3 The Technique of Supercritical Fluid Extraction

In contrast to the more-established sample preparation techniques SFE is a relatively new technique. No 'cookbook' of SFE methods is available, and appropriate conditions are often chosen by trial and error. Analytes that are sorbed, trapped, or imbibed by a solid matrix must be solubilized in the SF, and one must understand the relative affinities of analyte for the SF and the solid matrix, which all of the normal attractive forces (van der Waals, and hydrogen bonding, for example) can effect. Chemical properties such as the analyte's molecular weight, functional groups, polarity, solubility, volatility, thermal stability, pK_a, and concentration must be taken into account before selecting starting conditions. Equally important are the matrix characteristics such as particle size, homogeneity, amount, porosity, composition, solubility, density, and stability under SFE. If the analyte and matrix have similar

compositions, the matrix also can be dissolved by the SF, which can help (or hinder) enrichment or analyte removal . On the other hand, the analyte can be held by strong forces and not be extracted by the SF. With high-density polymeric materials, the matrix actually can physically obstruct analyte removal.

To develop a SFE method, the matrix's physical form must be considered. If the matrix is in bulk form (solid polymeric pellets, hard soils, or vegetable matter), some preliminary sample preparation such as grinding, sieving, drying, mixing, or even wetting may be required. For nonporous or semiporous solids, a smaller particle size allows faster extraction. In some cases, a pH adjustment or addition of solvent into extraction chamber may aid the SFE process. If the sample is a semisolid, gel, or liquid, it must be immobilized on a solid support. Application of the sample to a piece of filter paper, to a solid support such as diatomaceous earth, or to a drying agent can facilitate the extraction and prevent the matrix from being swept out of the extraction chamber. Particularly wet matrices, such as sludge, may require water removal for good recovery and reproducibility of analyte extraction. Adding sodium sulfate and diatomaceous earth with thorough mixing to make a free-flowing powder has produced excellent results (43,44).

Many variables affect the SFE efficiency and reproducibility. Controlling the density, composition, temperature, flow rate, and time is necessary to provide a reproducible extraction. The ability to control density, composition, and temperature sometimes enables one to selectively fractionate the sample. Fractionation of classes of compounds by discrete changes in solvent strength is called 'density stepping' or 'density programming'. Using this technique one can selectively remove

analytes in one or several fractions, leaving impurities behind of in another fraction. Consequently, fewer impurities will interfere with the detection of the analyte in the final analytical method (45). Also, when the optimum conditions are obtained, an increase in flow rate can accelerate analyte elution.

The following characteristics are desirable in any solvent which is to be employed for an extraction :

a. Preferential affinity of the solvent for analyte over other constituents in the matrix (selectivity)
b. High capacity to dissolve an analyte (small volume)
c. The solvent must not react with the analyte (e.g. degradation, isomerization etc.) which complicates the analysis.
d. Extraction should yield quantitative recoveries
e. Nontoxic, noncorrosive
f. Inexpensive
g. Ease of disposal

SFs incorporate most, if not all, of the qualities mentioned above.These properties enable SFs to provide excellent extraction efficiency and speed over conventional methods.

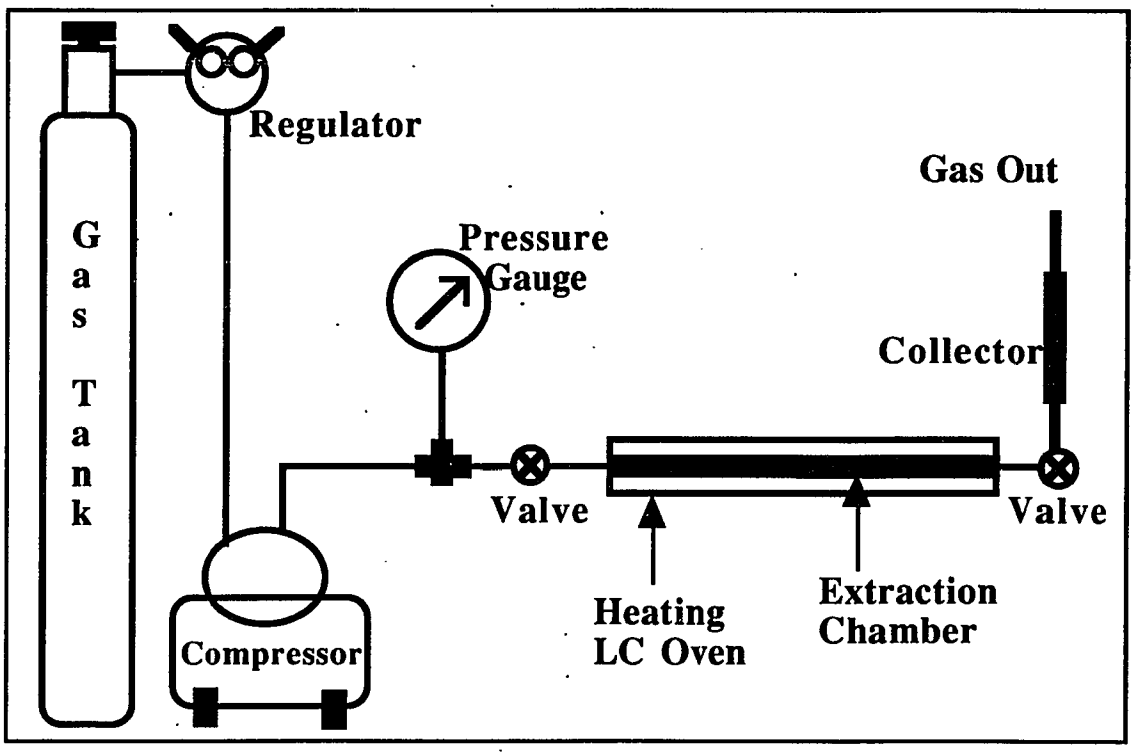
2.3.1 Instrumentation for SFE

The experimental arrangement is simple, and each extraction takes about 15-20 minutes. A schematic of the supercritical fluid extraction apparatus used in the present investigation is shown in Figure 2.2. The CO_2 is supplied through a single stage tank regulator. The gas is then

pressurized by a motor-driven single-end diaphragm compressor with a maximum output pressure of about 11,000 psi (749 atm). The gas then enters the extraction chamber, 12" long stainless steel tube (0.375"-o.d. x 0.236"-i.d.), which fits inside a thermostated LC heating oven (Scientific Systems Inc.; #CH-20-C0196). The temperature control module, attached to the oven, allows temperatures in the range of 30-99 °C with ±1°C accuracy. The extracted compounds are trapped in collector, usually 6"x1/4"(i.d.) stainless steel column packed with an adsorbent such as silica gel (60-200 mesh; T.J. Baker,Inc.), Tenax-TA (60-80 mesh, Alltech Associates Inc.) or Florisil (Thomas Scientific).

Although an SFE instrument can be constructed with separate pieces of hardware, many users prefer to buy commercial units. SFE instruments range from simple manual devices to sophisticated computer controlled units that provide automation, graphics, and other user-friendly features. A number of companies have entered the SFE market through SFC (Supercritical Chromatography) . Carl Erba (Valencia, California), Lee/Dionex (Salt Lake City, Utah), Suprex (Pittsburgh, Pennsylvania), Computer Chemical Systems (Avondale, Pennsylvania), and Jasco (Easton, Maryland) provide SFE-SFC coupled units; Isco (Lincoln, Nebraska), Hewlett-Packard (Palo Alto, California), and Suprex offer stand-alone instruments. Milton-Roy (Riviera Beach, Florida), one of the first companies to enter market, recently withdrew its units.

Figure 2.2
Schematic of the Supercritical Fluid Extraction Apparatus



2.3.2 Solvents Used in Supercritical Fluid Extraction

As in the industrial applications, carbon dioxide has been the solvent of choice in analytical supercritical extractions. Nearly two-thirds of the analytical scale SFEs have been performed with carbon dioxide (46). In addition to carbon dioxide, nitrous oxide and non-polar hydrocarbons such as ethylene, ethane and iso-butane have been used for the extraction of organics. Most of the solvents available for SFE are somewhat weak solvents compared to organic solvents. This weakness enhances the selectivity but limits their applicability. For example, supercritical carbon dioxide and nitrous oxide have sufficient polarity to extract relatively non-polar species such as alkanes, polycyclic aromatic hydrocarbons, and flavor and fragrance compounds, but they may not useful for the rapid extraction of more polar and higher molecular weight analytes. The ability to control the solvent strength and the poor solvent power towards some analytes has been cleverly used for selective fractionations. For example, poor extraction capabilities of carbon dioxide towards polar dioxins and furans has been effectively used to clean up fly ash prior to the extraction of the toxins (47). However, the solvent strengths of SFs can be markedly improved by using small amount of a polar modifier as discussed below.

2.3.3 Role of Modifiers in Supercritical Fluid Extraction

The solvent strength of SF, which is directly related to its density, is optimized by pressure-temperature programming. Even then some analytes especially polar ones are difficult to extract . The use of polar SF like ammonia is less practical due to corrosiveness and toxicity. Fortunately, the polarity of non-polar SFs like carbon dioxide can be

altered very easily to achieve the best results. The addition of a miscible polar solvent (modifier) is known to enhance the solvent properties of weak solvents. Most organic solvents such as alcohols, ethers, tetrahydrofuran, dimethyl sulfoxide, chloroform, etc. are miscible with carbon dioxide. Because the amount, usually under 10%, and type of modifier affects overall extraction time and elution order, in fractionation, it should be selected carefully to achieve sensitivity and selectivity. The use of modifiers demonstrates a significant improvement in the efficient removal of difficult extractable compounds from various matrixes. The introduction of these polar modifiers may produce a binary system with new critical parameters with no significant increase of the fluid density. But greater solvent power in as compared to the pure fluid itself. The polar modifiers can introduce specific interactions such as H-bonding with the analytes as well as weakening the interactions between the analyte and the matrix. This shows that the modified-solvent as well as the matrix plays an important role in the success of the experiment (48).

2.3.4 Comparison of Static, Dynamic, Recirculating SFEs

Quantitative analytical supercritical fluid extractions have been investigated in three different modes namely, static (49), dynamic (49), and recirculating (50). In the static equilibrium extraction mode, supercritical fluid is confined in a cell with fixed amount of sample. Pressure and temperature are optimized and sample allowed to equilibrate for a period of time, the equilibration time, with the SF to extract maximum analyte. After equilibrium, the extract-laden SF is released into a collector containing adsorbent. The sample thus extracted is collected for further analysis. In the dynamic extraction (flowthrough) mode, a continuous flow of the supercritical fluid is passed through the sample at

a constant or variable flow rates and the extract is continuously collected. The instantaneous equilibrium between SF and sample is assumed. The recirculating extractions, less commonly used, operates with a fixed fluid volume that recirculates continuously throughout the system until equilibrium is achieved. As far as the instrumentation is concerned, the static equilibrium mode employs a valve to create a closed system whereas a flow restrictor such as a capillary tube is used to maintain continuous flow in the dynamic extraction. Actually, most of the static equilibrium mode extractions are semi-continuous at the releasing step, unless the sample is separated within the extraction chamber.

Until recently, researchers measured equilibrium solubilities of compounds in SFs using laboratory-built devices. In 1988, LDC Analytical (Riviera Beach, Florida) introduced an instrument known as 'Sample Preparation Accessory' that enables such measurements to be made in either static, dynamic, or recirculating modes (51). This instrument gave good results if the compound to be extracted had moderate to high solubility in SF and a UV-Vis chromophore (52). For compounds that have low or wide range of solubility with or without chromophoric groups, a modified instrument has been described by Maxwell et al. (53).

2.3.5 SFE Vs Conventional Extraction Methods

As discussed earlier, SFE can be considered a hybrid of two most routinely used separation techniques namely distillation and extraction. A brief comparison of these two separation techniques with SFE is given below.

SFs are attractive solvents due their unique properties. SFE can be considered as an extension of distillation to high pressures but with

various differences. In distillation, a light phase is formed by vaporizing part of the mixture to be separated whereas in SFE, the light phase is usually a new component not present in the original, unseparated mixture. In distillation, the driving force for fractionation is a temperature gradient across the distillation column, whereas in SFE the driving forces can be temperature, pressure, or both. Thus, SFE can separate components with similar low vapor pressures, which are susceptible to heat degradation. In SFE, differences in solvent effects can enhance separations based on differences in vapor pressure.

SFE may be viewed as an extension of conventional liquid extraction to high temperatures, with some differences. To vary the solvent power of liquid solvents, it is necessary to change temperature significantly or to use additional chemical species. In SFE, separation based on differences in solvent effects is enhanced by differences in vapor pressure. Fractionations can be done easily by stepwise changes in solvent power in the loading or the release steps, or both, without the use of additional components. For a given solvent and solute, SFE capacity can vary from low values typical of poor solvents to high values typical of good solvents as temperature and pressure vary. The ability to drop loadings to low levels is important in the selective separation of chemically similar components. As solvent loadings decrease, the ability to discriminate between chemically similar components increases. The residual solvent in the extract can be easily eliminated without distillation in supercritical solvents because they are gases at ambient conditions. The favorable mass transport properties of supercritical solvents allow a more rapid approach to equilibrium. Hence they can penetrate deeper and more readily into substrates than can liquid solvents. Large differences in

densities between phases and the low viscosity of the SCF phase lead to easy phase separations. In SFE, it is relatively easy to obtain extracts that are free of entrained solids and liquid drops which is a common problem in conventional extraction processes.

2.3.6 Advantages of Supercritical Fluid Extraction

The option of having variable solvent power is one of the many important advantages of a SF solvent. The solvent strength of a pure SF is proportional to its density. Thus by changing pressure or temperature optimum extraction conditions for a target analyte can be achieved. By carrying out two step SFE, class-selective extractions of particular compound classes are possible . This simultaneous fractionation and extraction saves time and labor as compared to the partitioning processes of conventional extractions .

On the other hand, the poor solvating power of SFs compared to organic solvents for some analytes of very low volatility offers another important advantage. By choosing appropriate pressure and temperature a selective extraction is done for the analytes of medium to low volatility. The alternative separation technique, distillation, can not be used for such compounds which makes this feature of SFE very useful and important.

In liquid extractions, to recover analytes which are diluted in a large volume, solvent has to be distilled or rotovaporised which, of course, is time-consuming. Moreover, these concentration processes can cause loss or degradation of the analytes. In contrast, in SFE, complete separation of the analytes is achieved by isothermal decompression or isobaric heating depending on the nature of the analytes.

It is possible to perform SFE of thermally labile analytes at moderate temperatures due to the availability of SF-solvents with low critical temperatures.

SFE is about 10 - 100 times faster than conventional Soxhlet extraction in which distillation takes up most of the time. Also, SFE has been found to be faster and more selective as compared to sonication method.

SFE systems are normally made of metals which are airtight. Thus, SFE can be successfully overcome the common problems encountered while extracting photosensitive and easily oxidizable compounds.

In liquid extractions disposal of organic solvents is requires a great deal of time and money, whereas in majority of SFEs the solvents (SFs) can be simply vented to the atmosphere without any danger or may be recycled.

SF solvents are an economically favorable choice to conventional organic solvents due to low cost and high purity. SFs are easy to recycle as compared to time-consuming distillation processes used to gain back expensive organic solvents.

The solvent power of the SF can be modified depending on the nature of the analytes. Addition of a polar modifier like methanol, discussed earlier, to a non-polar SF increases the yield of extraction of polar analytes considerably. The modifiers facilitate extraction either by selectively interacting with the analytes or by displacing the analytes from an adsorptive matrix. Thus, SFs with wide range of solvent powers, needed for analytes with different polarity, can be prepared. The possibility of a similar advantage in Soxhlet extraction is very limited and restricted to only certain azeotrope conditions. Non-azeotrope

conditions result simply in the evaporation of the lower boiling liquid out of system reservoir.

Greater penetration power of the SFs due to favorable mass transport properties, as compared to liquids, results in greater efficiency even from highly sorptive matrices where liquid extractions yields are often poor.

SFE is free from experimental difficulties such as formation of emulsions, chemical reactions, often encountered in liquid extraction leading to the loss of analytes and thereby not giving quantitative yields.

Due to the nontoxic and nonflammable characteristics of carbon dioxide, the most commonly used supercritical fluid, it is highly acceptable in the food industries as a substitute for organic solvents such as halogenated hydrocarbons. Also, it is safer for laboratory and industrial workers.

On and off line compatibility of SFE with other analytical techniques, e.g. GC, HPLC, SFC, demonstrates the versatility and decisive superiority of this method over conventional ones. In summation, the salient features of SFE are :

Faster (minutes vs. hours)

Comparable or better recoveries

Lower extraction temperatures

No sample contact with boiling solvent.

Extraction done in absence of light and air

Solvent removal is simple

Gases are purer and cheaper than solvents

Solvent disposal is easier and harmless

CO_2 is nontoxic

Gas mixtures lead to selectivity

Pressure/temperature programming allows step-wise extractions

Transparency in the infrared and ultraviolet regions

Ease of disposal.

2.4 Applications of SFE

2.4.1 Analytical Applications of SFE

As described earlier, the phenomenon of supercritical fluids has been known for more than 100 years, but the implementation of SFE as an industrial scale extraction technique took place over last two decades or so. This development is independent of the progress in the field of high-performance liquid chromatography (HPLC), which is a separation method not only contemporary with SFE, but also with a similar history of development. In addition to their use in separation, these two techniques have many things in common such as instrumentation. Both use high-pressure pumps, sample introduction devices, packed or hollow separation columns, etc. Since 1960s, numerous reports on HPLC and SFE have been published. The unique properties of supercritical fluids has prompted their use for a variety of applications in the field of analytical chemistry. The largest number of applications occur in the field of chromatography, where these compressed gases are employed as highly interactive mobile phases.

In 1976 Stahl and Schiltz (54,55) published the first application of analytical SFE. They developed an extraction system in which SFE was coupled with TLC. Nieass et al. (56,57) used SFE connected to HPLC, for the first time, to examined solubility of organic compounds in SFs . Since

then the use of SF as mobile phase paved way for a large number of analytical applications resulting from off-line as well as on-line detection.

Off-line detection methods normally require concentration of extracted materials before the analysis, which can lower the achievable detection limits. Off-line detection methods are particularly suited for unknown compounds because several techniques can be employed to analyse the extracted analytes. Extraction of concentrated matrices, or analytical experiments such as solubility studies where a large quantity of the analyte is present, are performed using off-line methods. Extensive research in this area has been done by Dr. Locke's group at Queens College of the City University of New York (58,59).The off-line methods may suffer from ineffective trapping, incomplete recoveries from the trap, extra sample handling, dilution or concentration steps which can affect the results.

On-line SFE coupled with an appropriate chromatographic technique offers maximum sensitivity because all of the extracted analyte is quantitatively transferred into the chromatographic column. This feature is particularly attractive for trace analysis and small sample sizes. The ability to vary solvent power and polarity of SFs provides the basis for the selective extraction and an automated method where sample preparation and analysis can be instrumentally linked. The SFE-SFC, SFC-GC are more often used as compared to SFE-HPLC.

As a result of the extensive research carried out by several research groups, supercritical fluid chromatography (SFC) has emerged as one of the outcomes of the supercritical phenomenon known to date (60,61). It has been described as a technique that bridges the gap between GC and HPLC (62). Rapid mass transfer in SF mobile phase attracted researchers

as it offers high speed separation with high resolution on an open tubular capillary column and also on a packed capillary column. The low consumption of fluid as well as other features described earlier helped SFC to develop rapidly. In addition to commonly used UV-Vis detector, the SFC has been successfully coupled with mass spectrometry (63,64), Fourier transform infrared spectroscopy (65,66), flame ionization detection (67,68,69), and electron capture detection (70). Sophisticated SFC systems are available and have been described by Gere et al.(71) and Greibrokk et al (72). A major advantage of capillary SFC is the potential of yielding highly efficient chromatographic separations of polar-functional and non-volatile compounds. For example, mixtures of dyes is a class of compounds which is difficult to analyse otherwise. Up until the late 1970's, chromatographic analyses of dyes were performed primarily by paper and thin layer chromatography(TLC). More recently, high performance liquid chromatographic (HPLC) methods have been increasingly employed for the separation and analysis of dyes. Also, SFC has been applied to analyse coal-derived products, polymers, dyes, diesel exhaust particulate, erthromycins, glycerides, pesticides, cholesterol, mycotoxins etc. using one or more detectors described above (73).

The SFE-GC may be carried out on any commercial GC with or without minor adjustments. Hawthorne and Miller (74) used a simple yet effective set-up of dynamic SFE coupled to a capillary GC in which the extraction cell restrictor is inserted directly into capillary GC column using cryogenic focussing. Once the extraction is complete, the restrictor is removed from the injector and the GC oven is temperature programmed to elute the analytes. SFE-GC has been used for the analysis of a wide range of analytes such as caffeine, nicotine, PAHs,

PCBs, phenols, flavor and fragrance components, heterocyclic aromatics, etc. (75,76,77).

Comparatively fewer studies have been reported (78,79) which utilizes SFE-HPLC combination since nearly all the SFE-HPLC applications can be performed by SFC or SFE-GC.

2.4.2 Industrial Applications of SFE

It is apparent from above discussion that extraction with supercritical fluids is a powerful tool and due to this its development is receiving increasing attention from industry. It would be worthwhile to look into some of the successful applications of this superb technique.

Although the concept of supercritical fluids as solvents has been established for nearly a century but the first proposal for practical application of supercritical extraction was made in 1943 describing a method for deasphalting petroleum oils (80). In the USSR, Zhuze described a similar scheme based on a pressure-controlled process using supercritical propane. Supercritical methane has been used for fractionation of crude oil, extraction of lanolin from wool grease and ozocerite wax from ores (81). Since then there is a renewed interest in SFE, intensified due to increased government enforcement of stringent pollution-control laws and a change in process economics caused by rapidly increasing energy costs. In addition to these factors, increased performance demands on materials, which can not be met by conventional processes, created a strong background for these developments. The safety features of SFE make it an attractive solvent for the food and pharmaceutical industries. It is environmentally nonhazardous, nontoxic,

nonflammable, noncorrosive, inexpensive, and available in high purity. In the food industry, supercritical carbon dioxide is not regarded as a foreign substance or additive unlike organic solvents such as hexane or methylene chloride and it is an inert that does not react with food constituents. SFE does not leave residues in the product, which can be a problem in conventional processes. Due to these features, the Food and Drug Administration has classified carbon dioxide as Generally Recognized As Safe (GRAS) (82).

Some of the more important of these process scale applications are summarized in Table 2.3. Detailed discussions have been published in a number of reviews (19,20,25,15,83) and books (27,84).

Table 2.3

Industrial Applications of Supercritical Fluid Extractions

NATURAL PRODUCTS	
Decaffeination	raw coffee (85), tea (86)
Deodorization	vegetable oils (87)
	animal oils and fats (98)
	brewer's yeast (88)
Aroma Recovery	spices (89), hops (90,91)
	tobacco dust (92)
Oil Recovery	soya beans (98), jojoba beans (93)
	palm kernels (98), coconuts and olives (98)
	cotton seeds (98)
Fractionation	cod liver oil (98), fatty acid esters (98)
PETROCHEMICALS AND COAL INDUSTRIES	
Heavy Oil Recovery	residues from hydrogenation by the Bergius-Pier process of brown coal, bituminous coal, and petroleum vacuum residue (94,95)
	oil sands (96), oil shale (98)
Regeneration	used motor oil (98)
De-asphalting	residues from petroleum distillation (97)
	oil sand bitumen (98)
Fractionation	tall oil (98)
	brown coal high-temperature tar (98)

CHAPTER 3
Experimental, Results and Discussion

Despite extensive studies in recent years, SFE remains an empirical technique. There are no 'cookbook' methods available because solubility of analyte in the SF can't be precisely predicted on theoretical grounds alone. The situation becomes even more complicated due to the fact that analyte is not only the component present in the matrix being analysed.

In order to establish an effective analytical methodology, the effects of a wide variety of variables such as pressure, temperature, extraction time, nature of SF, type of trapping material on the efficiency of SFE were studied. The percentage recovery in an extraction (1) was calculated from the following equation :

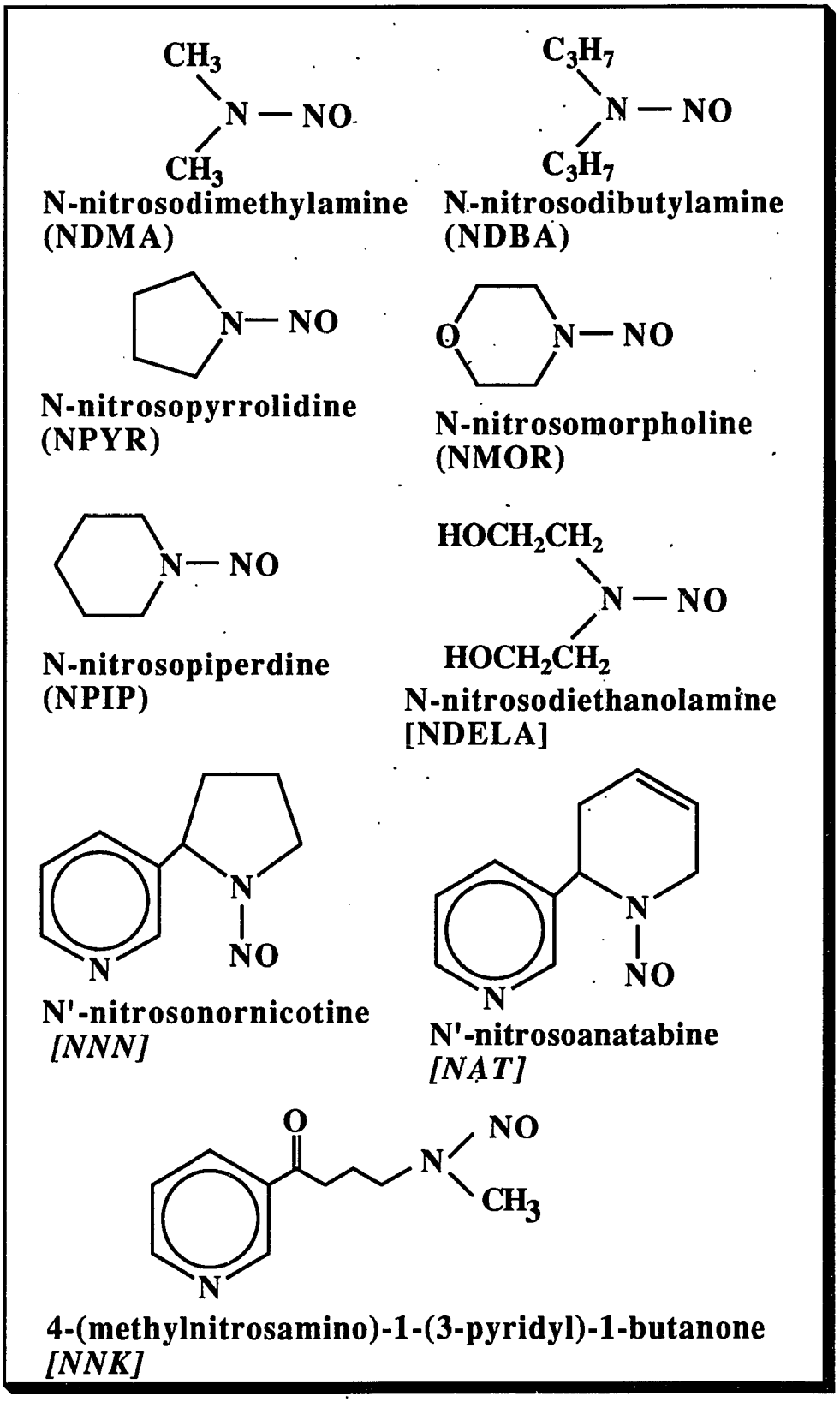
$$\% \text{ Recovery} = [W_e/W_s]x100 \qquad .....[1]$$

where C_e = weight of the spiked compound

C_s = weight of the compound recovered.

The concentration levels of N-nitrosamines (Fig. 3.1) chosen were kept similar to those found in naturally contaminated matrices. The efficiency of the SFE, expressed as the percentage recovery, is not only based on the optimization of SFE technique but also relies upon the quantification technique employed (GC-TEA in the present study). Therefore , the first task of this project was to calibrate GC-TEA to establish its reliability, reproducibility, and linearity. This was followed by the optimization of separation variables.

Figure 3.1 N-nitrosamines under investigation



3.1 Materials and Reagents

NDMA, NDEA, NDBA, NPIP, NPYR, NDELA were purchased from Aldrich (Milwaukee, WI). TSNA samples were obtained from American Health Foundation. The trapping materials such as silica gel (60-200 mesh), Tenax-TA were purchased from J.T.Baker (Phillipsburg, NJ), Fisher Scientific (Pittsburgh, PA), and Alltech Associates (Deerfield, IL) respectively. Linde bone-dry grade carbon dioxide (Union Carbide, Long Island City, NY) was used in all supercritical fluid extractions. HPLC-grade solvents (methylene chloride, methanol, hexane) were purchased from Fisher Scientific or J. T. Baker, Inc.

Because of potential carcinogenic nature of N-nitrosamines, all glassware, spatulas and tools were carefully cleaned and experiments were done under a hood. Fresh stock solutions were prepared under subdued light and stored in a cold room. Working standard solutions were prepared by serial dilutions in suitable solvent.

3.2 Extraction Techniques

3.2.1 Soxhlet Extraction

The filter containing TSNAs was placed into a glass extraction thimble (6.6 cm x 1.8 cm i.d.). The thimble containing the filter was then extracted in a standard Soxhlet extraction apparatus, containing 65 ml of methylene chloride, for 16 hours. The extract containing the analyte was evaporated either under a stream of nitrogen gas or using a rotavapor (Büchi model 110).

3.2.2 Supercritical Fluid Extraction (SFE)

A schematic diagram of the SFE apparatus is shown in Figure 2.2. Carbon dioxide supplied through a single stage tank regulator was first filtered through an Autoclave Engineers 5-μm cup-type line filter (AE model# CXF4-5) and then passed to a motor-driven single-ended diaphragm compressor (model 46-13411-2, Newport Scientific, Jessup, MD) where the carbon dioxide gas was compressed to various pressures up to a maximum of 10,000 psi. A Bourdon-tube pressure gauge was utilized to monitor the pressure of the supercritical carbon dioxide. The gas then entered the extraction chamber, 12" long stainless steel tube (0.375"-o.d. & 0.236"-i.d.), which fits inside a thermostated LC heating oven (Scientific Systems Inc.; #CH-20-C0196). The temperature control module, attached to the oven, allows temperatures in the range of 30-99 $^{\circ}$C with ±1 $^{\circ}$C accuracy. The extracted compounds are trapped in a collector, a 6"x1/4"(i.d.) stainless steel column packed with an adsorbent. In a typical SFE experiment , first, the extraction apparatus was washed with the methanol or methylene chloride and was dried in an oven. The extraction chamber was then packed with the sample with gentle tapping, if needed. Modifier, if any, was directly added onto the sample. Glasswool plugs on both ends were inserted, one inserted before adding the sample, to secure sample packing. The packed chamber was assembled into the heating oven. A low pressure of carbon dioxide gas purged the chamber for about 1 minute to remove air. The exit valve was then closed and extraction chamber brought to desired temperature which was maintained isothermal throughout the extraction. The system was then pressurized to the desired level and sample in the chamber was extracted for the desired period of time (5-30 minutes). After completion of extraction, the

compressor was turned off. The exit valve was opened slowly to achieve a linear depressurization of the system through the trap tube where the analyte was collected on the adsorbent. The trap and exit valve were washed with about 50 ml of solvent which was subsequently reduced to 1.00 ml, ready for further quantitation as described below.

3.3 Chromatographic Analysis

A Hewlett-Packard Model 5890 gas chromatograph (GC) interfaced to a Thermal Energy Analyser (TEA) (Thermo Electron Corp., Waltham, MA Model 543) was employed to analyse N-nitrosamines. The details of TEA (2) are given below.

A nitrosamine, mixed with the carrier gas, is introduced into a catalytic pyrolyzer. Here, all N-nitroso compounds, under vacuum, are cleaved at the N-NO bond, releasing the nitrosyl radical (NO^*). The products from this reaction then pass through a -150 $^{\circ}C$ cold trap. Here, most of the by-products of the pyrolysis reaction and any solvents are removed. A vacuum draws the NO^* radical into the reaction chamber where oxidation takes place with ozone, the product of this reaction being excited singlet-state nitrogen dioxide (NO_2). The excited NO_2 rapidly decays back to its ground state emitting near-infrared radiation at 600nm. This radiation is detected by a sensitive photomultiplier tube with readout by a strip-chart recorder. These processes are summarized in Figure 3.2. In these reactions, the yield of nitrosyl radical (NO^*) is stoichiometric and reproducible. Hence, the intensity of the near-IR radiation is a direct measure of the nitrosamine present. The principle of operation of TEA is also shown in Figure 3.2. In a typical analysis, an aliquot of 1 to 5 µl was injected into GC-TEA and results were obtained on a recording

integrator (Hewlett-Packard Model 3380). The experimental conditions, depending on the type of N-nitrosamines, were as follows:

(a) for VNAs- 14 ft x 1/8" o.d. stainless steel packed with 10% Carbowax 20M plus 0.5%KOH on Chromosorb W(80/100 mesh), Initial Temperature=175 °C(1min), programmed at 6 °C/min, Final Temperature = 205 °C (2 min), 31 ml Ar/min. The TEA pyrolyzer was held at 520 °C and the cold trap was kept at -150 °C.

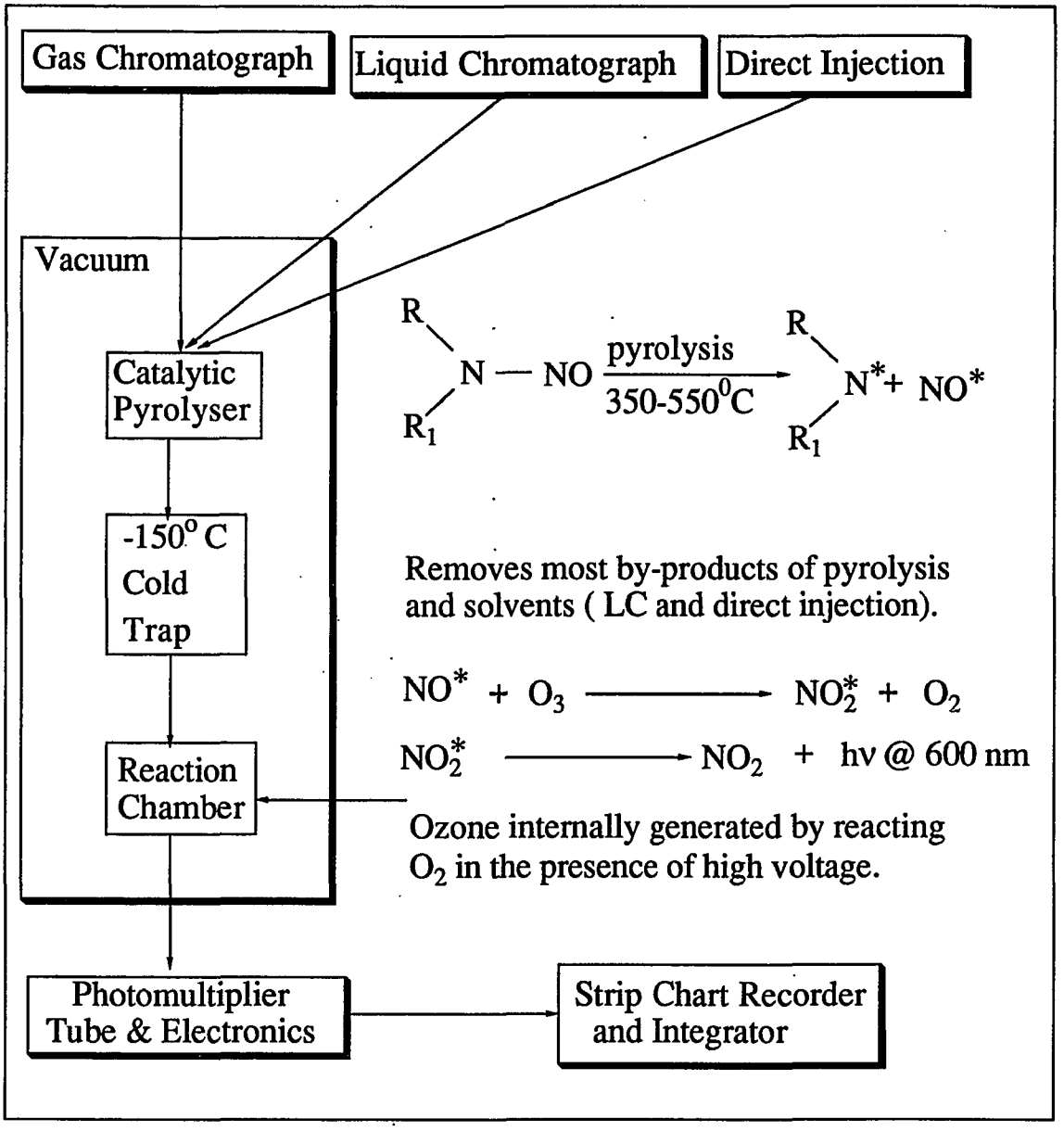
(b) for NDELA- 6'x1/4" o.d. (2 mm i.d.) glass column packed with 3% OV-225 on Chromosorb W HP, 80-100 mesh; isothermal at 180 °C, TEA interface 160 °C, TEA pyrolyzer was held at 520 °C. The carrier flow was 40 ml Ar/min, the TEA cold trap was kept at -150 °C.

(c) for TSNAs- 2m x 6.4mm(2mm i.d.) glass column packed with 3% XE-60, Initial Temperature=150 °C, programmed at 2 °C/min, Final Temperature= 220 °C. The carrier flow was 31 ml He/min. The column was baked overnight at 250 °C at least once in a week.

3.4 Analysis of the Standard Solutions

The GC-TEA system has high selectivity for N-nitrosamines (10^7 to 1) as compared to other compounds such as hydrocarbons, amines, common solvents, and other nitrogen-containing compounds which are known to interfere with the analysis of N-nitrosamines in other systems. It has been found to have a linear dynamic range of over 6 orders of magnitude with a sensitivity typically better than 0.2 ng NDMA at a S/N ratio of 3 to 1 (2).

Figure 3.2 Principle of Operation : Thermal Energy Analyser



The GC-TEA system was calibrated using standard solutions of N-nitrosamines, ranging from 0.5 ng to 16 ng, in methylene chloride. The curves were plotted based on the average of the measured peak areas obtained from integrator (Figure 3.3). The correlation coefficients for these plots range between 0.993 and 0.985. Rounbehler et al (3) found the correlation coefficients for samples containing about 25 ng of NDMA to be 0.99.

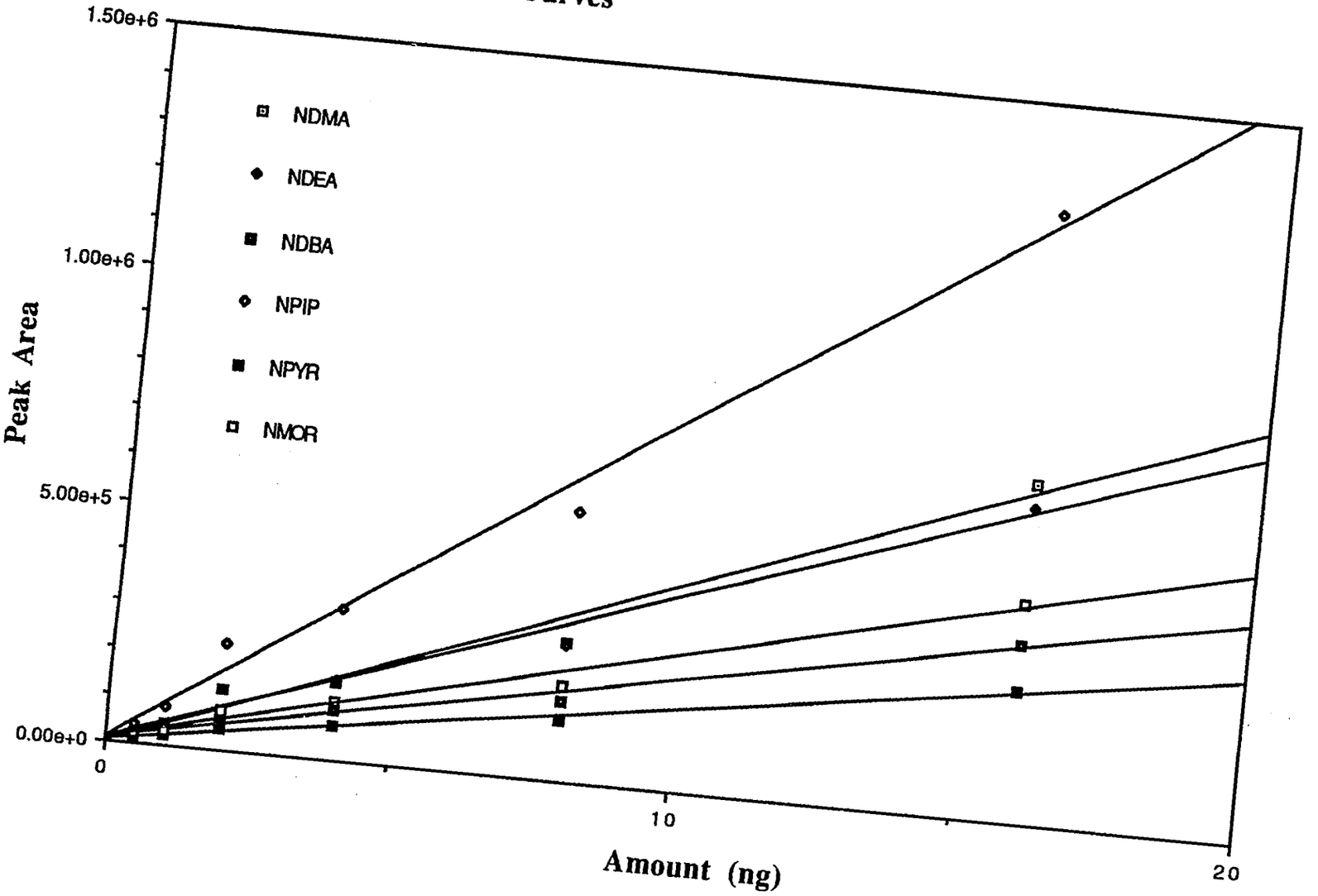
3.5 Optimization of the Efficiency of SFE

In a SFE, there are various experimental parameters which have to be optimized for a successful extraction. These variables include pressure, temperature, equilibration time (extraction time), material in the collector, and effect of a modifier (additive). Any change in the experimental conditions may alter the extraction efficiency quite markedly. Moreover, the selection and control of these parameters can pave the way for analysis of compounds similar to those in known samples.

3.5.1 Effect of Pressure

It is well-established that the solvating power of a SF is directly related to its density, which mostly depends on the pressure and temperature of the system (4). The selection of proper pressure and temperature is of greatest importance since both can affect the density (which alters efficiency) significantly. The lower boundary limits of pressure and temperature are determined by the critical values of the extracting fluids whereas the upper limits depend on various factors such

Figure 3.3 Calibration Curves



as quantitative recovery, instrumentation at hand, thermal lability of analyte, etc.

In a high density fluid the molecular interactions increase because of the shorter intermolecular distances as compared those in a lower density SF. A solute typically exhibits a threshold pressure, around P_c, above which solubility increases significantly; commonly, the near the critical pressure of extraction fluid maximum greatest change in the density of SF (5).

A filter paper (matrix) was spiked by pipetting 1 ml of standard solution containing known amount of VNAs (10-100 ng). This filter paper was dried and rolled, prior to insertion into the extraction chamber. Table 3.1 shows the effect of varying the system pressure on the recoveries of VNAs in the supercritical carbon dioxide extractions from a spiked filter paper, 100 ng each, at 50 °C for 20 minutes. It is apparent that any pressure above 5000 psi (340.2 atm) practically offers similar recoveries of VNAs which can be attributed to the volatile nature of these N-nitrosamines, and to the steeper increase of density at pressures close to P_c than at higher pressures.

An increase in pressure alone did not offer appreciable increments in the recoveries of TSNA and NDELA where the recoveries were relatively poor. However, higher recoveries, discussed later, resulted when the SF-CO_2 was modified with methanol.

3.5.2 Effect of Temperature

The competing effects of reduction in solvent density and increase in solute volatility with increase of temperature explain the necessity for evaluating the optimum temperature. Higher temperatures favor the extraction by increasing the escaping tendency of the solute from the

Table 3.1 Effect of Pressure on Percent Recovery

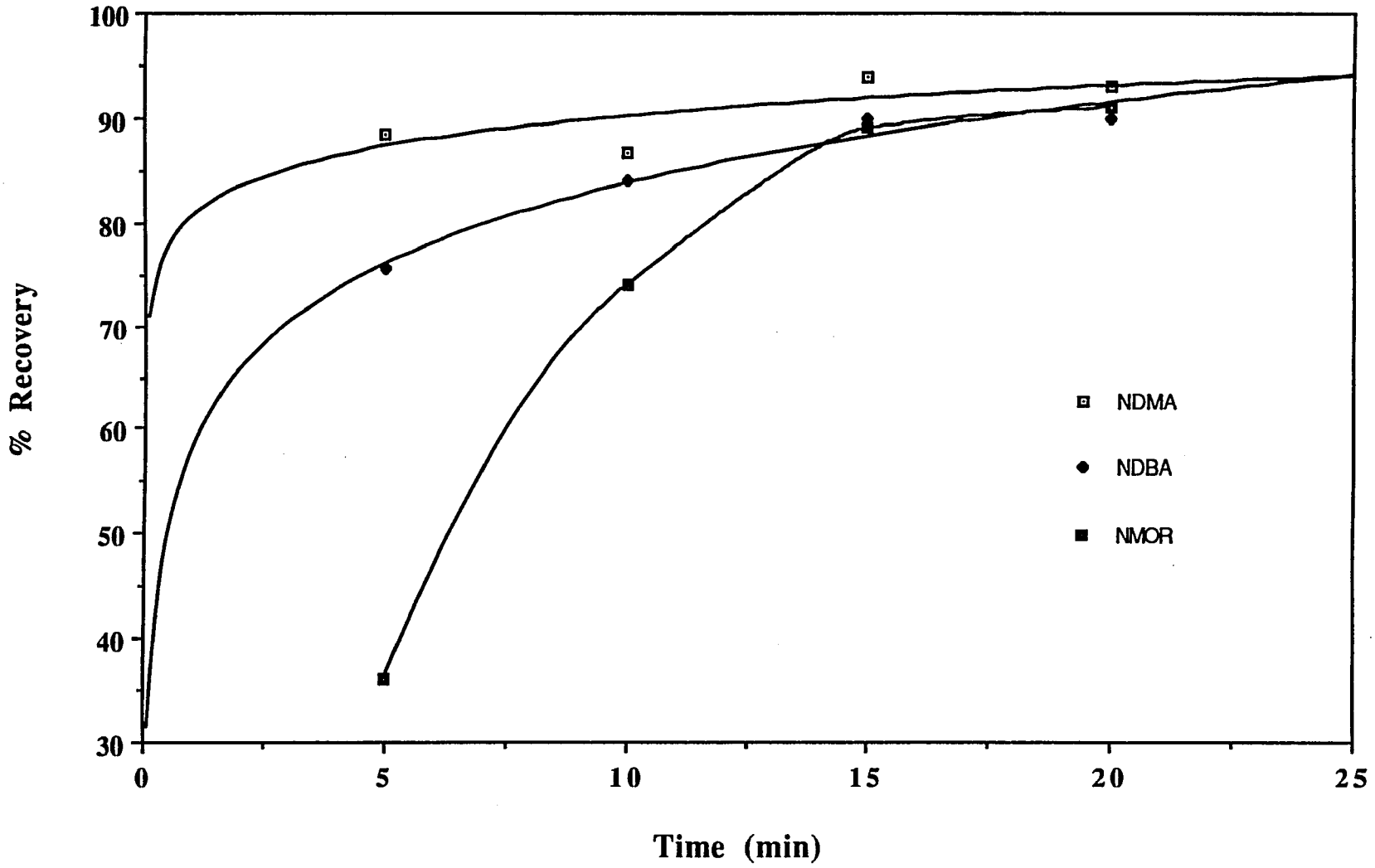
	Pressure (psi)		
	5000	7000	9000
NDMA	93	94	92
NDEA	90	92	90
NDBA	88	91	90
NPIP	100	92	91
NPYR	89	86	86
NMOR	89	93	86

condensed phase. In contrast to pressure, the effect of temperature on the success of a SFE is often complicated (6,7). The cmplications arise from the fact that not only the density of a SF, but also vapor pressure of solutes, matrix characteristics, etc. are also temperatute dependent.

As a general rule static equilibrium SFE experiments confine the upper temperature limit to no more than 100 °C above the critical temperature. The reasons for this are first, higher temperatures, at constant pressure, are accompanied by lower density of extracting fluids which means higher pressures must be applied to achieve liquid-like densities, and second, at higher temperatures the thermally labile compounds tend to decompose.

The extractions of VNAs spiked onto filter paper, 100 ng each, were carried out at five different temperatures, 35 °C, 40 °C, 45 °C, 50 °C, and 65 °C. The pressure was maintained at 9000 psi and equilibration time was 20 minutes. Figure 3.4 presents a typical scenario of the temperature dependence of percent recoveries of volatile compounds. The increments in the vicinity of the critical temperature of carbon dioxide (T_C=31 °C) are consistent with the general property of a SF that the solubility of a solute increases greatly when the system temperature just exceeds the critical temperature. The increase in the recoveries of VNAs could also be related to the volatile nature of these N-nitrosamines. It is observed that around 45 °C to 50 °C proves to be an ideal range for maximum recoveries and beyond which no further increments took place. Thus, all further extractions were carried out at 50 °C. Not only will a high temperature decrease the density of SF but will also decompose the thermally labile NAs.

Figure 3.5 Effect of Equilibriation Time on Percent Recovery

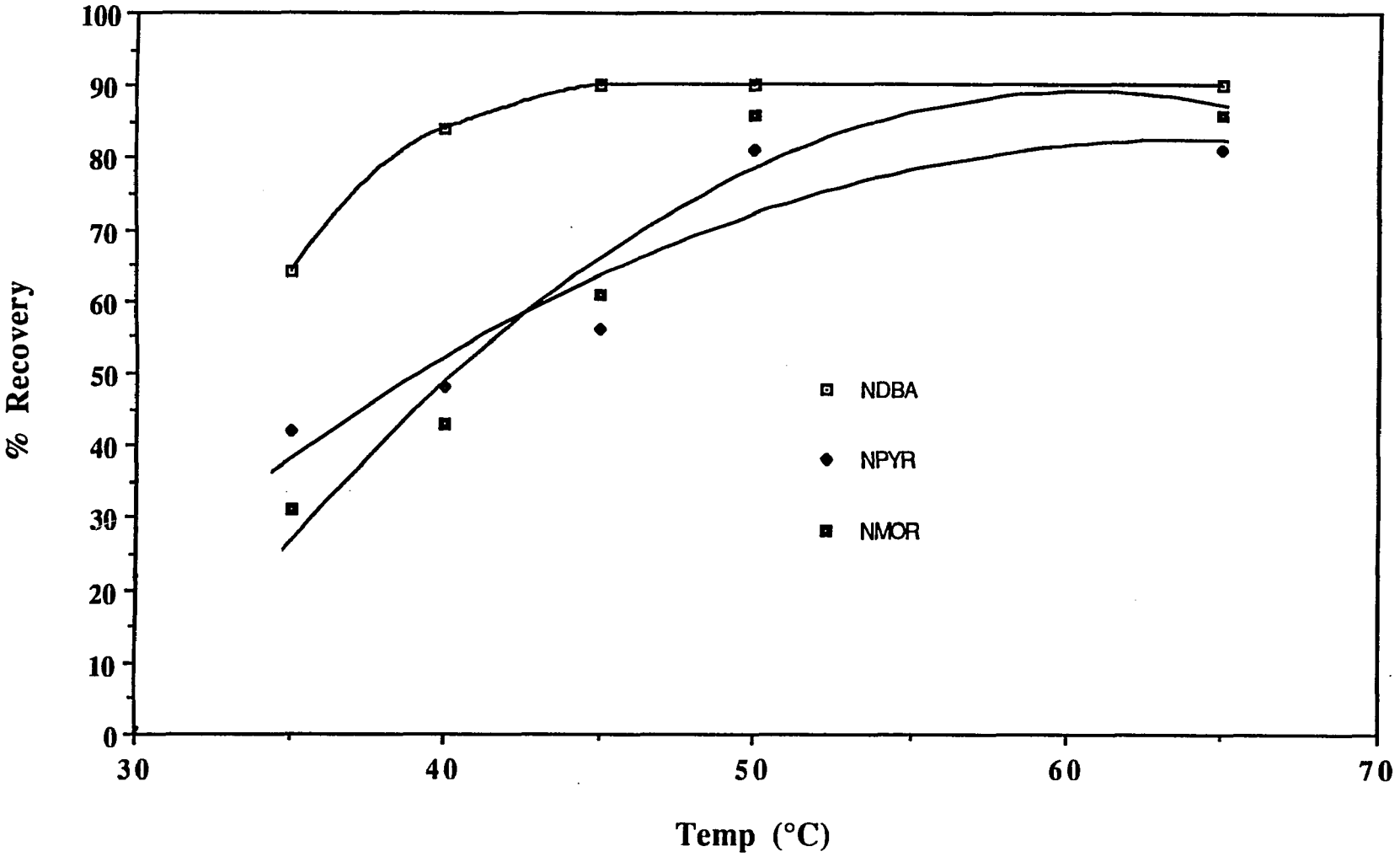


3.5.3 Effect of Equilibration Time (Extraction Time)

The equilibration time, another important analytical variable, signifies the length of time for a complete extraction of a particular sample. In general, it is preferred that the time required for an extraction to be as short as possible without compromising with the accuracy and efficiency of the method. This is accounted for by the kinetics of the extraction process. Comprehensive kinetic models have been developed (8,9,10), but the kinetics are still not well understood. However, a general mechanism can be used to explain the extraction process. The extraction process starts with the penetration of SF into extraction matrix. The penetrated SF interacts with the sample matrix and the analytes gets dissolved in SF. Finally, the analyte-loaded SF moves out of the sample matrix. It has been shown that penetration of the SF into the matrix is a diffusion controlled process (11). This implies that the equilibration time will depend on the particle size. Smaller porous particles will allow faster penetration and equilibration will be attained quickly as compared to large particles. During this study, spiked filter paper was initially used as the sample matrix. In later studies solid samples such as tobacco were powdered and sieved in order to get a small particle size, homogeneous matrix.

Figure 3.5 represents the effect of varying equilibration time (5, 10, 15, 20 minutes) on the recoveries of VNAs extracted with SF carbon dioxide at 9000 psi and 50 °C. These results demonstrate NAs can be quantitatively extracted within 15 minutes. NDMA, being the most volatile, is practically quantitatively extracted in 5 minutes followed by those with lower vapor pressures. However, to be on the safe side the

Figure 3.4 Effect of Temperature on Percent Recoveries



extraction time was chosen to be 20 minutes which ensures complete extraction in a given experiment.

3.5.4 Collector Efficiency

Depending upon the experimental conditions, it is possible for analyte molecules to nucleate and become entrained in the expanding gas, forming an aerosol which can be easily lost to atmosphere (12). The particle size of the resulting adsorbed compounds is dependent on the rate of depressurization. By creating a slow depressurizing process, relatively large particles can be produced instead of an aerosol, which makes it easier to collect the precipitated particles. Using a porous barrier to the gaseous flow, the solute particles can be filtered from the stream of gas. In addition, these particles can be efficiently trapped using a selective adsorbent material.

Three solid adsorbents- silica gel, Florasil, Tenax-TA, were used to collect the extracts after the depressurization step. The collector containing the solid adsorbent was transfered into a beaker and washed with about 40 ml of organic solvent. This step is crucial because incomplete elution or introduction of contaminants can cause erroneous results. To prevent loss of analyte in the concentration step, the rotary evaporator was used at slow speed and low temperature, around 40 ^{o}C. Finally, the sample volume was reduced to 1.00 ml by evaporating the solvent using a fine stream of nitrogen gas.

The results presented in Table 3.2 indicate silica gel to be more efficient collector material than Florasil and Tenax-TA, which may be attributed to its good adsorbing properties. It offers large specific surface area, easy elution, and simple cleaning process. It is also economical as

Table 3.2 Evaluation of Solid Adsorbents

	Collector Efficiency (% Recovery)		
	Tenax-TA*	Florasil	Silica Gel
NDMA	70	77	91
NDEA	71	78	89
NDBA	81	84	86
NPIP	71	79	78
NPYR	68	81	84
NMOR	85	82	89

Solvent: Methylene chloride (* Hexane)

Collector: 6"x1/4" stainless steel column

compared to other two. The polymeric Tenax-TA dissolves in halogenated or aromatic solvents (13); thus hexane was used to elute analyte.

It has been demonstrated that analyte is trapped within first two to three inches of collector. However, to prevent any loss and for safety considerations, a six inch long stainless steel tube was used which holds about one gram of adsorbent. The distance between outlet of the chamber and the trapping tube is minimized to ensure maximum adsorption, whereas a careful rinse of exit valve and union elbow ensures completeness of recovery as well as minimization of cross contamination between extractions.

3.5.5 Effect of Modifier (Additive)

The efficiency of SFE depends both on the chemical nature and quantity of modifier added. It has been hypothesized that the polarity and polarizability of supercritical fluid mixture should be optimized to match those of the analyte to achieve higher recoveries. This, perhaps, is an oversimplification of a complex phenomenon (14). The percent recoveries have been found to increase with increasing amount of modifier, and to reach a maxima, normally 10% modifier, beyond which further addition of additive have no effect. However, it can not be generalized that an increase in the volume of modifier will always result in higher recovery. Depending upon the quantity of sample matrix, addition of excess volume of additive may reduce the apparent recovery because solutes were extracted out of the extraction chamber with the excess modifier during sample loading process. Furthermore, excess amount of modifier has been found to saturate the carbon dioxide with the

modifier (15). Also, effects of modifiers on the recoveries vary from one system to another depending upon the type of matrix, nature of analyte, experimental conditions, critical parameters of binary SF/modifier fluid mixture, etc. Hence, it is apparent that the effects of modifiers on SFE are more complicated and less predictable as compared with those of pure SF systems. In a nutshell, the efficiency of SFE is a function of chemical nature and amount used of modifier which alters the fluid properties such as polarity, density, and therefore solvent power. The modifiers are capable of capillary condensation or adsorption into the matrix thereby changing pore environment and the diffusional situation (16).

The recoveries of NDELA, spiked onto Chromosorb-W, and TSNAs, spiked onto filter paper, using pure SF-CO_2 were relatively poor and generally increased by less than 10% in the pressure range of 8000 psi to 9500 psi at a temperature of 95 °C. To improve the recoveries a polar additive, methanol, and a non-polar additive, methylene chloride, were chosen. The introduction of a modifier to the extraction system was accomplished by pipetting 1 ml (~10%) of the desired liquid into the packed extraction chamber prior to the pressurization. The results, more pronounced in the case of TSNAs, are summarized in Table 3.3. Methanol proves to be the better choice, especially in the case of NDELA which may be due to the presence of two oxygen atoms present in analyte making it possible to be extracted in the SF/modifier binary mixture. However, for TSNAs both modifiers give comparable results.

3.6 Analysis for NDELA in Cutting Fluid

Cutting fluids are lubricants which are widely used in machine shops. The presence of NDELA results from reaction of nitrite ion,

Table 3.3 Effect of Modifier on Percent Recovery

	Modifier		
	None	Methanol	Methylene Chloride
NDELA	46	71	63
NAT	68	81	79
NNN	76	89	84
NNK	43	61	69

which is added as a rust inhibitor, with diethanolamine and triethanolamine, which are used as lubricants or emilsifying agents (17). Formation of NDELA by in vivo nitrosation has also been suggested (18). NDELA is of interest since this compound has demonstrated potent carcinogenic properties in animals (19). The spray-tap metal cutting fluid used in present study was procured commercially from Flushing, New York. This fluid was analysed for NDELA as per the procedure given below.

3.61 In-situ Derivatization of N-nitrosodiethanolamine

An aliquot of 1.0 ml containing NDELA was allowed to react with 0.5 ml of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA; Pierce Chemical Co.). The silylation reaction is very rapid and does not require additional heating (20). In the case of cutting fluids, each 1.00 ml of the fluid was first mixed with 0.5 ml BSTFA and then adsorbed on to 2.0 grams of inert support, Chromosorb-W, which then was transferred into extraction train for SFE. The extraction was carried out at 9500 psi at 95 ^{o}C for 20 minutes. The analysis of extract showed the concentration to be 1.87 ppm NDELA in this cutting fluid. This concentration is lower as compared to those reported (21) a decade ago which ranges from 3 to 29 ppm. NDELA has been found to be present in commercial cutting fluids if stored for 4 to 6 months or more even if it has been initially reported to be absent (17). The samples used in our laboratory were about month and half to two months old.

Another method, which however was not employed, is to make O-methyl ether derivative of NDELA by methylation using methyl iodide in the presence of sodium hydride as a catalyst (22).

3.7 Analysis for TSNA in Tobacco

Tobacco contains high levels of TSNAs; their concentration exceeds by more than 100 times the quantities of N-nitrosamines found in any other consumer products. Snuff dipping is associated with cancer of the oral cavity. Previous studies (23) have shown that TSNAs are powerful organ-specific carcinogens (24). In particular, people using Zarda in a pouch and keeping it under their lower lip (which is practiced especially in India) are in a high risk category. A majority of people affected by mouth cancer have been found to be heavy users of Zarda and other tobacco products which they keep in their mouth for a long time and swallow the juice. A cursory look at the results does present a scary relationship between TSNAs and users who are effected by cancer.

Sweet scotch snuff, Wintergreen chewing tobacco, Newport cigarette, and Zarda (India) were commercially obtained from the open market. These samples were finely powdered using a coffee mill and then seived. 1 gram of finely powdered (80/100 mesh) tobacco sample, with 1.00 ml of methanol as modifier, was extracted at 9500 psi at 95 °C for 20 minutes. The results are summarized in Table 3.4. Sweet scotch snuff are comparable to those reported in a detailed review (25).

3.8 Analysis for TSNA in Mainstream Smoke

Samples of mainstream smoke were obtained from a 20-port automatic smoker (H.Borgwaldt, Hamburg, GFR) with a rotating head. Every second port was connected with a nitrogen source, thus replacing air in the traps with nitrogen every 2 seconds (26). All experimental cigarettes (Newport) were stored in a humidity chamber

Table 3.4 Analysis for TSNAs in Tobacco

	Amount (ppm)		
	NAT	NNN	NNK
Cigarette, Newport	1.94	1.42	2.54
Snuff, Sweet Scotch	1.40	0.84	n.d.
Skoal, Wintergreen*	0.13	0.06	n.d.
Zarda, India*	0.07	16.0	0.13

* NMOR was also found to be present.

at 60% relative humidity. A volume of smoke of 35 ml/cigarette and 31 puff/4 cigarettes, with a blank puff between 2 cigarettes was used. The smoking was stopped 3 mm before the butt end. The smoke passed through a filter (4.5 cm diameter) which adsorbed the analyte. The weight of dark residue collected on filter was ~0.05 g per 4 cigarettes. The N-nitrosamines were then extracted from this filter by SFE and Soxhlet and analysed. The results are given in Table 3.5. indicating SFE and Soxhlet to be comparable. The presence of TSNAs in smoke indicates that non-smokers are effected by inhaling cigarette smoke (and TSNAs) from the environment polluted by smokers.

3.9 Conclusion

The SFE methodology has been developed and evaluated for analytical sample preparation and analyses of N-nitrosamines. Experimental parameters such as pressure, temperature, equilibration time, collector materials, and modifier were evaluated to optimize the recovery of analytes. As demonstrated by the essentially quantitative recoveries of VNAs, SF-CO_2 offers a powerful and environmentally safer alternative to organic solvents. In case of NDELA and TSNAs which are less volatile than VNAs, a binary mixture of SF-CO_2/modifier offers better recoveries as compared to pure SF-CO_2 . However, there still remains room for further improvement.

The SFE is inexpensive, simple, and easier to perform, offering substantial savings in terms of time as well as money. Also, the use of carbon dioxide as a solvent eliminates all the complications (disposal, hazardous etc) associated with routinely used organic solvents.

Table 3.5 Analysis for TSNAs in Mainstream Smoke

	Amount (ng/g)		
	NAT	NNN	NNK
SFE	1.19	0.51	n.d.
Sohxlet	1.12	0.67	n.d.

In SFE the photo-labile N-nitrosamines are not exposed to light, thereby assuring absence of photodecomposition of analytes. The GC-TEA determination step, already proven to be highly selective and sensitive to for quantitation of N-nitrosamines, is useful here as well. Overall this present project which is focused to develop a simple, rapid, accurate, reproducible and environmentally advantageous sample preparation technique for the estimation of N-nitrosamines at nanogram levels offers an interim alternative to existing analytical methodology which has only complex time- consuming procedures.

In the future experiments a combination of modifiers can be studied to improve further the recoveries of TSNAs and NDELA. The technique established in this project can be utilized to quantitate N-nitrosamines in various commercial products such as non-fat dry milk, fried bacon, canned food, cosmetics, etc.

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