DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS
51

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DETERMINATION OF SOME CARCINOGENIC CONTAMINANTS IN FOOD

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LIST OF ORIGINAL PUBLICATIONS

The thesis consists of a review and the six articles listed below. The articles are referred in the text by Roman numerals I–VI. The review summarizes and supplements the articles.

I S. Jurtchenko, T. Tenno, U. Mölder, and M. Reinik, Determination of volatile *N*-nitrosamines by gas chromatography/mass spectrometry (GC-MS) with positive-ion chemical ionization, *Proceeding of Estonian Academy of Sciences. Chemistry* **51** (2002), No. 3, 169–184.

II S. Yurchenko and U. Mölder, *N*-nitrosodimethylamine analysis in Estonian beer using positive-ion chemical ionization with gas chromatography mass spectrometry, *Food Chemistry* **89** (2005), No. 3, 455–463.

III S. Yurchenko and U. Mölder, The determination of polycyclic aromatic hydrocarbons in smoked fish by gas chromatography mass spectrometry with positive-ion chemical ionization, *Journal of Food Composition and Analysis* **18** (2005), No. 8, 857–869.

IV S. Yurchenko and U. Mölder, Volatile *N*-nitrosamines in various fish products, *Food Chemistry* **96** (2006), No. 2, 325–333.

V S. Yurchenko and U. Mölder, The occurrence of volatile *N*-nitrosamines in Estonian meat products, *Food Chemistry* (in press, available at www.sciencedirect.com).

VI M. Reinik, T. Tamme, M. Roasto, K. Juhkam, S. Jurtšenko, T. Tenno, and A. Kiis, Nitrites, nitrates and *N*-nitrosoamines in Estonian cured meat products: Intake by Estonian children and adolescents, *Food Additives and Contaminants* **22** (2005), No. 11, 1098–1105.

Author's contribution

The author's contribution to the **Papers I to V** has been essential. He has developed the method of determination of NA and PAH in food, performed all experiments, investigated factors affecting NA and PAH, writed articles.

In **Paper VI**, the author has obtained all results concerning NA content in meat products.

ABBREVIATIONS AND SYMBOLS

B[a]A	–	benz[a]anthracene
B[a]P	–	benzo[a]pyrene
B[b]F	–	benzo[b]fluoranhene
B[ghi]P	–	benzo[ghi]perylene
B[k]F	–	benzo[k]fluoranhene
DCM	–	dichloromethane
DMA	–	dimethylamine
EA	–	electron affinity
EI	–	electron impact
GC	–	gas chromatography
GC-MS	–	gas chromatography mass spectrometry
Hex	–	hexane
I[cd]P	–	indeno[1,2,3-cd]pyrene
LOD	–	limit of detection
LOQ	–	limit of quantitation
MeOH	–	methanol
MSD	–	mass selective detector
NA	–	*N*-nitrosamines
n.d.	–	not detected
NDBA	–	*N*-nitrosodibutylamine
NDEA	–	*N*-nitrosodiethylamine
NDMA	–	*N*-nitrosodimethylamine
NICI	–	negative-ion chemical ionization
NO	–	nitric oxide
NPIP	–	*N*-nitrosopiperidine
NPYR	–	*N*-nitrosopyrrolidine
PA	–	proton affinity
PAH	–	polycyclic aromatic hydrocarbons
PICI	–	positive-ion chemical ionization
ppb	–	parts per billion
SPE	–	solid-phase extraction
μg/kg	–	microgram per kilogram

INTRODUCTION

Volatile *N*-nitrosamines (NA) and polycyclic aromatic hydrocarbons (PAH) are some of toxic compounds, which are present in the environment, and traces of these substances have been found in various food products. NA and PAH investigated in this thesis (with the exception of benzo[ghi]perylene (B[ghi]P)) are reasonably anticipated to be human carcinogens based on sufficient evidence of carcinogenicity in experimental animals [62, 63, 116]. The International Agency for Research on Cancer categorize B[ghi]P into the group of unclassifiable as to the carcinogenicity to human [61].

NA constitute a large group of genotoxic chemical carcinogens, which occur in human diet and other environmental media, and can be formed endogenously in the human body [119]. NA occur as contaminants in different food categories and beverages including vegetable oil [34, 128], cheese [16, 19, 44], drinking water [66, 151], beer [3, 38, 65, 74, 113, 122, 127, 131, 136, 140, 155, 160], milk [53, 92, 132], fish [37, 39, 57, 64, 71, 164], and meat products [20, 25, 41, 69, 75, 126, 135, 163]. The acute toxicity of *N*-nitrosodimethylamine (NDMA) was demonstrated for the first time in 1954 by Barnes and Magee [5]. The first validated confirmation of the environmental occurrence of NA was provided in 1964 by Ender et al. [29].

The great interest in PAH compounds stems from the observations that some of these compounds may cause tumours in humans [63, 108]. In fact, the key event in this respect was the observation in 1775 by Percival Pott that scrotal cancer in chimney sweeps originates from occupational exposure to soot [111]. In the early 1900s it was widely recognized that soot, coal tar, and pitch are carcinogenic to man [23]. PAH occur as contaminants in different food categories and beverages including water [9, 17, 73], vegetable oil [6, 21, 22, 93, 94, 97], whiskey [90], fruit [14], tea [83], smoked meat [112, 138, 149, 152], and smoked fish [1, 72, 77, 85, 95, 137, 144]. Most of the studies conducted on environmental samples during the 1960s utilized ultraviolet and fluorescence techniques to estimate the PAH content [52]. The first method for determining PAH in foods was described at the beginning of the 1970s [55].

The maximum permitted levels (μg/kg wet weight) of the sum of NDMA and *N*-nitrosodiethylamine (NDEA) were limited in Estonia during 2000–2004 in raw and canned meat of 2 μg/kg, in smoked fish and beer – 3 μg/kg, and in smoked meat – 4 μg/kg [147]. At present in Estonia the content of volatile NA is not limited. Maximum level (μg/kg wet weight) of benzo[a]pyrene (B[a]P), as a marker for the occurrence and effect of carcinogenic PAH, is limited in oil and in smoked fish- and meat products of 2 and 5 μg/kg, respectively [18].

Present investigations were carried out during the period of 2001–2005 at University of Tartu. The results were published in six articles and used as a base

for the current thesis. The main position of this work has been presented at international conferences.

The purpose of this thesis was to develop a rapid and efficient method for clean-up and determination of NA and PAH in food products, and to study the influence of food processing on the NA and PAH formation and content in some Estonian food products.

The main contributions of the present thesis are the following: 1) the new method of determination of NA and PAH in food products was developed; 2) the validation of method used for determining the NA and PAH in some food products was performed; 3) the information on NA and PAH content in some kind of Estonian food was obtained; 4) the influence of food processing and storage conditions on NA and PAH content in food was established.

The conclusions of this thesis are the following: 1) some Estonian food products content a considerable amount of NA and PAH; 2) NDMA concentration in beer depends on percent of alcohol; 3) NA and PAH concentration depends on temperature and time of cooking; 4) the addition of sodium nitrite to meat rapidly increases the NA concentration in meat rapidly; 5) the NA concentration in fried fat is several times higher the concentration in lean pork.

1. LITERATURE REVIEW

1.1. Methods for determination of *N*-nitrosamines and polycyclic aromatic hydrocarbons in food

The determination of NA and PAH in environmental objects at the microgram and nanogram level is an extremely complex analytical problem, because such objects can contain very large number of substances.

The determination of volatile NA and PAH in food products has been carried out by different analytical methods, including gas chromatography (GC) [67, 74, 101, 136, 139, 142, 154, 155], gas-liquid chromatography with thermal energy analyser [15, 32, 34, 42, 102, 129, 133], micellar electrokinetic chromatography [121], liquid chromatography at atmospheric pressure [28], high performance liquid chromatography [8, 76], fluorescence and chemiluminescence methods [43, 162], and fluorometric method [96, 110]. Electron impact (EI) ionization [81], or positive-ion chemical ionization (PICI) mass spectrometry with methane [31, 36, 40] or ammonia [114] as reagent gases has been used to differentiate between volatile NA. The EI mass spectra of PAH are, unfortunately, almost always indistinguishable. However, other ionization strategies have been suggested for circumventing this limitation. PICI and negative-ion chemical ionization (NICI) mass spectrometry with methane, dimethylether, tetramethylsilane, ammonia [60, 86, 109, 117, 145] or a mixture of methane/argon, isobutane/oxygen, nitrogen/nitrous oxide, hydrogen [13] as reagent gases have been used to differentiate between isomeric PAH. The NICI using carbon dioxide alone or in a mixture with nitrogen and oxygen has been reported [143].

In literature, methods for sample preparation of NA and PAH are based on vacuum- [128, 146] and steam distillation [162], mineral oil distillation [47], liquid-liquid extraction [48, 130], thin-layer chromatography [161], supercritical fluid extraction [35, 76, 91, 106], solid-phase extraction (SPE) [12, 49, 54, 89, 99, 115, 118, 121], and solid-phase microextraction [2, 17, 73, 156].

The limit of detection (LOD) of reported analytical methods for NA and PAH ranging from 0,05 to 3,33 μg/kg, depending on the molecular mass of compound [6, 12, 14, 38, 42, 75, 78, 80, 101, 118, 139, 155]. In food, methods of analysis of NA and PAH involve many preliminary steps such as sampling extraction, clean-up, and concentration. Most of these procedures are time consuming and most of them require the use of toxic solvents. Therefore, it is necessary to develop simple, rapid, sensitive, economic, effective, and accurate method for NA and PAH analysis.

1.2. The formation of *N*-nitrosamines and polycyclic aromatic hydrocarbons in some food products

The main carcinogenic NA found in food throughout the world are NDMA, NDEA, *N*-nitrosopyrrolidine (NPYR), *N*-nitrosopiperidine (NPIP), and *N*-nitrosodibutylamine (NDBA). These volatile NA may be formed in certain foods from naturally occurring amines present in the food. These amines are nitrosated by agents derived from added nitrite or nitrogen oxides. The latter may be formed by combustion of ambient nitrogen and thus present in the drying air.

PAH are formed by the incomplete combustion of organic matter and are thus generated whenever fossil fuels or vegetation is burned. Because of the widespread distribution of PAH in the environment, most types of food contain measurable levels of PAH [79].

This part of thesis considers reasons of the formation of NA in beer and other alcoholic beverages, in fish- and meat products, and also the formation of PAH in food products.

NDMA in beer and other alcoholic beverages

It is well known that a beer may contain trace amounts of NDMA, a highly active carcinogen. NDMA can be formed during food processing, preservation, and preparation from precursor compounds already present in, or added to, the specific food items [154, 155]. Most malt beverages, including beer and most brands of whiskey, regardless of origin, contain NDMA. The presence of NDMA in beer was first reported in 1977 [127].

The NDMA concentration in malt depends on the type of the drying technique. High combustion temperatures (usually from 1500 to 1800°C) yield high NDMA concentration. When green malt was dried with direct-fired kilns, NDMA concentrations of 15–80 ppb in pale malt and 80–320 ppb in dark malt were detected. Green malt is usually dried with hot air produced by burning coal in a stove and the temperature of the air is usually lower than 100°C so the concentrations of nitrogen oxides in the air may be lower. Lower concentrations of nitrogen oxides in the air and alkaloids in (hordenine and gramine) the malt may thus prevail, reducing the formation of NDMA in malt [139]. These alkaloids were derivates of dimethylamine (DMA) and were easily nitrosated, in this case, by nitrogen oxides as the nitrosating agent. Reaction of nitrogen oxides with alkaloids during the direct-fire drying step of the malting process has been established as the major pathway for the formation of NDMA in beer [11, 82, 88]. The nitrosation mechanism of gramine is shown in Fig. 1. The possible importance of DMA in the malt as a precursor for NDMA in beer is underlined by the evidence that DMA concentrations in dark lager, dark strong

lager and dark ale are relatively high and correspond with higher NDMA values [142].

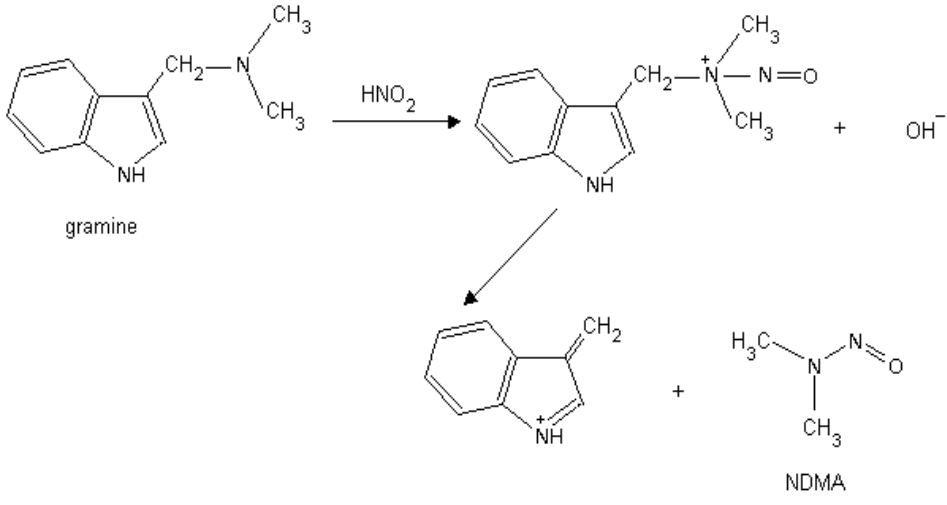


Fig. 1. The mechanism for the nitrosation of gramine to yield NDMA [119].

N-nitrosamines in fish

The main precursors of NDMA in the fish are believed to be DMA, diethylamine, trimethylamine, and trimethylamine oxide all of which are abundant in various fish, especially marine fish. The presence of other secondary amines has not yet been established. However, since DMA is a strongly basic amine, its rate of nitrosation is very low, and trimethylamine and trimethylamine oxide are even slower [133]. Cooking of food can be another source of secondary amines as pyrolysis products of protein. It can release amino acids like proline, hydroxyproline and arginine as well as nitrosable amines, like pyrrolidine and piperidine. Assuming the presence of nitrate and nitrite, high temperature accelerates NA synthesis in the fish [64].

In fish itself there are rich sources of secondary and tertiary amines, while in the crude salt used to pickle the fish, there is nitrate and possibly nitrite. All these factors suggest the possibility that NA might be formed in the salted fish [37, 164]. The possible reason of variation of NA content in salted fish was related in part to the degree of contamination by nitrate-reducing *Staphylococcus aureus*. This organism isolated from fish has been shown to increase the NDMA content in salted fish broth [164].

NA are formed by the reaction of nitrogen oxides with, mainly, secondary amines present in the fish (Fig. 2). The nitrogen oxides are generated from nitrites, and they are also present in wood smoke. The concentration of amines in fish products depends on various factors such as species, age, environment,

bacterial flora, and storage conditions [134]. Amines are usually present in larger quantities in marine fish than in fresh-water fish. It is, therefore, difficult to predict whether a particular sample of fish, especially a marine fish, will be free of these amines, and be safe to treat with nitrite [123, 133].

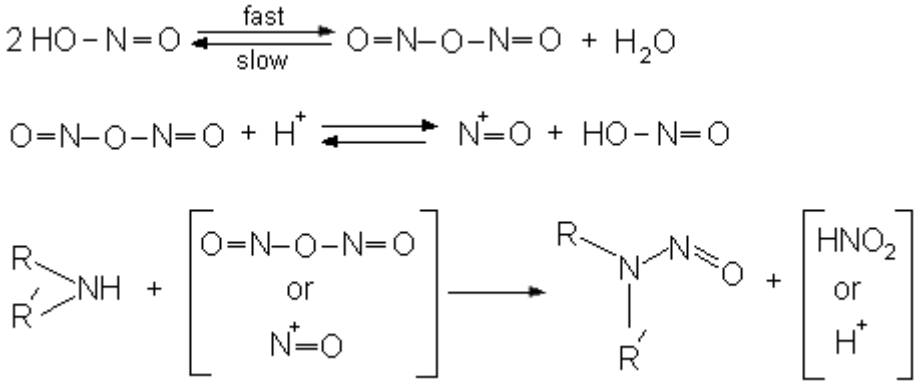


Fig. 2. The formation of NA from nitrogen oxides in fish products [134].

N-nitrosamines in meat

The formation of NA in meat is a complex process and a large diversity of substances could influence nitrosation reaction. Starting materials for NA formation in meat products are nitrate, nitrite, primary, secondary and tertiary amines, amides, proteins, peptides and amino acids or precursors of these, which are transformed into NA precursors by microbial action. Microorganisms could take part in NA formation by nitrates reduction to nitrites, degradation of proteins to amines and amino acids [154]. NA are formed after cooking, by an oxygen-dependent mechanism, the key step being the oxidation of nitric oxide (NO) and the formation of higher nitrogen oxides which could act as direct nitrosating agents. The nitrosating agent responsible for the formation of NA in fried meat might be N_2O_3, formed during heating of nitrite in meat, or NO radical formed by dissociation of N_2O_3 at high temperature [134]. The structure of the nitrosating agent is not known, but evidence suggests that it is a reaction product of nitrite and lipids in the meat [84]. It is unlikely that all of NA formed in food by the reaction of nitrite with amines are carcinogenic, but, surely, some of them are and others might by easily converted into carcinogens. For example by heating of proline forms NPYR, a powerful carcinogen. This is one of the sources of the NPYR found in fried bacon [98, 153].

NDBA, which induces tumours of the urinary bladder in various species, was traced to rubber netting used to tie up the cured meat after processing. Derivates of dibutylamine are used in rubber manufacture and apparently

become nitrosated at some stage and the NDBA then migrates into the meat. Any time food comes into contact with a rubber product, it can be expected that NA in the rubber will migrate into the food [134].

Sodium nitrite is normally added to meat products such as canned sausages, ham, and salamis to prevent the formation of toxins produced by *Clostridium botulinum*. Nitrite is also responsible for the development iron reaction with some meat pigments to produce the desirable red colour, and flavour characteristic for these products. Nitrite, however, is converted to nitrosating agents that may react with amines and amino acids in meat to produce carcinogenic NA [41, 121, 158]. Since nitrite is highly reactive, its concentration in meat product gradually decreases with storage and depends on many factors such as processing conditions, and cooking method [134].

Indeed, the concentration of NA in meat products depends on the method of cooking [103], cooking temperature and time, residual and added nitrite concentration [120, 124, 125], presence of nitrosation catalysts and inhibitors [33, 45, 46], and storage conditions [24, 26, 27].

Polycyclic aromatic hydrocarbons in food

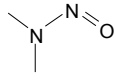
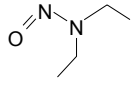
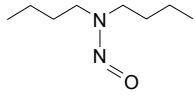
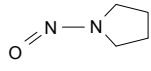
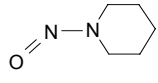
Before beginning a description of the results obtained by several authors concerning distribution of PAH in food, some aspects should be considered. However, in some studies only the concentration of B[a]P has been determined, because this compound is considered an indicator of other PAH. Because foods are a complex matrix, determining their PAH content is very time-consuming and cumbersome [56].

The possible sources of PAH in food are environmental contamination, as well as thermal treatment of varying severity which is used in the preparation and manufacturing of food [50], the absorption and deposition of particulates during food processing such as smoking, grilling, boiling and toasting, the pyrolysis of fats and the incomplete combustion of charcoal [50, 78, 94]. Regarding food of animal origin, one hypothesis suggests that the lipophilic character of PAH is responsible for the accumulation into the fat of animals which eat contaminated plants [51]. The actual level of PAH in smoked food depend on several variables in the smoking process, including type of smoke generator, combustion temperature, and degree of smoking [77, 94]. This, together with the control of some important parameters such as temperature, humidity, smoke concentration, and circulation rate, can contribute to the minimization of PAH contamination [95].

2. EXPERIMENTAL

In this study was developed the method for determination the most commonly encountered volatile NA and PAH in food samples. Structures and other properties of the studied compounds are given in Tables 1 and 2.

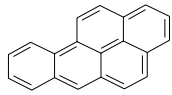
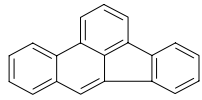
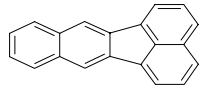
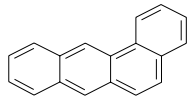
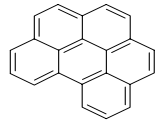
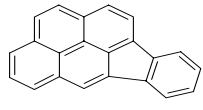
Table 1. Physicochemical properties of some NA [119, 156]

Nomenclature	Structure	MW[a], g/mol	BP[b], °C	WS[c], g/L	log K_{ow}[d]
N-nitrosodimethylamine (NDMA)		74,1	151	1000	–0,57
N-nitrosodiethylamine (NDEA)		102,1	177	106	0,48
N-nitrosodibutylamine (NDBA)		158,2	—	1,2	2,63
N-nitrosopyrrolidine (NPYR)		100,2	214	1000	–0,19
N-nitrosopiperidine (NPIP)		114,2	220	76	0,36

[a] molecular weight; [b] boiling point; [c] water solubility at 25°C; [d] polarity expressed as log $K_{octanol–water}$.

All considered NA are yellow oily liquids which are moderately soluble in water and soluble in many organic solvents as methylene chloride, chloroform, alcohols, etc. These compounds are sensitive to light, especially ultraviolet light, and undergo relatively rapid photolytic degradation. NA are combustible, and when heated to decomposition, they emit toxic fumes of nitrogen oxides. The dipole moment of aliphatic NA is 3,9–4,4 D, which indicates the polarity of their molecules, determined by the possibility of the delocalisation of the unshared electron pair [134].

Table 2. Physicochemical properties of some PAH [51, 83]

Nomenclature	Structure	MW[a], g/mol	BP[b], °C	WS[c], μg/L	log K_{ow} [d]
Benzo[a]pyrene (B[a]P)		252	496	4,0	6,0
Benzo[b]fluoranthene (B[b]F)		252	481	1,5	6,6
Benzo[k]fluoranthene (B[k]F)		252	481	0,8	6,8
Benz[a]anthracene (B[a]A)		228	435	14,0	5,6
Benzo[ghi]perylene (B[ghi]P)		276	—	0,3	7,0
Indeno[1,2,3-cd]pyrene (I[cd]P)		276	536	—	7,7

[a] molecular weight; [b] boiling point; [c] water solubility at 25°C; [d] polarity expressed as log $K_{octanol–water}$.

The six PAH listed occur as needles, plates, crystals, leaflets, or prisms ranging from colourless to pale yellow or golden yellow. Their boiling point is high and their volatility is low. Solubility characteristics vary for each PAH, but in general, they are slightly soluble in ethanol, acetone, and water, but readily soluble in toluene [116].

Much attention has been given to develop the method for clean-up of volatile NA and methods for determination of NA and PAH.

2.1. Development of methods for clean-up and determination of *N*-nitrosamines and polycyclic aromatic hydrocarbons

In principle, all analysis of organic pollutants follows the same procedure, consisting of extraction, purification, concentration, and instrumental analysis. The basic purpose of this thesis was to develop a rapid and efficient analytical methodology for the analysis of NA and PAH in food products.

2.1.1. Solid-phase extraction of *N*-nitrosamines

The SPE method is based on the interaction between NA molecule and the functional groups of the adsorbent as a function of solvent. To extract polar NA from non-polar organic solvents were employed "normal phase" retention mechanism. The properties of the sorbent determine extraction efficiency and the overall quality of the separation. Table 3 provides a list of the typical physical properties of some "normal phase" SPE sorbents.

The retention mechanism is based on hydrogen bonding, dipole-dipole and π-π interactions between polar analyte and polar stationary phase. Highly specific normal phase extraction can be obtained by carefully optimizing the polarity of the conditioning solvent and the solvent used to dilute and load the sample/matrix. The aim of this stage is to define the optimum conditions in terms of recovery for an extraction procedure, based on solid-phase clean-up. The possible way to determine the efficiency of extraction (recovery) is to spike test portions with the analyte at various concentrations, and extract the fortified portions. The major goal is to optimize the extraction efficiency (maximize the recovery of the target analytes and minimize the amount of co-eluted contaminants) under conditions that provide reproducible results in a simple, economic manner.

Table 3. Physical properties of some "normal phase" SPE sorbents [150]

Solid phase	Surface area range, m^2/g	Particle size range, microns	Pore size range, angstrom
Cyanopropyl	450–550	50–60	60–75
Extrelut (kieselguhr)	250–600	50–60	60–75
Silica gel	250–600	50–60	60–75
Aminopropyl	450–550	50–60	60–75
Florisil	300–600	50–200	60–80
Alumina	100–150	50–300	100–120

Several methods have been described in literature for the analysis of NA using SPE clean-up. For example, J. T. Baker [4] reported a method for the determination of NPYR in bacon. In this method the sample was extracted with hexane (Hex)-dichloromethane (DCM) (9:1, v/v), followed by a clean-up step through a Cyanopropyl (CN) cartridge and final elution with methanol (MeOH)-DCM (95:5, v/v). Other authors for isolation of volatile NA were used dry column extraction on Extrelut with DCM [54, 79, 102, 142]. Results of our work indicated that the elution with Hex using different sorbents quantitatively recovered the most apolar NA, such as NDEA, NPIP, and NDBA. The obtained results showed that the efficiently of extraction of NA depends upon the polarity of the solvent and the sorbent, on the nature of the matrix, and on the preparation of the sample. The low recoveries were obtained for NDMA and NPYR using Hex as eluent. Therefore it was decided to use Hex-DCM (50:50, v/v) as solvent in experiments with different SPE sorbents.

The first step in the development of the SPE method was the selection of the most appropriate SPE cartridges. During method development, different types of SPE cartridges containing different solid phases were tested to determine the NA. Extrelut, Florisil, CN, silica gel, aminopropyl, alumina, and a combination of Extrelut-Florisil were checked as solid supports for SPE. Clean-up with Florisil by one-step SPE extraction gave the highest recovery of NA. Table 4 lists the recovery (mean of three replicates) by two fortification levels, relative standard deviation obtained by SPE from raw meat samples using different solid sorbents.

Table 4. Recovery and relative standard deviations obtained by SPE using different sorbents

Compound	Concentration, µg/kg	Recovery, % (RSD[a], n=3)						
		Cyanopropyl	Extrelut	Silica gel	Aminopropyl	Florisil	Alumina	Extrelut-Florisil
NDMA	0,5	35 (5,3)	50 (6,2)	46 (5,8)	53 (7,5)	67 (6,9)	64 (7,8)	82 (6,2)
	5,0	31 (6,1)	46 (7,8)	44 (8,3)	55 (8,9)	64 (8,4)	66 (9,3)	80 (9,1)
NDEA	0,5	25 (6,1)	50 (6,6)	47 (5,4)	46 (7,1)	64 (6,4)	61 (7,3)	84 (6,3)
	5,0	23 (8,3)	48 (7,9)	49 (7,4)	44 (8,2)	62 (7,6)	59 (9,2)	79 (8,8)
NPYR	0,5	42 (6,3)	54 (6,8)	42 (7,1)	62 (7,1)	65 (5,8)	57 (6,6)	85 (7,8)
	5,0	39 (7,7)	52 (82)	40 (9,3)	58 (9,5)	67 (8,9)	55 (8,8)	82 (9,5)
NPIP	0,5	34 (6,6)	58 (6,1)	37 (5,8)	64 (6,1)	61 (7,2)	53 (6,2)	76 (7,1)
	5,0	33 (7,1)	56 (9,3)	36 (8,5)	66 (8,9)	61 (9,4)	50 (8,4)	74 (8,9)
NDBA	0,5	47 (6,8)	53 (6,2)	56 (5,3)	55 (6,9)	59 (5,3)	57 (5,8)	83 (6,8)
	5,0	44 (8,2)	51 (9,6)	54 (9,4)	56 (8,7)	56 (9,1)	55 (9,6)	78 (8,7)

[a] relative standard deviation in percent.

It is evident, that tandem Extrelut-Florisil SPE is an efficient to isolate NA from meat samples. In this first SPE step using Extrelut, the elution was achieved with a Hex-DCM (60:40, v/v) mixture. A second step was required for further purification of NA. The elution of NA from Florisil cartridge was achieved with a DCM-MeOH (95:5, v/v) mixture. The recoveries ranged between 74 and 85% depending on the molecular mass of the NA.

As elaborate study indicated the combination of different solvent mixtures tends to improve the solvation of the analytes of interest and thus improve overall efficiency of SPE. NA can be eluted with the use of the non-polar organic solvent such as Hex, in combination with medium-polar solvent such as DCM. We investigated different solvents such as Hex and DCM, and their mixtures as Hex-DCM 40:60, 50:50, 60:40, 70:30, 80:20 (v/v) for 20 min extraction using Extrelut sorbent. The Hex-DCM (60:40, v/v) mixture provided the best eluting efficiency and removal of impurities (Table 5). Among the solvents studied, the Hex-DCM (60:40, v/v) mixture gave recoveries in the range of 68–77% while that of (40:60, v/v) yielded 40–55% recovery. These differences in the recovery can be explained by different solubility of NA in different extraction solvents. Elution with Hex recovered only the most apolar NA, NDEA, NPIP, and NDBA, while NDMA and NPYR retained on the SPE column. The DCM was necessary for the recovery of most polar NA: NDMA and NPYR.

Table 5. The effect of solvent on Extrelut SPE of NA

Compound	Recovery, %						
	Hex	DCM	Hex-DCM (40:60, v/v)	Hex-DCM (50:50, v/v)	Hex-DCM (60:40, v/v)	Hex-DCM (70:30, v/v)	Hex-DCM (80:20, v/v)
NDMA	20	65	55	50	68	39	29
NDEA	55	27	46	50	72	71	73
NPYR	25	61	53	54	70	54	42
NPIP	62	24	42	58	73	74	75
NDBA	68	20	40	53	77	80	83

The next step in the method development was the determination of the breakthrough volume of the selected cartridges. The elution was achieved with a Hex-DCM (60:40, v/v) mixture. The minimum volume required to elute the NA was 40 mL. Whereas NDEA, NPIP, and NDBA were eluted within the first 25 mL, and an additional 15 mL of mixture was required to elute NDMA and NPYR.

In order to determine the optimum extraction time, SPE with a Hex-DCM (60:40, v/v) mixture was carried out for 5, 10, 15, 20, 25 and 30 min, respectively. The extraction time beyond 20 min did not show any significant improvement in the extraction yield, therefore it was used in all subsequent experiments.

The extract of Hex-DCM (60:40, v/v) contained large amount of impurities. To avoid saturation and performance degradation of the capillary column the clean-up of the extract was necessary before gas chromatographic injection. Thus the extract was applied to a Florisil cartridge after selective removal of DCM. A subsequent washing step with Hex removed most lipophilic compounds. The DCM is not sufficiently polar to elute the all NA. The elution of the polar NA from Florisil cartridge was tested with different solvents such as Hex and DCM, and their mixtures as Hex-DCM 40:60, 50:50, 60:40 (v/v), and as DCM-MeOH 95:5, 90:10 (v/v). The DCM-MeOH (95:5, v/v) mixture provided the best eluting efficiency and removal of impurities (Table 6).

Table 6. The effect of solvent on Florisil SPE of NA

Compound	Recovery, %						
	Hex	DCM	Hex-DCM (40:60, v/v)	Hex-DCM (50:50, v/v)	Hex-DCM (60:40, v/v)	DCM-MeOH (95:5, v/v)	DCM-MeOH (90:10, v/v)
NDMA	14	69	68	67	30	82	83
NDEA	26	65	65	64	42	79	68
NPYR	18	67	66	65	33	80	81
NPIP	31	54	62	61	45	77	62
NDBA	34	49	61	59	50	76	57

The method of preparation described by Raoul [115], with some modifications, was used. The two-step SPE with Extrelut (biogenic amorphous silica) and Florisil (activated magnesium silicate) sorbent was applied to isolate these analytes from meat, fish, beer, and oil products. A sample of fish (6,0±1,0 g), meat (6,0±1,0 g), beer (25,0±1,0 mL), or oil (10,0±1,0 mL) was mixed with 0,1 N NaOH (6 mL).

As the first step, about 6 g of Extrelut was placed at the bottom of the glass column (30 cm × 1,5 cm) and wetted with 20 mL Hex-DCM (40:60, v/v). After that, the sample was eluted with two 20 mL portions of Hex-DCM solution. The eluate was collected in a 50 mL concentrator flask and evaporated in water bath at 60ºC. As the second step, about 1 g Florisil was placed at the bottom of the Florisil cartridge (6,5 cm × 1,3 cm), wetted with 6 mL Hex, and eluted with 6 mL DCM-MeOH (95:5, v/v). The solution was evaporated at 60ºC to 1 mL. The prepared solution was transferred to the GC injector vial. Extractions were performed in duplicate [see Paper I].

The results obtained by an Extrelut-Florisil extraction method for NDMA determination in beer were compared with those obtained by an Extrelut extraction method [102]. In both cases the determination was done by GC using a mass selective detector (MSD). A comparison of these methods indicates that our two-step SPE method has 10% better recovery.

The proposed method was validated and data of this are given in Tables 12–14 (see Section 2.2). The method of analysis was accredited by the Estonian Accreditation Body.

2.1.2. Gel-permeation chromatography for the selective isolation of polycyclic aromatic hydrocarbons

For the smoked fish and oil samples preparation, the method described by Ojaveer and Tanner [100], with some modifications, was used. This method implies gel permeation chromatography for efficient lipid removal. The fish sample (10,0±0,5 g) or the oil sample (3,0±0,5 g) was hydrolyzed in 42 mL of concentrated hydrochloric acid. After 12 hours, 40 mL DCM-Hex (25:75, v/v) solution was added and mixed during 45 min. After that, 4 g Na_2SO_4/$NaHCO_3$ 1:1 was placed at a filter of a glass funnel and eluted with 20 mL DCM-Hex (25:75, v/v) solution. The eluate was evaporated to dry in water bath and 20 mL ethyl acetate-cyclohexane (50:50, v/v) solution was added. Five millilitres of the sample was injected into a gel chromatograph equipped with a column (Pharmacia Fine Chemicals 1000 mm x 25 mm) and UV detector (LDC SpectroMonitor II 1202). A fraction was collected in a 100 mL concentrator flask and was evaporated to dry in water bath. After solvent evaporation, the residue was dissolved in 1 mL of MeOH. The prepared solution was transferred to the injector vial. The proposed method was validated and data are given in Paper III.

2.1.3. Gas chromatography mass spectrometry method for determination of *N*-nitrosamines

The purpose of this part of thesis was to develop a rapid and efficient gas chromatography mass spectrometry (GC-MS) method for determination of NA in food products. NA was separated by GC-MS using EI and PICI with ammonia and methane as reagent gases and tested in order to evaluate the performance of each one.

The standard mass spectrometry uses EI. Ionization is achieved through the interaction of an analyte with an energetic electron beam that results in the loss of an electron from the analyte and production of a radical cation. As samples are thermally desorbed to the gas phase and subjected to the high energy of EI, analytes must be both thermally and energetically stable. The EI ionizing energy typically used is 70 eV to promote the greatest sensitivity and to produce molecular and fragment ions used for chemical characterization and identification. A summary of the mass spectra of the NA, obtained in this work by EI is given in Table 7. The spectra of NDMA, NDEA, NPYR, and NPIP are compared with those published in NIST Standard Reference Database [7]. The mass spectrum of NDBA is not available in NIST and it is compared to the spectrum published in [81].

Table 7. Intensities (in % of relative to the most intensive peak) of the NA ions m/z observed with EI in present work and in NIST Database

Compound	Present work, m/z, Rel. intensity (in parentheses)	NIST Database [7], m/z, Rel. intensity (in parentheses)
NDMA	74(100), 42(98), 43(44)	74(100), 42(72), 43(45)
NDEA	102(100), 44(25), 42(20), 56(21)	102(100), 44(87), 42(83), 56(54)
NPYR	100 (100), 41(80), 42(61), 68(18)	100 (100), 41(69), 42(45), 68(14)
NPIP	114(90), 41(25), 42(100), 55(54)	114(100), 41(28), 42(94), 55(83)
NDBA	158(38), 84(100), 116(43), 99(36), 57(54)	158(18), 84(100), 116(35), 99(19), 57(70)

PICI as a soft ionization method has been developed. The probability of proton exchange depends on the relative gas phase basicities (proton affinities (PA)) of the analyte and the reagent gas. Although PICI is capable of generating molecular weight information from labile species, it still requires the sample to be volatile, which could hinder the detection of thermally unstable analytes. Thus NA molecules (M), for which PA is greater than that of methane (PA (CH_4) = 543,5 kJ/mol [58]) give mass spectra with $[M+H]^+$ as the base peak, and adduct ions at $[M+C_2H_5]^+$ and $[M+C_3H_5]^+$.

As expected, the use of ammonia as reagent gas led to lower fragmentation than observed with methane. The best sensitivity was achieved at ammonia flow of 1 mL/min. Usually, NA molecules, for which the PA is lower than that of ammonia (PA (NH_3) = 853,6 kJ/mol [58]) give mass spectra with $[M+ NH_4]^+$ as the base peak, and adduct ions at $[M+H]^+$ and $[M+N_2H_7]^+$. The details of mass spectra obtained by PICI mass spectrometry using methane and ammonia as the reagent gases are summarized in Table 8.

Table 8. Intensities (in % of relative to the most intensive peak) of the NA ions m/z observed with PICI with methane and ammonia used as reagent gases

Compound	m/z, Rel. intensity (in parentheses)					
	Methane			Ammonia		
	$[M+1]^+$	$[M+29]^+$	$[M+41]^+$	$[M+1]^+$	$[M+18]^+$	$[M+35]^+$
NDMA	75(100)	103(0)	115(4)	75(16,6)	92(100)	109(1,6)
NDEA	103(100)	131(10)	143(8)	103(27)	120(100)	137(0,6)
NPYR	101(100)	129(0)	141(0)	101(30,9)	118(100)	135(0,6)
NPIP	115(100)	143(7,2)	155(4,7)	115(32,6)	132(100)	149(0,6)
NDBA	159(100)	187(0)	199(0)	159(86)	176(100)	193(1,3)

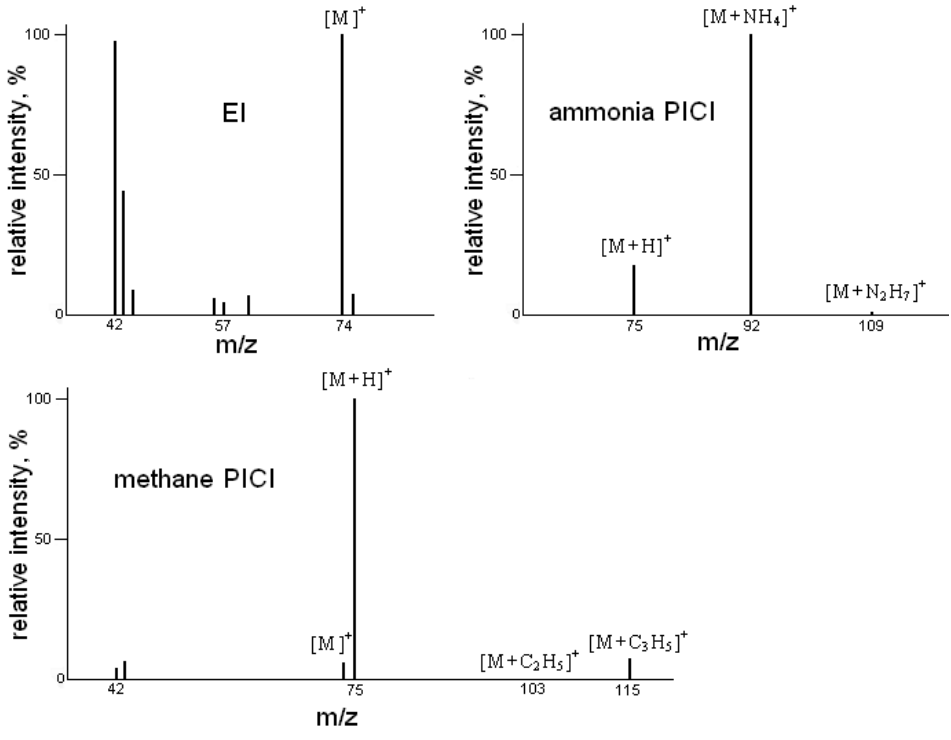


Fig. 3. Comparison of mass spectra of NDMA obtained by EI and PICI with methane and ammonia as reagent gases.

Five volatile NA were separated by GC with MSD and recognized by PICI using ammonia and methane as reagent gases. The sensitivity increased and LOD was better when ammonia was used as reagent gas (Table 9). EI ionization for NA in most cases giving up to 10^2 times higher LOD, except for NPYR. Fig. 3 shows the comparison of mass spectra of NDMA obtained using EI, and PICI with methane and ammonia as reagent gases.

Table 9. The LOD reached for NA with PICI and EI

Compound	LOD, ppb		
	Methane PICI	Ammonia PICI	EI
NDMA	0,50	0,10	10,0
NDEA	0,02	0,01	2,0
NPYR	0,20	0,20	2,0
NPIP	0,01	0,01	1,0
NDBA	0,01	0,01	1,0

The following analysis of NA was performed by PICI mass spectrometry using ammonia as reagent gas. GC analysis was carried out using Hewlett-Packard (HP) Model 6890 gas chromatograph equipped with a split/splitless injector. Five microlitre of the sample solution were injected into the gas chromatograph using pulsed splitless injection in the selected ion monitoring mode. Detection was done by a HP 5973 MSD mass spectrometer.

Sample portions were injected into a chromatograph column (30 m HP-1701 MS; 0,25 mm i.d., 0,25 μm film thickness) containing 14% cyanopropylphenyl and 86% methyl polysiloxane. For the GC separation of NA oven programme started at 35°C (held 1 min), set at 50°C/min from 35°C to 240°C and held isothermally at 240°C for 1 min; the velocity of He carrier gas (99,9996%) was 1 mL/min.

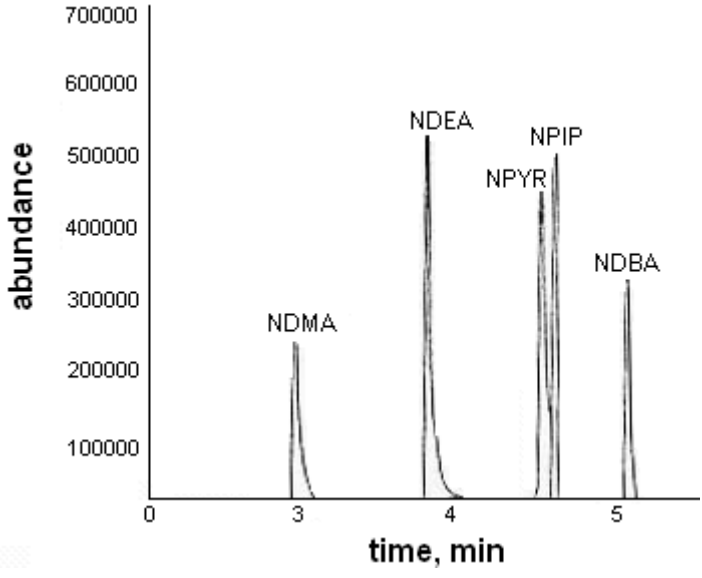


Fig. 4. Total ion chromatogram of five volatile NA extracted from smoked sausage sample after analytical clean-up (spiked with 5 μg/kg of each single NA).

To calibrate the GC-MSD spectra, six different standard solutions were prepared which cover the concentration range 0,1–60 ng/mL. In this study the squared correlation coefficient for different calibration curves of NA was found to be 0,9994–0,9998. This calibration curve enables to calculate NA concentration using the GC-MS peak area measurements.

Fig. 4 shows the chromatogram of five NA extracted from smoked sausage spiked with 5 μg/kg of each single NA. The selectivity of the method was assessed evaluating the purity of NA chromatographic peaks, and comparing the

spectra of each NA peak in the chromatogram of the different food samples assayed with those obtained with standard solutions.

The SPE-GC-MS method was applied to determine of NA in beer, fish and meat samples.

2.1.4. Gas chromatography mass spectrometry method for determination of polycyclic aromatic hydrocarbons

PAH were separated by GC-MS using PICI and NICI with ammonia and methane as reagent gases.

PICI mass spectrometric method with ammonia as reagent gas was carried out. Since PAH molecule (M), for which the PA is greater than that of ammonia give mass spectra with $[M+H]^+$ as the base peak corresponding to protonation of the molecule, and a much smaller peak for the $[M+NH_4]^+$ and $[M+N_2H_7]^+$ adduct (except for B[b]F). The PA of PAH and details of their ammonia PICI mass spectra are summarized in Table 10. In the positive mode, all of PAH peaks can be identified.

Table 10. Ammonia PICI mass spectra of six PAH[a]

Compound	PA[b], kJ/mol	Relative intensities, %			
		$M^{+\cdot}$	$[M+1]^+$	$[M+18]^+$	$[M+35]^+$
B[a]A	867	25	100	—	—
B[b]F	848	100	33	54	60
B[k]F	857	33	100	32	60
B[a]P	882	11	100	—	5
I[cd]P	—	18	100	12	10
B[ghi]P	871	21	100	12	9

[a] all relative intensities are expressed as a percentage of the base peak; [b] the PA values obtained from [117].

PICI mass spectrometry with methane as reagent gas of all PAH studied in this work shows little or no fragmentation and contents from $[M+H]^+$ as the base peak, and adduct ions at $[M+C_2H_5]^+$ and $[M+C_3H_5]^+$. The methane could not be used to distinguish between B[ghi]P and I[cd]P. These isomers required high reagent gas selectivity for ion-molecule reactions to be differentiated. Therefore, all PAH were differentiated by PICI using ammonia as reagent gas.

NICI is a useful sensitive technique for substances having a high electron affinity (EA), such as PAH. Table 11 summarizes the fragment ions in the methane NICI mass spectra of the six studied PAH. PAH with EA values greater than 49 kJ/mol produce $[M]^-$ as the base peak, and B[a]A with EA value less than 49 kJ/mol produces $[M–H]^-$ as the base peak. The $[M–H]^-$ ions can be attributed to the deprotonation of PAH by anions, such as, OH^-, $O^{-\cdot}$, and H^-.

The $[M+15]^-$ ion has been attributed to $[M+CH_3]^-$, formed by fast radical addition reaction between the PAH molecule and CH_3 radicals present in the plasma of the hydrocarbon reactant gas [86].

Table 11. Methane NICI mass spectra of PAH[a]

Compound	EA[b], kJ/mol	Relative intensities, %			
		$[M-1]^-$	$[M]^-$	$[M+1]^-$	$[M+15]^-$
B[a]A	40,53	100	22	21	40
B[b]F	—	—	100	18	1
B[k]F	—	—	100	24	1
B[a]P	61,76	—	100	28	4
I[cd]P	—	—	100	27	2
B[ghi]P	49,22	—	100	30	4

[a] all relative intensities are expressed as a percentage of the base peak; [b] the EA values obtained from [86].

As shown in Paper III, the LOD for I[cd]P by NICI is lower than by PICI. The B[a]A is not detected by NICI mass spectrometry. It appears that the LOD for B[a]P, B[a]A, B[k]F+B[b]F, and B[ghi]P by PICI detection with ammonia as reagent gas is lower. Fig. 5 represents the mass spectra of B[a]P obtained with EI, PICI and NICI with methane, and PICI with ammonia as reagent gas.

The following analysis of PAH was performed by PICI mass spectrometry using ammonia as reagent gas. 10 μL of the sample solution was injected into the gas chromatograph using pulsed splitless injection in the selected ion monitoring mode. A HP Model 6890 gas chromatograph equipped with a Model 5973 MSD was employed for the analysis. Operating conditions were as follows: 30 m × 0,25 mm i.d. silica capillary column with film thickness of 0,25 μm (HP-5); helium carrier gas 0,9 mL/min; injector and detector temperatures 275°C and 260°C, respectively; temperature programme: 80°C (1 min), 80–115°C (20°C/min), 115°C (1 min), 115–130°C (10°C/min), 130°C (0 min), 130–295°C (25°C/min), 295°C (0,50 min), 295–300°C (1°C/min), 300°C (0 min). The ionizing voltage was 150 V. It was unable to separate B[b]F and B[k]F by this methodology. These compounds were determined together as the sum.

To calibrate the GC-MSD spectra, six different standard solutions were prepared which cover the concentration range 0,1–60 ng/mL. The squared correlation coefficient r^2 for different calibration curves of PAH was found to be 0,9990–0,9996. This calibration curve enables to calculate PAH concentration using the GC-MS peak area measurements.

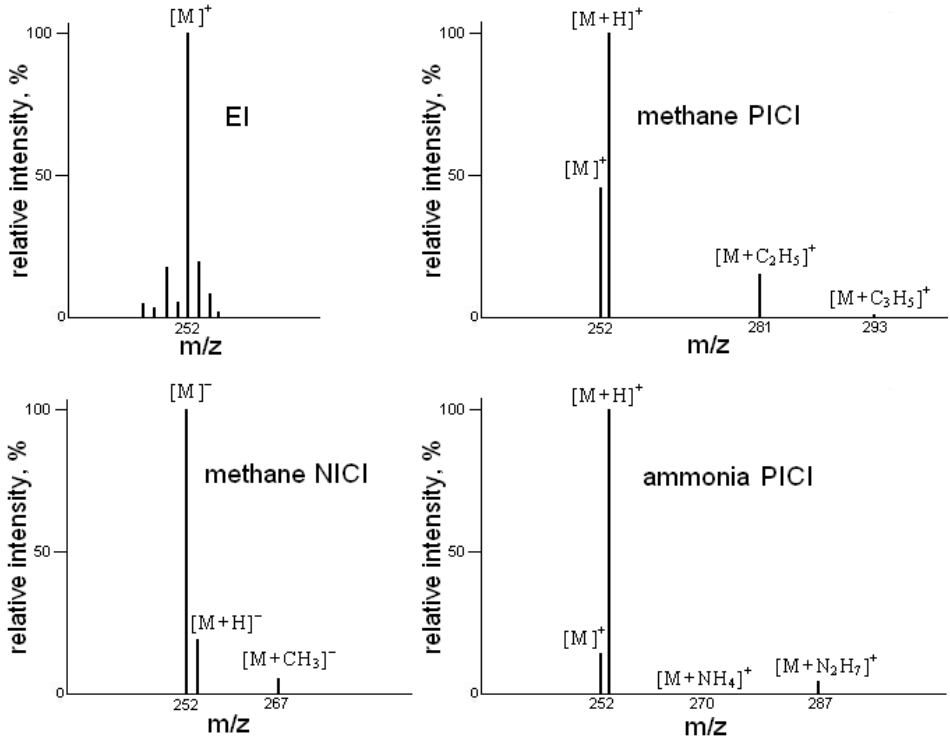


Fig. 5. Comparison of mass spectra of B[a]P obtained by EI, PICI and NICI with methane, and PICI with ammonia as reagent gas.

2.2. Validation of methods for determination of *N*-nitrosamines and polycyclic aromatic hydrocarbons in food

To demonstrate the method was under analytical control the LOD, the limit of quantitation (LOQ), and recovery experiments were performed. The LOD and the LOQ have been established using spiked samples. The LOD was measured as the lowest amount of the analyte that may be detected to procedure a response which is significantly different from that of a blank. The value of the LOD was calculated as follows:

$$LOD = X_{bl} + K \cdot SD_{bl},$$

where X_{bl} is the mean of the blank measures and SD_{bl} is the standard deviation of the blank measures, and K is a numerical factor chosen according to the confidence level desired. If confidence level is 95%, the K is 3,36. The LOQ of an analytical procedure is the lowest amount of analyte in a sample which can

be quantitatively determined with a certain confidence. The LOQ is then 3,3 times the LOD [148].

Reference standards of the PAH and the NA are used not only to identify the peaks detected in the chromatograms, but also to determine the efficiency of the methods for extracting of compounds from the food samples. For the recovery experiment a sample with a low content of NA was chosen, and fortified with two different amounts of NA standard mixture. The sample was analyzed by GC-MS and recovery of NA was calculated as follows [30]:

$$\text{Recovery (\%)} = [(C_1\text{-}C_2)/C_3] \times 100,$$

where C_1 is the concentration determined in the fortified sample, C_2 is the concentration determined in the unfortified sample, and C_3 is the concentration of fortification.

Method for determination of NA was validated for meat, fish, and beer samples (see Papers II, IV, V). Validation was carried out in terms of LOD, LOQ, linearity, and recovery. For example, the values of the LOD and the LOQ of this method for raw mutton are shown in Table 12. The LOD and the LOQ for this method were approximately 0,10 and 0,35 μg/kg, respectively.

Table 12. The LOD and the LOQ data (μg/kg) of NA in raw mutton

Com-pound	Unfortified sample	Fortified samples[a]					
		Mean concentration (n=6) of NA, μg/kg	Rec[b], %	SD[c]	RSD[d], %	LOD	LOQ
NDMA	n.d.[e]	0,40	79	0,023	5,75	0,08	0,26
NDEA	n.d.	0,42	82	0,027	6,43	0,09	0,30
NPYR	n.d.	0,44	88	0,032	7,27	0,11	0,35
NPIP	n.d.	0,40	86	0,022	5,50	0,07	0,24
NDBA	n.d.	0,41	88	0,027	6,59	0,09	0,30

[a] 0,50 μg/kg of NA added; [b] average recovery of NA in percent; [c] sample standard deviation; [d] relative standard deviation in percent; [e] not detected.

The approach employed to evaluate the accuracy of the method was based on the recovery of known amounts of each NA spiked into meat samples in two levels. Table 13 lists the NA amounts found in meat samples for different fortification levels, the percentage recovery (mean of six replicates), the standard- and relative standard deviation of the replicates. The relative standard deviation is expressed in percent and is obtained by multiplying the standard deviation by 100 and dividing this product by the average concentration. These results show the good efficiency of the developed method in terms of extraction recovery with the standard addition method.

Table 13. Validation data of five NA spiked at two levels in raw mutton

Com-pound	Unfortified sample	Fortified samples							
		Level I[a]				Level II[b]			
		Mean concentration (n=6) of NA, μg/kg	Rec[c], %	SD[d]	RSD[e], %	Mean concentration (n=6) of NA, μg/kg	Rec, %	SD	RSD, %
NDMA	n.d.	1,13	75	0,09	8,14	2,90	73	0,223	7,69
NDEA	n.d.	1,18	79	0,09	7,20	3,01	75	0,206	6,84
NPYR	n.d.	1,21	81	0,11	9,34	3,12	78	0,323	10,35
NPIP	n.d.	1,20	80	0,13	10,92	3,08	77	0,311	10,10
NDBA	n.d.	1,23	82	0,12	10,08	3,15	79	0,324	10,29

[a] 1,5 μg/kg of NA added; [b] 4 μg/kg of NA added; [c] average recovery of NA in percent; [d] sample standard deviation; [e] relative standard deviation in percent.

The repeatability data, resulting from six replicate analyses of the same sample of canned pork are reported in Table 14.

Table 14. Analytical repeatability of the proposed method (sample of canned pork)

Compounds	Mean concentration (n=6) of NA, μg/kg	SD[a]	RSD[b], %
NDMA	1,01	0,071	7,03
NDEA	n.d.	–	–
NPYR	2,20	0,192	8,75
NPIP	1,08	0,091	8,43
NDBA	0,52	0,045	8,65

[a] sample standard deviation; [b] relative standard deviation in percent.

Method for determination of PAH was validated for fish samples. Results of the validation studies for cold-smoked mackerel are documented in Paper III. The LOD and the LOQ for this method were approximately 0,30 and 0,85 μg/kg, respectively with about 80% recovery.

The obtained results characterize the suitability of method for determination the NA and the PAH in various samples of food products.

3. OCCURRENCES OF *N*-NITROSAMINES AND POLYCYCLIC AROMATIC HYDROCARBONS IN SOME ESTONIAN FOOD PRODUCTS

3.1. *N*-nitrosodimethylamine analysis in alcoholic beverages

The mean level of NDMA in 158 samples of Estonian beer was found to be 0,20 μg/L and in 106 samples of imported beer – 0,21 μg/L. The other NA are not detected in beer. The highest NDMA level in domestic beer was found in porter (0,57 μg/L, the average of 12 samples) and in dark beer (0,48 μg/L, the average of 15 samples). It was found, that the NDMA concentration depends on percent of alcohol in beer, what can be explained by the assumption that ethanol is an inhibitor of nitrosation (Fig. 6) [119, 157].

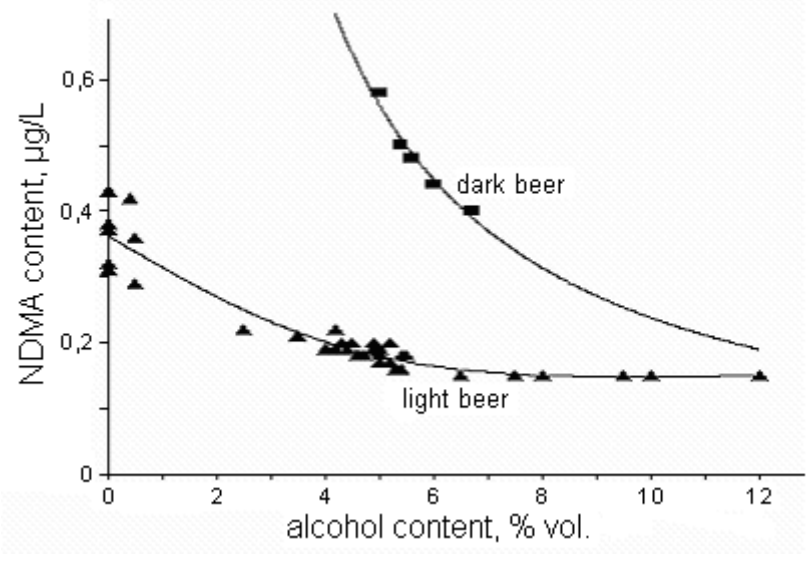


Fig. 6. The graphs showing percent of alcohol versus NDMA content in light and dark Estonian beer.

In dark beer, the level of NDMA is higher than in light beer with the same percent of alcohol. In light beer, the highest concentration was observed in alcohol-free beer, the NDMA concentration decreasing continuously with increasing alcohol content. At alcohol percent around six, the NDMA concentration in light beer reaches the LOD (see Paper II).

The temperature of storage has a significant effect on the concentration of NDMA. During transport, beer samples may be exposed for short duration to

higher temperature. After two weeks storage at 4ºC the samples showed no detectable changes, but at higher temperatures was observed a loss of NDMA (Fig. 7). The decrease in NDMA level after storage can be caused by volatility of NDMA.

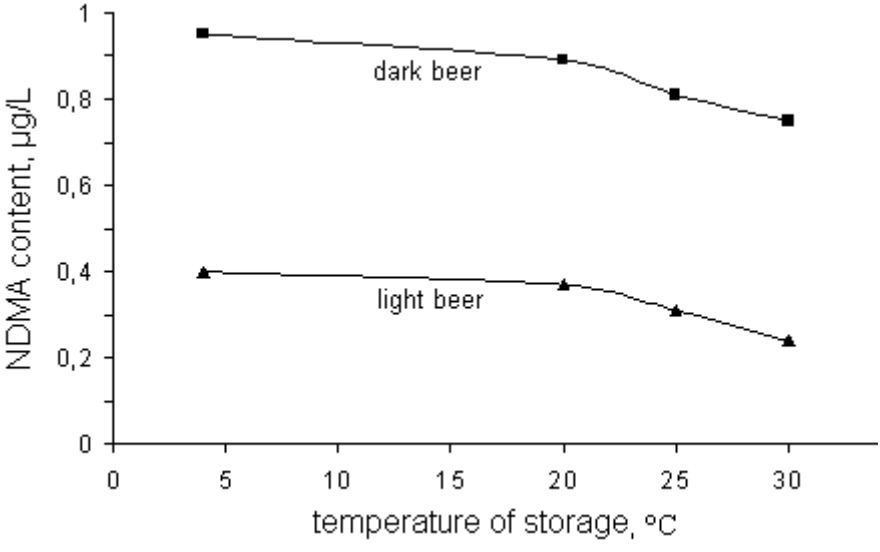


Fig. 7. The influence of storage temperature (during two weeks) on the content of NDMA in dark and light beer.

In the studies of foreign beers carried out during 1991–1992 and 1994, the respective averages were 0,71 µg/L (106 samples) and 0,15 µg/L (36 samples) [136]. We repeated some of these measurements during 2003–2004 using our two-step SPE method and supplemented it by extensive measurements of NDMA content in Estonian beer (see Tables in Paper II). The significant difference in level of NDMA is probably caused by difference in beer technology.

NA was not detected in alcoholic beverages such as red and white wine, liquor, rum, tequila, vodka, brandy, cognac, and gin samples. However NDMA was also present in whiskies of various kinds, no doubt arising from the same source, but at lower concentrations than in beer and posing a lower cancer risk, because whisky is consumed by most people in smaller amounts than beer. The samples of Scotch whisky contained NDMA from 0,50 to 2,20 µg/L, and Irish – from not detectable to 0,50 µg/L. Note, that the alcohol percent in whisky is about 40% vol. Compared with fried and smoked food products which may contain several micrograms of NA per kilogram, NA concentration in beer and whiskies is low.

3.2. *N*-nitrosamines and polycyclic aromatic hydrocarbons in fish products

The GC-MS method was applied to determine of carcinogenic contaminants such as NA and PAH in fish products.

In this work, the level of six PAH in 108 samples of various fish products, and in 32 samples of oil purchased from Estonian market was determined. Also, the content of five NA in 294 samples of fishery products, and in 77 samples of vegetable oil was determined. The samples with the high PAH and NA content were repeatedly analyzed.

The details about data on PAH and NA level in various samples of fish products, and in the oil samples are given in Papers III and IV, respectively. In samples of raw fish the level of PAH and NA was not detected. The highest PAH level was found in samples of smoked sprats in oil. In fish products, although PAH accumulate preferentially in the lipid tissue, they diffuse also into the muscles, where they may be bound by some structural elements. Most of PAH in smoked foods come from the wood smoke.

The highest level of NA was found in samples of fried- and pickled fish. Relatively high level was found in hot-smoked fish, in sprats in oil, in salted- and in salted/dried fish. The formation of significant amounts of volatile NA in the fish is probably caused by the interaction of nitrites and amines in the fish. In 8 of 294 samples of various fish products the concentration of the sum of NDMA and NDEA exceeded the tolerance limit of 3 μg/kg. The samples with the high level of NA and PAH have the potential risk to human health. Hot-smoked fish contains about two times more NA than the cold-smoked product, depending on the species of the fish, methods and parameters of smoking. As well, in samples of cold-smoked fish the level of PAH was not detected, and in hot-smoked samples was found detectable amount.

The level of PAH in 34 samples and NA in 13 samples of smoked sprats in oil were determined. In these samples, the ratio of smoked sprats to vegetable oil was 7:3. In 15 of 34 and in 11 of 13 samples of smoked sprats in oil contained B[a]P and NDMA at level exceeding 1 μg/kg. Moreover, the samples of neat vegetable oil contain sum of NA at level not exceeding 1,18 μg/kg, whereas the oil of sprats contains an average 1,15 μg/kg of NA. The samples of neat oil contain the sum of PAH at level not exceeding 6 μg/kg, and some oil of sprats contains on an average 58 μg/kg of PAH. The content of PAH in oil of sprats was about nine times higher than in canned smoked sprat. However, in smoked fish canned in oil, the contamination may be carried by spices added to vegetable oil.

Increased level of NA was observed after baking and frying indicating formation of these compounds during cooking. The concentration of NA in the fish after baking in pre-heated electrical oven depends on the fish cooking temperature. To check it we divided approximately 300 g of silver hake

(*Merluccius bilinearis*) into six roughly equal parts. One portion of fish was reserved for analysis in a fresh state. The other five portions were baked separately in pre-heated electrical oven in open oven-proof glass dishes with 50 mL oil at regulo 50°C, 100°C, 150°C, 200°C, and 250°C, during 30 min. The test was repeated for rape- and olive oil. The cooked portions were allowed to cool but before becoming cold the excess fat and oil were separated from the sample. Since the aim was to measure NA in the fish and in the oil as normally consumed, this excess fat was analysed. The level of NA was determined also in baked fish without oil. The rape- and olive oils had been analysed after baking in electrical oven at different temperatures as well. There was significant increase in all NA level in the fish and in the oil after baking with electrical oven at temperature around 150ºC (Fig. 8). In repeatedly baked rape oil samples was found insignificant increase in NA concentration.

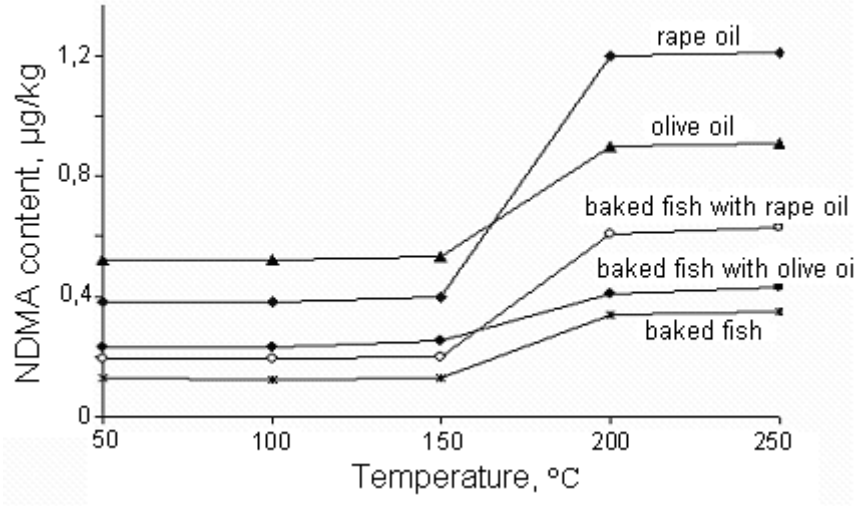


Fig. 8. The influence of temperature on the content of NDMA in fish and oil.

After frying for 30 min per side in a pan using natural gas, NA was found in 5 of 5 samples of fish. The amount detected in the fried oil was comparable to the amount present in the fried fish. In most cases the level of NDMA in the gas-frying sample was higher than that in the electrical oven-baking fish. Apparently, cooking temperature and time have a significant effect on the concentration of NA.

For comparison, the content of PAH and NA found in various fish products was confronted with data from literature. The level of NA in Estonian fish products was lower than that found in various fish products from Japan [70], and China [164], but show good agreement with results from France [10], Sweden [77, 102], Canada [104], Russia [72], Italy [95], and Denmark [105].

The highest level of B[a]P has been found in smoked fish as was reported by Russian investigators. For example, Petrun [107] reported findings ranging from 7 to 60 μg/kg. Karl [68] has found that the content of PAH in samples of smoked fish from modern smoking kilns with external smoke generation was lower than the content from traditional smoking kilns.

3.3. *N*-nitrosamines in meat products

In this part we concern with various meat products which are a very popular dish in Estonia. The level of volatile NA, namely NDMA, NDEA, NDBA, NPYR, and NPIP in 386 samples of meat products purchased from Estonian market were determined.

The level of NA found in studied samples of meat products is shown in Paper V. The raw meat contents no detectable amount of NA. NDMA was noted in 88%, NDEA – 27%, NPYR – 90%, NPIP – 65% and NDBA – 33% of heated meat samples. The highest level of NA was found in samples of fried meat. Relatively high level was found in grilled meat, smoked pork, half-smoked sausage, and in ham.

The concentration of all NA observed in smoked sausages was lower than in half-smoked sausages and fried meat products. This may be due to the variation in nitrite level in various meat products. One may anticipate that in smoked sausages the nitrite concentration is lower than in half-smoked sausages. Among the analysed assortments the high level of NDMA was recorded in half-smoked sausage, in grilled poultry and pork, and in fried pork. The highest level of NDEA with the mean concentration of 0,88 μg/kg was found in fried poultry. The very high level of NPYR with the mean concentration of 20,67 μg/kg was detected in fried poultry with paprika. The paprika may contain precursors of NPYR [59]. Indeed, the level of NA in meat without paprika is lower than in meat with paprika. Differences in the NPIP content may be the results of applied spices and different degree of meat crumbling, thus different penetration of spices into the whole capacity of meat pieces [25]. The black pepper, which contains piperidine, could be the main source of NPIP. We may anticipate that the concentration of NA in grilled meat without spices is lower than in grilled meat with spices. Cadaverin, the product of lysine decarboxylation during thermal processing of meat or its maturing could be also the precursor of NPIP [125]. As pyrolysis of protein by cooking can be a source of secondary amines, the formation of significant amounts of volatile NA in the meat may be also caused by the interaction of nitrites and amines in the meat.

One may anticipate important role of added sodium nitrite on the formation of volatile NA in meat products. Sodium nitrite solutions with 50, 100, 150, and 200 mg/kg concentrations were added to raw mutton. After adding, the samples were stored during 24 hours at 5±1ºC. After that, part of samples was fried in a

frying pan during 30 min using natural gas. All samples with added nitrite fried or not, contained detectable levels of NA (see Paper V). For example, Fig. 9 shows the effect of nitrite concentration on the content of NDMA in fried mutton samples. We observe roughly linear increase in concentration of NA in fried and raw meat with the addition of sodium nitrite. The sample of mutton processed with high level of added nitrite and fried in a conventional frying pan contains the high level of NA. The process of frying causes increase of slope i.e. the sensitivity of all NA to nitrosation increases about 10 times compared to raw mutton samples. Most sensitive for sodium nitrite addition is NPYR (about 0,16 μg pro 1 mg $NaNO_2$). The other NA were produced in less amounts, the concentration increase pro 1 mg added $NaNO_2$ was about 0,020 μg for NDMA, 0,014 μg for NPIP, 0,005 μg for NDEA and NDBA.

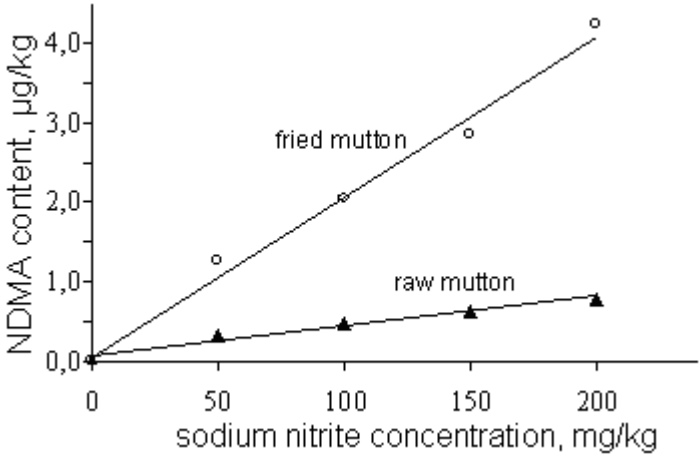


Fig. 9. The level of NDMA in fried and raw mutton samples prepared with different amount of sodium nitrite added.

The effect of baking in an electrical oven on the volatile NA level was investigated as described before (see p. 33) for fish. Neat olive oil has been analysed after baking in electrical oven at different temperatures. The level of NA was determined also in baked mutton without oil. All these data are presented in Paper V. There was significant increase in NA level in the meat and in the oil after baking with electrical oven at temperature around 150ºC. The concentration of NA in baked mutton parts with olive oil is equal to the sum the concentration of NA in mutton baked without oil and in neat olive oil. Among that, the concentration of NDMA and NDEA at all observed temperatures in neat olive oil prevails remarkable the concentration in mutton. Particularly important a role of fat in baked mutton. It appears that about 73% of NA are concentrated in fat of baked mutton. Thereby at baking temperatures lower than 200ºC about 90% of NDMA is concentrated in fat, whereas NPYR

abundance in fat reaches 30% by these temperatures. At baking temperature 250ºC the abundance of NDMA in fat is 73% and NPYR 64%.

To determine the influence of cooking method on the growth of volatile NA in meat products the sample of mutton (thin slices) was baked in an electrical oven at 150ºC, cooked in a microwave oven without oil at 120ºC, and fried in a frying pan with olive oil at 150ºC. The results are given in Table 15. The level of NA in gas-frying sample of mutton exceeded remarkably the level in the electrical- and microwave oven-baking mutton.

Table 15. The effect of cooking (30 min) in an electrical oven, in a microwave oven, and in a frying pan on the level of volatile NA in mutton

Compound	Mean concentration (n=4) of NA, μg/kg		
	Microwave oven (120ºC)	Electrical oven (150ºC)	Frying pan (150ºC)
NDMA	0,48	0,71	1,16
NDEA	0,36	0,55	0,72
NPYR	0,51	0,62	3,17
NPIP	0,19	0,22	1,14
NDBA	n.d.	n.d.	0,35

The formation of NA increases with time and temperature of frying. The sample of mutton was fried in a frying pan using natural gas during 30 minutes. The temperature of mutton slices fried in a frying pan with olive oil maintained at 150±1ºC (determined by thermocouples inserted into the slices). After frying for 10 min the temperature of mutton maximized to 102ºC. As shown in Fig. 10, the concentration of NA increases rapidly in range from 40 to 100ºC and then stay stabile. Hence the formation of NA takes place in temperatures below 100ºC.

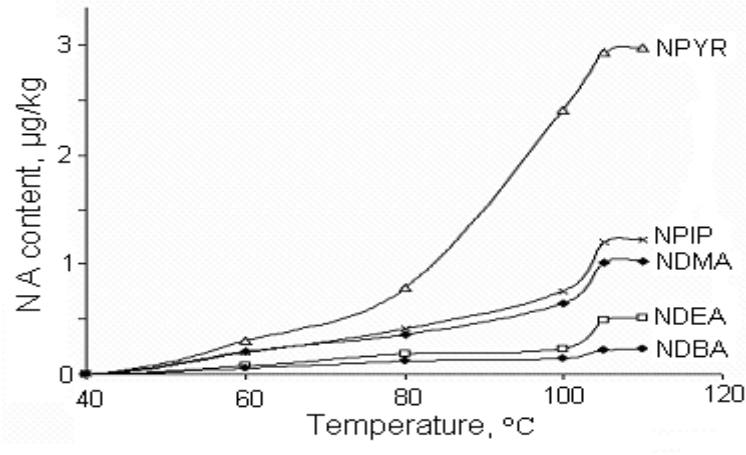


Fig. 10. The influence of temperature on the content of volatile NA in mutton.

The pork was fried in a frying pan with olive oil using natural gas, and the total NA amount in whole rashers and in separated lean and fat components is given in Paper V. After frying for 30 min per side, NA were found in 5 of 5 samples of pork. The total yield of NA from the fat of pork was much greater than that from either the lean or even whole rasher. In fried pork with paprika the concentration of NA in fat exceeds the concentration in lean 6 times (among them NDMA 8 times, NDEA 2,5 times, NPYR 6,7 times, NPIP 3,2 times, and NDBA 1,9 times). In fried pork without paprika the concentration of NA in fat exceeds the concentration in lean 5,4 times. Hence, one can conclude that the effect of paprika in meat doesn't prevail in formation of NA in frying process.

The effect of different storage conditions on NA content in fried meat products was demonstrated. 33 samples of fried mutton was stored in different conditions e.g. to 120 hours at 5±1°C and at 20±2°C. After storage period the NA content was determined. It reveals that temperature and time of storage have a significant effect on the formation of NA. The changes of NA concentration after different storage conditions can be explained by results of chemical reactions between precursors of NA present or formed in meat products. No significant changes are observed for samples stored at 5°C for 120 hours. Higher storage temperature (20°C) is optimal for bacterial growth and their metabolism, thus the decrease in NA level after storage can be caused by bacterial action or vaporation as well [26]. A different effect appeared by storage of canned pork purchased from market. As shown in Fig. 11, we observe the surprising increase of NA concentration with time of storing at 5±1°C, and the decrease at 20°C. The latter effect can be explained as before but the increase of NA amount at low temperatures may be due to different reaction paths of precursors of NA introduced in samples (sodium nitrite, spices).

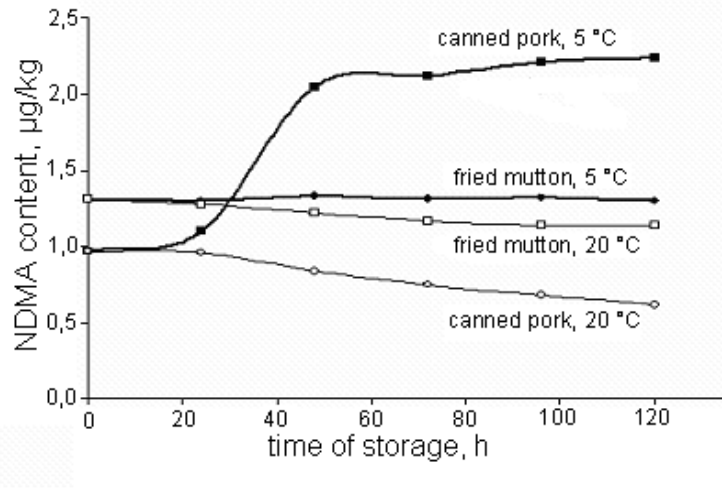


Fig. 11. Mean concentration of NDMA in studied samples of fried mutton and canned pork stored in different conditions.

For the comparison, the content of NA found in various meat products was confronted with data from literature. The level of NA in Estonian meat products in the present study shows good agreement with results from France [10], Russia [110, 163], Germany [75, 141, 154], Poland [25], Sweden [102], and Japan [87, 159].

CONCLUSIONS

In the first part of the thesis method for determination of NA and PAH in food was developed (see Papers I, III). In the present study six PAH and five NA were separated by GC and differentiated by EI, PICI, and NICI mass spectrometry using two different reagent gases – ammonia and methane. It was found that PICI with ammonia as reagent gas is the most selective technique for determination of isomeric PAH and NA. The developed method showed a high correlation coefficient (0,999), sufficient sensitivity and reproducibility. Our GC-MS method for determination of NA and PAH have the LOD of 0,10 μg/kg and 0,30 μg/kg, respectively. The recovery of PAH and NA in some food products varied from 75% to 88%.

We have chosen to use SPE technique, since this is a way to minimize the use of chemicals and it is easy to automatize if a large number of samples are to be analysed. We showed that extraction at Extrelut-Florisil sorbent gives higher recovery of NA compared to other sorbents. SPE method was found to be superior to other extraction methods for high recovery and rapidity of analysis.

Two-step SPE with Extrelut-Florisil sorbent was applied to isolate of NA from meat, fish, beer, and oil products. However, for the selective isolation of PAH from the samples of fish and oil were used the method of gel-permeation chromatography. Further advantages of proposed method are low time needed for sample preparation and decreased possibility of pollution of GC-MS system by additives. In conclusion, we developed a simple, rapid, effective and accurate method to evaluate NA and PAH content in food products.

In the second part the developed methods were applied to determine NA and PAH in beer, fish and meat samples (see Papers II–VI). With fish and meat products as a food model, different factors affecting the NA and the PAH formation were tested. It was shown that the addition of nitrite to meat, storage conditions, the temperature and time of cooking have a large impact on the NA formation. With the addition of sodium nitrite, one can observe roughly linear increase in concentration of NA in fried and raw meat. About 73% of NA are concentrated in fat of baked mutton. In fried pork, the concentration of NA in fat is six times higher than in lean. The results reveal that NA and PAH level in food is strongly dependent on the method of cooking. The amount of NA formed in cooked meat depends on the duration and temperature of frying, but it is very little in microwave cooking (see Paper V). It was found that the NDMA concentration depends on percent of alcohol in beer, since ethanol is an inhibitor of nitrosation.

Comparison of the results of our analyses with the results obtained by other authors encourages us to use this highly sensitive method for identification and quantitative analysis of NA and PAH.

The level of NA also depends on species of fish and meat. The highest level of PAH was found in samples of smoked sprats in oil. The content of PAH in oil of sprats was about nine times higher than in canned smoked sprat. However, in smoked fish canned in oil, the contamination may be carried by spices added to vegetable oil. The high level of NA was found in some food products such as fried-, grilled meat, smoked pork, half-smoked sausage, ham, fried-, pickled fish, hot-smoked fish, sprats in oil, salted fish, dark and alcohol-free beer. Hot-smoked fish contains much more PAH and NA than the cold-smoked product.

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SUMMARY IN ESTONIAN

Mõningate kantserogeensete saasteainete määramine toidus

Meie tähelepanu koondus toidu uurimisele, mis on kirjanduse andmetel üks levinumaid analüüsiobjekte. Tasemel uurimismeetodid eeldavad tänapäeval mass spektromeetria kasutamist. Püüdsime välja töötada usaldusväärsed analüüsimeetodid *N*-nitrosoamiinide (NA) ja polütsükliliste aromaatsete süsivesinikute (PAS) määramiseks toidus, mis ühtlasi võimaldaks lühendada proovi ettevalmistus- ja mõõtmisaega. Eesmärgiks oli määrata nimetatud kantserogeenide tekkimist mõjutavad faktorid.

Töö koosneb kolmest osast: kirjanduse ülevaade, eksperimentaalne osa, kantserogeensete saasteainete sisalduse määramine toidus ja nende tekkimist soodustavate faktorite uurimine.

Käesoleva väitekirja esimeses peatükis antakse ülevaade analüüsimeetoditest NA ja PAS määramiseks erinevates toiduproduktides ja analüüsitakse nende ainete võimalikke tekkemehhanisme.

Teises osas esitatakse uus meetod NA ja PAS määramiseks (artiklid I, III). Analüüsiks kasutati massiselektiivset detektorit HP 5973 MSD ja gaasikromatograafi HP 6890 GC. NA määramise meetodi väljatöötamiseks kasutati erinevaid sorbente proovi puhastamiseks ja võrreldakse ionisatsiooni meetodeid. Leiti, et kaheetapiline (Extrelut-Florisil) tahke faasi ekstraktsioon võimaldab paremat NA regeneratsiooni kui teised sorbendid. Ammoniaak annab keemilise ionisatsiooni protsessis "pehme" ionisatsiooni ja vähe fragmente, mistõttu on ta sobiv reagentgaas NA määramiseks. Kasutades kaheetapilist ekstraktsiooni, keemilist ionisatsiooni ja ammoniaaki reagentgaasina on NA avastamispiir 0,10 μg/kg ja regeneratsioon on ligikaudu võrdne 80%. Väljatöötatud analüüsi meetodi testimisel saavutati hea kokkulangevus analüüsil määratud ning eelnevalt lisatud NA kontsentratsiooni vahel.

PAS analüüsil kasutati proovi puhastamiseks geelkromatograafilist meetodit. Võrreldi positiivse- ja negatiivse-iooni keemilist ionisatsiooni, kasutades erineva prootonafiinsusega reagentgaase (metaani ja ammoniaaki). Osutus, et positiivse-iooni keemiline ionisatsioon reagentgaas ammoniaagiga annab paremad avastamispiiri väärtused. Selle meetodi avastamispiir on 0,30 μg/kg ja regeneratsioon on ligikaudu võrdne 80%.

Väitekirja kolmandas osas rakendati väljatöötatud metoodikat erineva struktuuriga NA ja PAS sisalduse määramiseks õlles, kala- ja lihatoodetes ning uuriti nende tekkimist mõjutavaid faktoreid (toote säilitamise tingimused, nitrite lisamine, temperatuuri mõju) (artiklid II–VI). Tootjad lisavad liha säilitamise ja väljanägemise parandamiseks naatriumnitriteid. Ilmnes, et naatriumnitritite lisamisel kasvab NA kontsentratsioon lihas lineaarselt. Leiti et *N*-nitrosodimetüül-

amiini kontsentratsioon sõltub õlle alkoholisisalduse protsendist ja säilitamise tingimustest.

Väljatöötatud analüüsimeetodiga määratud NA ja PAS sisaldused on väga heas kooskõlas kirjandusest avaldatud tulemustega. Kokkuvõtteks võiks öelda, et antud töös on välja töötatud kaks erinevat meetodit kantserogeensete saasteainete määramiseks toidus.

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