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NITRATES, NITRITES, N-NITROSAMINES AND POLYCYCLIC AROMATIC HYDROCARBONS IN FOOD: ANALYTICAL METHODS, OCCURRENCE AND DIETARY INTAKE

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Publication III: The author is responsible for analytical methods, data sets and preparation of part of the manuscript describing analytical methods and data

Publication V: The author is responsible for gathering data and preparation of the manuscript

ABBREVIATIONS AND SYMBOLS

ADI	–	acceptable daily intake
ALARA	–	as low as reasonably achievable
BaA	–	benzo(a)anthracene
BaP	–	benzo(a)pyrene
BbF	–	benzo(b)fluoranthene
BcL	–	7H-benzo-(c)-fluorene
BgP	–	benzo(g,h,i)perylene
BjF	–	benzo(j)fluoranthene
BkF	–	benzo(k)fluoranthene
CEN	–	European Committee for Standardization
CHR	–	chrysene
CI	–	chemical ionization
CPP	–	cyclopenta(cd)pyrene
DeP	–	dibenzo(a,e)pyrene
DhA	–	dibenzo(a,h)anthracene
DhP	–	dibenzo(a,h)pyrene
DiP	–	dibenzo(a,i)pyrene
DlP	–	dibenzo(a,l)pyrene
EC	–	European Commission
EI	–	electron impact
EU	–	European Union
GC	–	gas chromatography
GC/MS	–	gas chromatography mass spectrometry
HPI	–	Health Protection Inspectorate
HPLC	–	high performance liquid chromatography
IARC	–	International Agency on Research of Cancer
IcP	–	indeno(1,2,3-cd)pyrene
JECFA	–	Joint FAO/WHO Expert Committee on Food Additives
LC/MS	–	liquid chromatography mass spectrometry
LD50	–	lethal dose for 50% of test animals
LOD	–	limit of detection
LOQ	–	limit of quantification
MRL	–	maximum permitted residue level
MSD	–	mass-selective detector
NA	–	N-nitrosamines
NDBA	–	N-nitrosodibutylamine
NDEA	–	N-nitrosodiethylamine
NDMA	–	N-nitrosodimethylamine
NMKL	–	Nordic Committee on Food Analysis
NPIP	–	N-nitrosopiperidine
NPYR	–	N-nitrosopyrrolidine

PAH	–	polycyclic aromatic hydrocarbons
SPE	–	solid phase extraction
SPME	–	solid phase microextraction
U	–	expanded measurement uncertainty
WHO	–	World Health Organization
5MC	–	5-methylchrysene

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1. INTRODUCTION

Interest in the dietary intakes of nitrates and nitrites has arisen from the concern about their possible adverse effect on health. The natural occurrence of nitrates in plants is a consequence of the nitrogen cycle whereby mineral nitrogen is assimilated by the plant as nitrates to use them in the synthesis of plant proteins. Nitrates and nitrites are also used as food additives in cured meats due to their ability to protect products from *Clostridium botulinum* and other *Clostridium* species, and for their red colour fixing properties. Nitrates and nitrites are found in drinking water due to both natural occurrence and contamination of water supplies, mostly from agricultural sources and municipal wastewater.

The concern over nitrates and nitrites in the diet has two aspects: they may create an excess of methaemoglobin possibly leading to toxic effects such as cyanosis and they may cause the endogenous formation of carcinogenic N-nitroso compounds.

Nitrate represents the stable oxidation state of nitrogen and can be reduced to nitrite in the environment by microorganisms and within human tissues. Nitrite represents a less stable oxidation state of nitrogen and therefore can be further reduced to various compounds or oxidized to nitrate. Nitrite may endogenously react with secondary amines to form N-nitrosamines (NA) at low pH values, as is the case in the gastric environment of mammals. Nitrosoamines may also be pre-formed in foodstuffs during certain biological, chemical and physical processes in crops, industrial transformation or even at the time of consumption (Knekt *et al*.1999; Pegg and Shahidi 2000).

Polycyclic aromatic hydrocarbons (PAHs) are numerous group of potent carcinogenic compounds consisting of two or more fused aromatic rings present in the environment. PAHs are formed in incomplete combustion processes or high-temperature pyrolysis of coal, oil and other organic materials. The most important sources are identified as coal coking; production of aluminum, iron, and steel; heating in power plants and residences; cooking; motor vehicle traffic; environmental tobacco smoke; and the incineration of refuse (WHO 1998). Over 100 PAHs have been identified in the environment as pollutants and occur as complex mixtures (Mottier et al. 2000).

Many of PAHs are carcinogenic in experimental animals. They are widely believed to make a significant contribution to the burden of cancer in humans (Phillips 1999). The International Agency of Research on Cancer has categorized 15 PAHs, including the PAHs possibly occuring in food – benzo(a)pyrene, benz(a)anthrazene, benzo(b)fluoranthene, benzo(j)fluoranthene, benzo(k)fluoranthene, dibenz(a,h)anthracene, dibenzo(a,e)pyrene, dibenzo(a,i)pyrene, dibenzo(a,h)pyrene, dibenzo(a,l)pyrene, indeno(1,2,3-cd)pyrene and 5-methylchrysene – as reasonably anticipated to be human carcinogens (IARC 1973, 1983, 1987). In mammalian cells PAHs undergo metabolic activation to diol epoxides that bind covalently to cellular macro-

molecules, including DNA, thereby causing errors in DNA replication and mutations that initiate the carcinogenic process (Janoszka *et al.* 2004).

Food contaminant and additive intake estimates are needed for food safety assessment, for the targeting of official food control and monitoring programmes. Different types of methods for estimating food contaminant intake have been used. The method should not be too expensive, because both the food analysis and consumption data are extremely costly. Membership of Estonia in the European Union has furthered these demands, because the estimation of intake is going to be compulsory. Information on intake is needed for setting maximum permitted residue levels (MRL) for contaminants. The annual food safety monitoring programme was initiated in 1998 by Estonian Ministry of Agriculture in collaboration with the laboratories of Health Protection Inspectorate to get information about the concentrations of food additives and contaminants in the food marketed in Estonia and to obtain data for the risk assessment (Reinik *et al*. 2001).

The purpose of the thesis was to 1) develop and introduce into routine practice efficient analytical methods for the determination of nitrates, nitrites, N-nitrosoamines and PAHs for the purposes of official food control and monitoring, 2) validate the methods for the analyses of most important food matrices, 3) obtain information about the contents of nitrates, nitrites, N-nitrosoamines and PAHs in food and drinking water and 4) estimate the exposure of abovementioned compounds through the consumption of food and drinking water by Estonian population.

2. LITERATURE REVIEW

2.1. Nitrates and nitrites in food and water

Nitrates and nitrites can be found in food as naturally occurring compounds, drinking water and vegetables being substantial sources of nitrate intake. An acceptable daily intake (ADI) of 0 to 3.7 mg/kg body weight for nitrate and of 0 to 0.06 mg/kg body weight for nitrite has been established by EU Scientific Committee for Food (EU Scientific Committee 1995).

It has been estimated that vegetables constitute a major source of human exposure to nitrates contributing approximately 40 to 92% of the average daily intake (Penttilä 1995, Dich *et al*. 1996, Ximenes *et al*. 2000, Eichholzer and Gutzwiller 2003). Nitrate concentrations in vegetables can vary from 1 to 10 000 mg/kg depending on biological properties of cultivars, light intensity, soil composition, air temperature, growth density, moisture, maturity of plant, duration of growth period, harvesting time, size of the vegetable, storage time, edible plant portion and nitrogen source (Walker 1990, WHO 1995, Fytianos and Zarogiannis 1999). Even among different samples of the same vegetable varieties, the range of concentrations can be manifold. De Martin and Restani (2003) showed that leafy green vegetables accumulate the highest amounts of nitrates, concentrations reaching up to 6000 mg/kg. The nitrite content of most fresh, frozen or canned vegetables is relatively low and usually of the order of 0–2 mg/kg (Siciliano *et al*. 1975, Corré and Breimer 1979).

The levels of nitrates in fruit are low compared with the vegetables, usually remaining under 10 mg/kg. Nitrite contents in fruit are found to be below 1 mg/kg (White 1976, Gajewska *et al.* 1989, Nabrzyski *et al*. 1994, Susin *et al.* 2006).

It has been estimated that the contribution of milk and dairy products to overall nitrate/nitrite ingestion is very low (Blüthgen *et al.* 1997, Amariglio and Imbert 1980, Nikolas *et al*. 1997, Luf 2002). Most milk samples contain nitrates in amounts not exceeding 2 mg/kg, while nitrites are present in trace amounts or were not detected at all (Przybylowski *et al*. 1989).

The nitrate concentrations in bread and miscellaneous cereals are in the range of <4–20 mg/kg, nitrite concentrations remain under 1 mg/kg (Nabrzyski *et al*. 1990, Belitz and Grosch 1999, Ysart *et al.* 1999). Fresh uncured meat products may contain nitrates up to 10 mg/kg and nitrites up to 1,7 mg/kg (Ysart *et al.* 1999).

In order to protect human health and taking into account the possible association of nitrates and nitrites in food with the formation of carcinogenic N-nitrosamines, the level of these compounds should be reduced to as low as reasonably achievable (ALARA principle). At present time, regulatory limits for nitrates in food have been established in the EU only for spinach, lettuce and

baby foods. The maximum level of nitrates in baby foods and processed cereal-based baby foods for infants and young children should not excced 200 mg/kg. The content of nitrates in spinach is limited to 2000–3000 mg/kg, lettuce 2500–4500 mg/kg and "iceberg" type lettuce 2000–2500 mg/kg (EC 2006). The regulatory limit depends on the harvesting season and place of growth of the vegetables – highest concentrations are permitted in plants grown in winter period and/or greenhouse conditions.

Nitrate and nitrite are used as food additives in the processing of meat products because of their antimicrobial action and their ability to give meat characteristic pink colour, texture and flavour. The colour change to pink is the result of conversion of myoglobin to nitric oxide myoglobin (nitrosomyo-globin), and occurs if a source of nitric oxide is provided. It is generally accepted that approximately 40 μg/g of added nitrite is sufficient to attain and maintain a stable cure colour but considerably more nitrite has been regarded to be necessary to inhibit the growth of *Clostridium botulinum* (Roberts and Dainty1996).

Cured meat products are the major source of nitrites and N-nitrosoamines in human dietary intake in many countries. Many papers report on the results of the analyses of the content of nitrates, nitrites and N-nitroso compounds in different types of meat products demonstrating a great variability in the concentrations (Hill 1996, Penttilä 1995, Sen and Baddoo 1997). The maximum permitted concentrations of residual nitrite (as $NaNO_2$) and nitrate (as $NaNO_3$) in commercial meat products established in Estonian legislation on food additives (VVM 2000) are 100 and 250 mg/kg, respectively. The legislation is harmonized with the EC Directive 95/2/EC.

Drinking water is regarded to be the second-largest source of nitrate in the diet after vegetables (Belitz and Grosch 1999, Fytianos and Zarogiannis 1999, Knobeloch *et al.* 2002, Caballero Mesa and Rubio Armendáriz 2003, WHO 2004). According to the results of several studies 20% of the total nitrate intake comes from the consumption of drinking water (White 1983). Nitrate and nitrite can occur in drinking water mainly as a result of intensive agricultural activities. Contamination of soil with nitrogen-containing fertilizers, including anhydrous ammonia as well as animal or human natural organic wastes can raise the concentration of nitrate in water. Nitrate-containing compounds present in the soil are generally soluble and readily migrate into groundwater. As nitrite is easily oxidized to nitrate, nitrite levels in water are usually low, and nitrate is the compound predominantly found in groundwater and surface waters. Water in highly polluted wells may also contain nitrites at elevated levels. To guarantee drinking water safety, maximum allowable concentrations have been established for nitrate and nitrite, being 50 mg/l and 0,5 mg/l, respectively (SoM, 2001).

Nitrate ion has a low level of acute toxicity, but if transformed into nitrite, it may constitute a health problem. Reduction to nitrite may take place in the

presence of bacteria or enzyme nitrate reductase, and in contact with metals. Nitrite is unstable at acidic pH values at which it can disproportionate to yield nitrate and nitrogen oxide and/or react with food components including amines, phenols and thiols (Hill 1996). It has been estimated that 5 to 8% of the nitrate from the diet may be reduced to nitrite by the microflora in the oral cavity (Mensinga *et al.* 2003). Although nitrates and nitrites have been used for centuries, it has only recently been discovered that nitrate is manufactured in mammals by the oxidation of nitric oxide and that the nitrate formed has the potential for disinfecting the food we eat (Benjamin 2000, Archer 2002). Consumption of vegetables containing high level of nitrates and incorrect storage of home-made vegetable purees has been found to be potential causes of infant methemoglobinemia (Sanchez-Echaniz and Benito-Fernández 2001).

Nitrite has higher acute toxicity than nitrate and ADI of 0.06 mg nitrite per kg of body weight (EU Scientific Committee for Food 1995). As an unstable ion nitrite undergoes series of reactions as soon as it is added to food. In acidic environment nitrite is converted into nitrous acid, which decomposes into nitric oxide. Nitric oxide, being an important product from the standpoint of colour fixation in cured meat, reacts with myoglobin to produce a red pigment – nitrosomyoglobin (Merino *et al*. 2000). The intake of nitrite is normally low compared with the dose that is acutely toxic, but nitrite in food is primarily considered to be a health problem because its presence both in food and body may lead to the formation of carcinogenic nitrosoamines (JECFA 1996, Vermeer *et al.* 1998) and the clinical symptom of methemoglobinemia (WHO 1995, Sanchez-Echaniz and Benito-Fernández 2001). Time, temperature, pH and additives have an important effect on the depletion of nitrite in cured meat (Merino *et al.* 2000). Processed infant foods may also contain increased levels of nitrites (Hill 1996).

Short-term exposure to drinking water with a nitrate level at or just above the health standard of 10 mg/l nitrate-N is a potential health problem primarily for infants. Infants and small children consume large quantities of water relative to their body weight, especially if water is used to mix powdered or concentrated milk formulae or juices. Also, the immature digestive system of infants is more vulnerable to the reduction of nitrate to nitrite. During many years, studies in different countries have reported thousands of cases of children with nitrate-nitric methaemoglobinemia and more than hundred children have died. Mild toxicoses were reported when the nitrate concentration was 80–100 mg/l in water used for infant food preparation (Spalding and Exner 1993).

2.2. N-nitrosamines in food and water

N-nitroso compounds can be divided into two categories: the class of nitrosoamines and the class of nitrosoamide-type compounds, including N-nitrosoureas, N-nitrosocarbamates and N-nitrosoguanidines. Compounds of both groups differ considerably in their chemical formation and biological effectiveness.

Although the carcinogenicity of N-nitrosoamines in humans cannot be tested, epidemiological studies have suggested a possible link to the incidence of various cancers in humans (Pegg and Shahidi 2000, Eichholzer and Gutzwiller 2003). It is possible that other factors such as the intake of vegetables, fruit and nitrosation inhibitors, or some component of cured meat and salted fish other than N-nitroso compounds could in part be responsible for the observed associations (Eichholzer and Gutzwiller 2003). Liu *et al.* (2006) showed in their recent work that consumption of cured meat or fish more than a few times a month, and vegetables rarely or occasionally, was significantly associated with increased risk of acute leukemia.

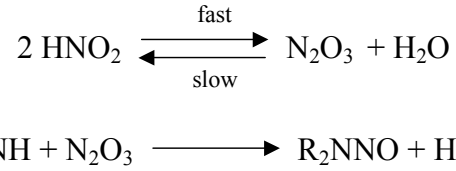


Figure 1. Formation of nitrosamines from secondary amines in food (Sen 1986)

Starting materials for nitrosamine formation in food are nitrate, nitrite, primary, secondary and tertiary amines, amides, proteins, peptides and amino acids, which are transformed into nitrosamine precursors by microbial action. NA are formed after cooking, the key step being the oxidation of nitrous oxide and the formation of higher nitrogen oxides, for example N_2O_3, which could act as direct nitrosating agents (Figure 1) (Sen 1986). The key representatives of N-nitrosoamines that are found in some thermally cured products include N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA). These compounds are known to be carcinogenic, mutagenic and teratogenic in experimental animals. Over 90% of more than 300 N-nitroso compounds tested on different animals have been shown to cause cancer (Tricker and Preussmann 1991).

Diets of different countries vary significantly in the content of N-nitrosoamines, many are low and others relatively high, depending on the types and consumed amounts of nitrosamine-containing food. NDMA has been detected in cured meats, especially in fried bacon, salted and smoked fish, cheese, pickled vegetables, beer and some dried products (Österdahl 1988, Penttilä

1995). The levels of N-nitrosamines, if present in cured meat are in μg/kg range, maximum concentrations reaching 10–90 μg/kg (Walker 1990, Pegg and Shahidi 2000, Zhukova et al. 1999, Lijinsky 1999).

Until recently, concerns about N-nitrosamines mainly focused on the presence of NDMA in food, consumer products, and polluted air. NDMA may also be found in drinking water as a contaminant resulting from reactions occurring during chlorination or via direct industrial contamination. Because of the relatively high concentrations of NDMA formed during wastewater chlorination, the reuse of municipal wastewater is a particularly important area of concern (Mitch 2003).

2.3. Polycyclic aromatic hydrocarbons in food and water

Diet is the primary source of PAHs contributing to more than 90% of total human exposure to PAHs (WHO 1998, SCF 2002). PAHs occur as contaminants in different food categories and beverages including drinking water (Chen 2004, King *et al.* 2004), vegetables, fruit, cereals, oils (Dennis *et al*.1991, Moret and Conte 2000, Moret and Conte 2002, Rojo Camargo and Toledo 2003, Tao *et al*. 2006, Voutsa and Samara 1998), fish (Šimko 1991, Lodovici *et al.* 1995, Moret and Conte 2000, Yurchenko and Mölder 2005, Karl and Leinemann 1996, Stołyhwo and Sikorski 2005) and meat (Kazerouni *et al*. 2001, Elhassaneen 2004, Šimko 2002, Mottier *et al.* 2000, Janoszka *et al.* 2004). The possible sources of PAHs in food are environmental contamination from the atmosphere, soil or water, contamination from packaging materials and thermal treatment which is used in the preparation and manufacturing of food (Guillen 1994). The levels of PAH found in unprocessed foods reflect the background contamination which originates from long distance airborne transportation of contaminated particles and natural emissions.

PAHs are lipophilic and have very low aqueous solubility so they accumulate in lipid tissue of plants and animals. PAH will not tend to accumulate in plant tissues with a high water content and limited transfer from the soil to root vegetables will occur. Heavier PAH tend to be found on particulate matter so atmospheric fall-out is a principal route of contamination. Consequently, vegetables with large leaves, grazing cattle and poultry which may injest particulate matter from soil are susceptible to contamination by PAH adsorbed to particles. In vegetables and fruits, the presence of PAHs originates mainly by deposition of air pollution particulates on their surfaces. The levels found are dependant on the location of the growing sites and on the product (Rojo Camargo and Toledo 2003). PAH concentrations are generally greater on plant surface than in internal tissue (Larsson 1986). Careful washing may remove up to 50% of the total PAH (SCF 2002).

Processing procedures, such as smoking and drying, and cooking of food are commonly thought to be the major sources of contamination of food by PAH. Formation of PAHs in meat products is affected by several factors: the methods used for preparation of food (grilling, frying, roasting etc.), temperature and time of cooking, distance from the heat source and drainage of fat (SCF 2002, Kazerouni *et al.* 2001). Several mechanisms of formation of PAH have been proposed, such as melted fat that undergoes pyrolysis when dripping onto the heat and pyrolysis of the meat due to the high temperature (Lijinsky 1991). Because PAH form on or near the surface of meats, foods cooked without being exposed to smoke do not show significant levels of PAH. The levels of PAHs in smoked foods depend on several variables in smoking process, including type of smoke generator, kind of wood, combustion temperature and degree of smoking (Moret *et al.* 1997). Lowering the temperature of smoke formation to 300–400°C coupled with the used of filters, the PAH content of smoke can be decreased about ten-fold (Sikorski 2005).

PAH levels are not high in raw cereals, contamination is due to aerial deposition which is in agreement with occurence of PAH in higher concentrations in bran than in flour (Dennis *et al.* 1991). Drying techniques used for cereals preservation can increase their PAH concentrations. Although PAH levels in cereals are often low, cereals have been found to account for around one third of the total exposure to PAHs in diet (Dennis *et al.* 1983, de Vos *et al.* 1990). The presence of PAHs in oils can be attributed both to the environmental contamination and to contamination during processing. Molluscs and fish accumulate light PAHs to a similar degree, heavy PAHs seem to accumulate more in molluscs. The ability of some seafoods to accumulate PAHs is why the concentration of PAHs in these organisms has been considered as an indicator of the contamination of their habitat (Guillen and Sopelana 2003).

PAHs containing up to four fused benzene rings are known as light PAHs and those containing more than four benzene rings are called heavy PAHs. Heavy PAHs are more stable and more toxic than light ones.

PAHs highlighted to be genotoxic and carcinogenic by the Scientific Committee on Food for which further investigation of the levels in food is required are the following: benz(a)anthracene, benzo(b)fluoranthene, benzo(j)fluoranthene, benzo(k)fluoranthene, benzo(g,h,i)perylene, benzo(a)-pyrene, chrysene, cyclopenta(c,d)pyrene, dibenz(a,h)anthracene, dibenzo(a,e)-pyrene, dibenzo(a,h)pyrene, dibenzo(a,i)pyrene, dibenzo(a,l)pyrene, indeno(1,2,3-cd)pyrene, 5-methylchrysene (European Commission 2005). JECFA has added 16th analyte to the list – 7H-benzo-(c)fluorene (JECFA 2005). In view of the non-threshold effects of genotoxic substances the levels of PAH in foods should be reduced to as low as reasonably acchievable. In order to protect health, maximum levels have been established for BaP in certain foods containing fats and oils and in foods where smoking or drying processes or

environmental pollution may cause high level of contamination. Lower maximum levels are valid for infant food. (European Commission 2006). Current EU legislation sets also maximum allowed concentrations for BaP amd benzo(a)anthracene in liquid smoke flavouring primary products of 10 μg/kg and 20 μg/kg, respectively (European Commission 2003).

Benzo(a)pyrene (BaP) is the most known and studied of the PAHs because it is one of the most potent animal carcinogens, its relatively easy to analyse and it is present in wide variety of food items. BaP is regarded to be a good marker for other PAHs in food items (Kazerouni *et al*. 2001).

Intake of PAHs has been estimated in some European countries: United Kingdom, Italy, The Netherlands and Austria, BaP intakes are available for Sweden, Germany and USA (SCF 2002). According to several studies the major dietary contributors are cereals, oils and vegetables although PAH levels in cereals are often low (Dennis *et al.* 1983, de Vos *et al.*1990, Tao *et al*. 2006). Grilled and barbequed meat contributed to the total mean daily BaP intake in USA significantly (21%) according to the data obtained by Kazerouni *et al*. (2001).

The levels of benzo(a)pyrene up to 1 μg/l have been detected in drinking water (IARC 1983). Atmospheric pollution contaminates the surface of open-air water supplies and the runoff from waste deposits may contaminate ground water. Specific maximum limits were set at the EU level for 5 PAHs in drinking water by Council Directive 98/83/EC. Maximum perimitted limit of 0,010 μg/l for benzo(a)pyrene and 0,10 μg/l for the summed content of benzo(b)fluoranthene, benzo(k)fluoranthene, indeno(1,2,3-cd)pyrene and benzo(ghi)perylene have been established (SoM 2001).

2.4. Methods of analysis

2.4.1. Nitrates and nitrites

A variety of analytical methods, including spectrophotometry, high performance liquid chromatography (HPLC), ion chromatography (IC), gas chromatography (GC), polarography and capillary electrophoresis (CE), for the determination of nitrate and nitrite in food have been developed. Using of HPLC methods has gained more popularity in last decades since they are more rapid than classic methods based on reduction process followed by colorimetry.

Many analytical methods for nitrite and nitrate determination in foodstuffs employ the same extraction procedure for both anions. In general the sample is extracted into hot water or sodium tetraborate solution and treated with protein precipitation reagents prior to filtration and measurement. Several nitrate extraction methods from plant material have been evaluated by Farrington *et al.*

(2006). The hot water extraction method described in European Standard EN 12014–2:1997 (CEN 1997) was found to give most reliable results. Extraction has to be performed under alkaline conditions, as nitrite may react with other matrice components if the extraction is carried out in even mildly acidic environment (Massey 1996).

Classic methods for the determination of nitrite are based on variations of the Griess diazotisation procedure, in which azo dye is produced in a reaction of diazonium salt with an aromatic amine or phenol (Kirk and Sawyer 1991). The most widely used colorimetric method is based on the reaction of nitrite, sulphanylamide and N-1-naphtylethylenediamine under acidic conditions to form a red azo dye. The red colour produced in the reaction is measured by a spectrophotometer at 538 nm. Nitrite present in the original sample is determined before nitrate reduction in cadmium column and nitrate content is obtained by the difference of the analysis of unreduced and reduced extract (Slack 1987, Zanardi *et al*. 2002, Ruiter and Bergwerff 2005). The use of cadmium columns to reduce nitrate to nitrite is widely applied in the analysis of cured meats (Oliveira *et al*. 2004), cheese and baby food (AOAC 2000), fruit and vegetables (CEN 1998, Petersen and Stoltze 1999). Detection limits for nitrate and nitrite by reduction methods are generally around 1 mg/kg (Dennis *et al*., 1990).

Flow-injection spectrophotometric detection for simultaneous analysis of nitrite and nitrate has been used both for food and water samples by Petersen and Stoltze (1999), Monser (2002), Andrade *et al*. (2003), Gal *et al*. (2004) and Ensafi *et al*. (2004). The injected sample is split into two streams. One of the streams is transported through a reductor microcolumn containing copperized cadmium, where nitrate is reduced to nitrite. The total nitrite concentration initially plus that produced in cadmium column is measured spectrophotometrically. A continuous flow method for the analysis of vegetables and vegetable products using spectrophotometric detection has also been adopted as a European standard EN 12014–7:1998 (CEN 1998).

Many attempts have been made to avoid using the toxic and carcinogenic metal cadmium. Other nitrate-reduction techniques include chemical treatment with vanadium(III)chloride for baby food samples (Casanova *et al*. 2006), microbial reduction with *E.coli* for analysis of plants (Cruz and Martins Loução 2002) and enzymatic reduction for the analysis of meat samples (CEN 2005a).

HPLC/UV methods have been reported for the nitrate and nitrite in vegetables (Cheng and Tsang 1998, Chou *et al*. 2003), dairy products (Reece and Hird 2000, Gapper *et al*. 2004), cured meats (Dennis *et al*. 1990) and beer (Massey *et al*. 1990).

Reverse-phase HPLC has been used both in the analysis of water and food (Frohlich 1987, Mullins 1987, Cheng and Tsang 1998), although ion-exchange HPLC is preferred in foodstuff analysis (Pentchuk *et al*. 1986, Dennis *et al*. 1990, Siu and Henshall 1998, Reece and Hird 2000, Stalikas *et al*. 2003, Gapper

et al. 2004). Normal phase ion-pair chromatography was used for water and vegetable samples by Butt *et al*. (2001). UV-detection is not suitable for multi-ion measurement due to the poor UV absorbance of chloride and phosphate. In such cases the analytes can be measured by indirect UV in which a UV-absorbing compound is included in the composition of mobile phase and anions owing a lower absorbance give a signal in the form of a negative peak on elution from the column (Mullins 1987). Alternatively, the conductivity detector may be applied (Pentchuk *et al*. 1986, De Martin and Restani 2003, Kissner and Koppenol 2005, Masson *et al*. 2005, McMullen *et al*. 2005, Dugo *et al*. 2007).

Various types of solid phase extraction cartridges have been tested to clean the sample extract prior to HPLC measurement: C18 SPE columns have been used by De Martin and Restani (2003) for analyses of leafy vegetables and by Vaessen and Schothorst (1999) for total diet samples, Dennis *et al*. (1990) have used cyclohexyl Bond Elut cartridges in the analysis of cured meats. Hunt and Seymour (1985) treated vegetable extracts with activated charcoal.

Three ion chromatographic methods, including two present European Standard methods EN 12014-2 (CEN 1997) and EN 12014-4 (CEN 2005b), were tested in a NMKL collaborative study (Merino *et al*. 2000) and compared to spectrophotometric method. In the analysis of nitrite, no statistically significant difference was found between the spectrophotometric method and EN 12014-4. It was concluded that the use of a strong anion exchange column is necessary to ensure the reliability of results. Nitrite determination with the weak anion exchanger column used in standard method EN 12014-2 for the analysis of vegetable samples, is not suitable for the determination of residual nitrate and nitrite in meat products. The detection limits for HPLC-based methods are typically between 0.1 and 10 mg/kg for foodstuffs (Eggers and Cattle 1986, Dennis *et al*. 1990, Vaessen and Schothorst 1999, Merino *et al*. 2000, Chou *et al*. 2003).

Gas chromatographic methods for the measurement of nitrate and nitrite in water and foodstuffs involve the formation of a volatile derivative, extraction into organic solvent and measurement by GC using a selective detector (Wu *et al*. 1984, Funazo *et al*. 1980, Ross and Hotchkiss 1985). In recent years capillary electrophoretic (CE) methods have been developed for the simultaneous detection of nitrite and nitrate in foodstuffs (Marshall and Trennery 1996, Öztekin *et al*. 2002). Nitrate-selective electrodes have found little application in the analysis of foodstuffs because of their potential positive interference from several commonly occuring anions (Pentchuk *et al*. 1986).

2.4.2. N-nitrosamines

US Environmental Protection Agency Method 521 has been developed recently for the analysis of NDMA and 6 additional *N*-nitrosamines in drinking water at ng/L concentrations (Munch and Bassett 2006). Standardized methods for the analysis of volatile N-nitrosamines in food are not available up to now.

The determination of volatile nitrosoamines in food and water samples has been carried out by different analytical methods, including gas chromatography with mass-selective detection (Jurchenko *et al*. 2002, Charrois *et al.* 2004) gas chromatography with thermal energy analyzer (Hamburg 1995, Domanska and Kowalski 2002, Byun *et al.* 2004, Andrade *et al.* 2005), GC with phtoionization detection (Meili *et al.* 2005), LC/MS/MS (Eerola *et al.* 1998), micellar electrokinetic chromatography (Sanches Filho *et al.* 2003), high performance liquid chromatography (Bellec *et al.* 1996, Komarova and Velikanov 2001, Perez-Ruiz *et al.* 2005) and high performance planar chromatography (Cardenes *et al.* 2002) and flow-injection spectrophotometric (Luque-Perez *et al.* 2001) methods. Electron impact ionization (Libbey and Scasnlan 1981) or positive-ion chemical ionization mass spectrometry with methane or ammonia as reagent gases has been used to differentiate between volatile nitrosamines (Prest and Hermann 1999, Jurtchenko *et al.* 2002, Charrois 2004). Chemical ionization (CI) techniques for the analysis of N-nitrosamines have proven to be more sensitive than EI mass spectrometry (Jurtchenko *et al.* 2002, Munch and Bassett 2006). The methods of sample preparation are based on supercritical fluid extraction (Fiddler and Pensabene 1996, Maxwell *et al.* 1993), liquid chromatographic cleaning (Meili *et al.* 2005), solid phase extraction (Raoul *et al.* 1997, Sanches Filho *et al.* 2003). In recent years, SPME method for the extraction of nitrosamines from food and water samples has been described by several authors (Andrade *et al.* 2005, Ventanas *et al.* 2006, Grebel *et al*. 2006).

2.4.3. Polycyclic aromatic hydrocarbons

Major problems associated with the determination of PAHs in food samples are the low analyte level and the diversity of potential interferences present. Despite the fact that BaP may be used as a marker of the occurence and the effect of the carcinogenic PAHs in food, the analytical method used in official food control and monitoring scheems should preferably cover the whole range of 15+1 EU-priority PAHs (Wenzl *et al.* 2006).

Following homogenization of the sample the widely used procedures for the extraction of PAHs are liquid–liquid partition, caffeine complexation and saponification (Moret and Conte 2000). The recovery of PAH by the method of solvent extraction can be high when samples soluble in the organic solvents are used for the extraction. In the case of meat and fish samples PAHs are known to

6

form covalent bonds with nucleic acids (SCF 2002). It has been shown that alkaline hydrolysis of samples previously extracted with boiling methanol increased the total recovery of PAHs from meat (Grimmer and Böhnke 1975). Saponification by alcoholic KOH followed by liquid-liquid extraction has been used by Pupin and Figueiredo Toledo 1996, Vásques Troche *et al.* 2000, Win *et al.* 1998, Rojo Camargo and Toledo 2003, supercritical fluid extraction by Järvenpää *et al.* 1996, Moret and Conte 2000, Lage Yusty and Daviña 2005. Solid phase extraction (Nazarkina *et al.* 2001, Mottier 2000, Guillén 1994, Chiu *et al.* 1997) and gel permeation chromatography (Cejpek *et al.* 1995, Martinez-López *et al.* 2005, Yurchenko and Mölder 2005) have been used for additional sample cleanup.

The determination of PAHs in food has been carried out by different analytical methods, including thin layer chromatography (Kazerouni *et al.* 2001, Moret and Conte 2000), high performance liquid chromatography with UV (Chen *et al.* 1996) or fluorescence detection (Šimko 1991, Cejpek *et al.* 1995, Chiu *et al.* 1997, Rojo Camargo and Toledo 2003, de Boer and Law 2003), gas chromatography with mass-selective detector (Mottier *et al.* 2000, Jira 2004), ion trap detector (ITD) (Chiu *et al.* 1997) or flame ionization detector (Grimmer and Böhnke 1975, Moret and Conte 2000). The only standardized method available is ISO 15753:2006 which describes two methods for the determination of 15 polycyclic aromatic hydrocarbons (PAHs) in animal and vegetable fats and oils: a general method, and a method specific for coconut oil and short-chain vegetable oils.

Three ISO methods for the analysis of PAHs in water samples are available: thin-layer chromatographical method ISO 7981–1:2005 and HPLC methods for the determination of 6 PAHs (ISO 7981–2:2005) and for the determination of 15 PAHs (ISO 17993–2002). Analyte enrichment is a prerequisite for the analysis of PAHs in water for which several techniques, such as liquid-liquid extraction, solid-phase extraction, solid-phase microextraction or stir-bar sorptive extraction have been used (Wenzl *et al.* 2006).

2.5. Assessing the consumer exposure to food chemicals

Risk assessments are required in order to establish whether the presence of any of food components (additives, contaminants, pesticides etc.) poses an unacceptable risk to the health of the consumer. Risk assessment process consists of the following steps: hazard identification, hazard characterization, exposure assessment and risk characterization (Ruut and Lilleväli 2004). Exposure assessement is the qualitative and/or quantitative evaluation of the likely intake of chemical agents via food as well as exposure from other sources if relevant. For the evalutaion of oral exposure, information on the amount of each food type consumed and the concentrations of chemicals present in food

and drinking water is required. In risk characterization step the exposure is compared with the reference dose.

There are a number of ways to assess food consumption, including food supply data, data from household consumption surveys, data from dietary surveys among individuals and the collection of duplicate diets. Biomarkers form a fifth type of exposure data, whereby these measures reflect both the consumption of food and the concentration of the chemical in consumed foods (Kroes *et al.* 2002). Each of these sources has advantages and limitations.

Trace levels of materials in foods often fall below the analytical limit of detection and are typically reported as "non-detects". Values of zero, half of the limit of detection, limit of detection, or some other derived distribution of values have been assigned to non-detects in statistical data analyses (CFSAN 2006).

For non-genotoxic food chemicals the estimated exposure is compared to Accepatble Daily Intake (ADI) value. ADI is defined as an estimated maximum amount of an agent, expressed on a body mass basis, to which a subject may be exposed daily over his lifetime without appreciable health risk. For the calculation of ADI value, 100-fold uncertainty factor is applied to the maximum non-effect dose determined in long-term animal studies in order to derive an acceptable intake for humans. Approaches to carcinogen risk assessment have differed between countries. The linearized mulitistage (LMS) model has been used to extrapolate from the dose-response data obtained in animal experiments to the "virtually safe" dose (VSD) expected to be associated with a "tolerated" risk of one excess cancer in one million people with lifetime exposure (Renwick *et al.* 2003, Ruut and Lilleväli 2004).

3. MATERIALS AND METHODS

3.1. Methods of analysis

The aim of the method development described in this part of the thesis was to introduce into practice analytical methods which are:

1) specific, accurate and reproducible
2) applicable for the analysis of different food matrices
3) meet the method performance characteristics required by the legislation
4) reasonably rapid and not too expensive

Analyses of the samples were carried out at Tartu Laboratory of Estonian Health Protection Inspectorate. All the methods described in this chapter are accredited by Estonian Accreditation Centre.

3.1.1. Nitrates and nitrites

Nitrites and nitrates concentrations in products of animal origin, infant vegetable purees and in ready-made food were determined by the HPLC method based on the NMKL (Nordic Committee on Food Analysis) method No. 165 (NMKL, 2000), the method described by Jackson *et al*. (1984) and standard EN 12014–2:1997 (CEN 1997).

Nitrates and nitrites were extracted from the homogenized samples by hot water. Two different ways have been used for the removal of interfering substances:

1) addition of acetonitrile, filtering through 0,45 μm membrane filter or
2) ultrasonication of the solution, centrifugation and filtering through 0,45 μm membrane filter, additional cleanup with C_{18} columns (Sep-Pak Vac RC 500 mg) for the removal of interfering compounds, another filtration if the solution is not clear.

The second way of sample treatment gave chromatograms containing less additional or disturbing peaks.

100 μl of the solution was injected to the Shimadzu LC10 chromatograph, nitrates and nitrites were separated by the Waters IC-Pac Anion HC column (4,6×150 mm) and detected by the UV-detector at the wavelength of 205 nm. Final determination of nitrates and nitrites has to be performed during the same day as sample preparation.

The method was validated on sausage, cheese and baby food matrices (Table 1). Using the described method, the laboratory has successfully participated in a collaborative study of the method (Merino *et al.* 2000) and intercomparison tests by FAPAS. The Z-values for the analysis of meat sample were –0,1 for nitrite and +0,6 for nitrate, respectively.

Nitrate concentrations in vegetables were determined by potentiometric method based on Russian standard GOST 4228-86 (Gosudarstvennõi agro-promõšlennõi komitet 1986). The advantages of the method are being cheap and rapid compared to HPLC method, which makes it possible to analyse large amounts of vegetable samples. Prior to the analysis, non-edible parts of the sample were removed. In case of small vegetables the whole sample was chopped. Large sample units (e.g. head cabbage) were cut vertically into four pieces. One quarter from each unit was taken for the analysis. Vegetable juice was prepared using a juice press. Leafy and low juice-containing vegetables were finely chopped. Nitrates were extracted by $KAl(SO_4)_2$ solution and determined potentiometrically by ion-selective electrode Volta-4000. The method has been validated on most frequently analysed matrices – potato, beetroot, carrot, cabbage and cucumber (Table 2). Results of intercomparison tests have been satisfactory, for the lettuce sample Z-scores 0,7 and 1,8 have been achieved in FAPAS scheme.

Table 1. Method performance characteristics in nitrite and nitrate HPLC analysis

Compound	Recovery, %	LOD, mg/kg	LOQ, mg/kg	Measurement uncertainty U (k=2,norm)
nitrite in animal and plant products	92–96	2	6	8%
nitrate in animal products	98–99	3	8	9%
nitrate in plant products	98–99	4	10	5%

Table 2. Method performance characteristics in potentiometric analysis of nitrates

Sample matrice	Recovery, %	LOQ, mg/kg	Measurement uncertainty U (k=2,norm)
potato	90	30	15%
beetroot	91	32	7%
carrot	102	32	6%
cabbage	99	33	15%
cucumber	102	35	8%

Nitrates and nitrites in drinking water

Nitrate content of drinking water samples was measured by ion chromatographic method based on the standard ISO 10304–1:1992. The method enables to determine simultaneously chloride, nitrate and sulfate ions. The samples are filtered and diluted with eluent before injecting 100 µl of the solution into the chromatograph. Anions are separated by Alltech AN-1 column (4,6×250 mm,

8 μm). Limit of quantification is 1,5 mg/l for nitrate ion. The measurement uncertainty at the limit of quantification is 20%.

Concentration of nitrite ions in drinking water was determined by colorimetry. The method is based on the standard ISO 6777:1984. Nitrite ions react with 4-aminobenzenesulfoneamide in the presence of ortophosphoric acid to yield diazonium salt. The latter reacts with N-(1-naphtyl)-1,2-diaminoethane, intensity of the formed pink colour is measured spectrophotometrically at 540 nm. The method enables to determine nitrite concentrations in the range of 0,002–0,2 mg/l. The analyses have to be performed as soon as possible after sampling (in 24 hours). Measurement uncertainty at the limit of quantification is 30%, in higher concentration region 5%.

The laboratory participates in intercomparison test for drinking water analyses twice a year. The results for nitrite and nitrate analysis are satisfactory.

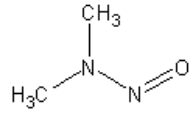
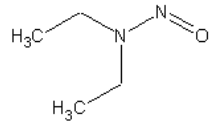
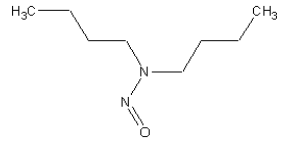
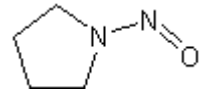
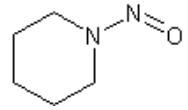
3.1.2. N-nitrosamines

The GC/MS method with positive ion chemical ionization was used for the quantification of 5 N-nitrosoamines: N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N- nitrosopiperidine (NPIP), N-nitrosopyrrolidine (NPYR), N-nitrosodibutylamine (NDBA).

Beforenamed analytes were chosen as NDMA, NDEA, NPYR and NPIP are carcinogenic compounds that may be formed in certain foods from naturally occuring amines present in food. The food amines can be nitrosated by added nitrites or nitrogen oxides (Figure 1). The latter may be formed by combustion of ambient nitrogen in air used for drying the food. NDBA can be found occasionally when food comes into contact with rubber containing dibutylamine derivates (Lijinksy 1999). The structures and toxicity data of analysed nitrosamines are presented in Table 3.

The method of analysis is based on the works by Raoul *et al.* (1997), Prest and Hermann (1999) and Spiegelhalder *et al.* 1983. During the method development stage different solid phase extraction sorbents for sample purification, SPE eluents, GC/MS ionization modes and reagent gases were tested (Jurchenko *et al.* 2002, 2006).

Table 3. Structure and toxicity of the analysed N-nitrosoamines (IARC 1978)

Compound	Structure	IARC classification	LD50 (rat), μg/kg
N-nitrosodimethylamine (NDMA)		2A, probable human carcinogen	40
N-nitrosodiethylamine (NDEA)		2A, probable human carcinogen	280
N-nitrosodibutylamine (NDBA)		2B, possible human carcinogen	1200
N-nitrosopyrrolidine (NPYR)		2B, possible human carcinogen	900
N- nitrosopiperidine (NPIP)		2B, possible human carcinogen	200

The method of sample preparation described by Raoul *et al.* (1997), with some modifications was used. 6 grams of the homogenized sample was taken to Erlenmeyer flask and 0,1 M NaOH solution was added for the inhibition of nitrosation reaction. Nitrosoamines were extracted from the sample by using 2-stage solid phase extraction by sorbents Extrelut NT and Florisil. 6 g of Extrelut was placed onto the bottom of the glass column and wetted with 40:60 dichloromethane/hexane mixture. The sample was eluted from the column twice with 20 ml of dichloromethane/hexane solution. Dichloromethane was evaporated at 60°C. The sample extract in hexane was eluted through the cartridge containing about 1 g of Florisil, the column was washed twice with dichloromethane/methanol 95:5 solution. The eluate was evaporated at 60°C to the volume of 1ml, the residue was transferred to the GC vial.

Two-step SPE with Extrelut (biogenic amorphous silica) and Florisil (activated magnesium silicate) gave the best recovery (Table 4). Satisfactory elution efficiency for all analysed nitrosamines was achieved using mixtures of solvents with different polarity. Polar nitrosamines NDMA and NPYR could not be eluted by nonpolar solvent, so dichloromethane addition to hexane enabled to recover all five analytes at acceptable rate.

Purified samples were injected to the HP GC 6890 gas chromatograph with MS-detector HP 5973 MSD and the analytical column 30 m HP-1701 MS. Ammonia was used as a reagent gas. For the GC separation of nitrosamines the column oven temperature was programmed from 35°C (stay 1 min), 50°C/min up to 240°C (isothermal 1 min). The velocity of He carrier gas was 1 ml/min. Five nitrosamines were separated during 6,5 min of run time (Figure 2). Nitrosamine molecules give mass spectra with $[M+NH_4]^+$ as the most intensive peak and adduct ions at $[M+H]^+$ and $[M+N_2H_7]^+$. Six standard solutions containing 5 nitrosamines covering the concentration range of 0,1–60 ng/ml were used for the calibration. The method has been validated for the analysis of smoked meat and fish products. Method performance characteristics are shown in Table 4.

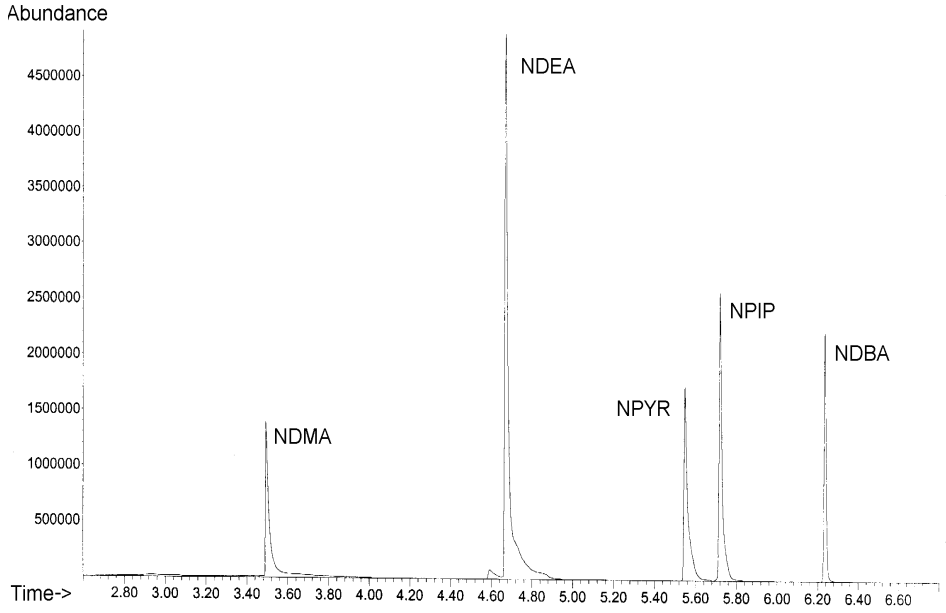


Figure 2. Total ion chromatogram of volatile N-nitrosamines

Table 4. Method performance characteristics in N-nitrosamine analysis

Compound	Recovery, %	LOD, mg/kg	LOQ, mg/kg	Measurement uncertainty U (k=2,norm)
NDMA	85	0,05	0,15	13%
NDEA	83	0,05	0,15	14%
NPYR	75	0,07	0,2	25%
NPIP	82	0,07	0,2	20%
NDBA	80	0,07	0,2	25%

3.1.3. Polycyclic aromatic hydrocarbons

Development of the GC/MS method started in 2004, when 5 PAHs were quantified, in 2005 the list composed already of 12 PAHs and from 2006 15 EU-priority PAHs were analysed (Table 5). Samples in which only benzo(a)pyrene content was measured were analysed by HPLC with fluorescence detection.

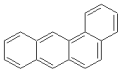
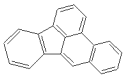
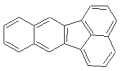
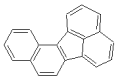
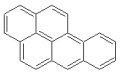
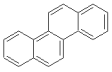
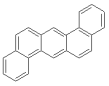
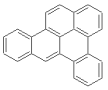
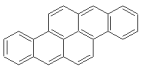
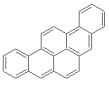
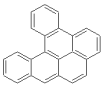
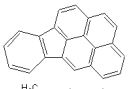
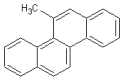
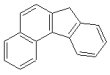
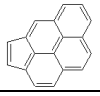
The method described by Ojaveer and Tanner (1996) with some modifications was used for the sample preparation. The sample was homogenized and hydrolyzed in concentrated hydrochloric acid overnight. Dichloromethane/hexane solution 25:75 was added. The solution was filtered through the layer of Na_2SO_4 and $NaHCO_3$ 1:1. The eluate was evaporated to dryness, ethyl acetate/cyclohexane 50:50 solution was added. Portion of the extract was injected into a gel chromatograph. The fraction containing PAHs was collected and evaporated to dryness. The residue was dissolved in methanol.

A Hewlett Packard 1100 HPLC equipment with Agilent 1100 fluorescence detector was used for benzo(a)pyrene analysis. Limit of quantification was 0,3 μg/kg, recovery 80% measurement uncertainty U 30% (k=2,norm.).

A Hewlett Packard Model 6890 gas chromatograph equipped with mass selective detector and chromatographic column HP-5 (30m x 0.25 mm i.d. silica capillary column with film thickness of 0.25 μm) was used for the analysis of PAHs. Temperature programme from 80° to 300°C was used. EI (electron impact) ionization was performed for the identification of 15 PAHs. Benzo(b)fluoranthene, benzo(k)fluoranthene and benzo(j)fluoranthene are determined as a sum of isomers (Figure 3). Analysis of 16th compound in EU priority list – cyclopenta(cd)pyrene – is problematic in the case of food matrice, satisfactory results have been achieved only for standard solutions and oil samples up to now.

Analytical method performance criteria in benzo(a)pyrene analysis have been established by a Regulation by European Commission (EC 2007) according to which LOD has to be at least as low as 0,3 μg/kg, LOQ 0,9 μg/kg and recovery 50–120%.

Table 5. Structure and toxicity of EU-priority PAHs (IARC 1987, EC 2005), analysed compounds at Tartu Laboratory of HPI in different years

PAHs	Structure	IARC classification*	2004	2005	2006
Benzo(a)anthracene (BaA)		2A	X	X	X
Benzo(b)fluoranthene (BbF)		2B	X	X	X
Benzo(k)fluoranthene (BkF)		2B	X	X	X
Benzo(j)fluoranthene (BjF)		2B	–	X	X
Benzo(g,h,i)perylene (BgP)		3	–	X	X
Benzo(a)pyrene (BaP)		2A	X	X	X
Chrysene (CHR)		3	–	X	X
Dibenzo(a,h)anthracene (DhA)		2A	–	X	X
Dibenzo(a,e)pyrene (DeP)		2B	–	X	X
Dibenzo(a,h)pyrene (DhP)		2B	–	–	X
Dibenzo(a,i)pyrene (DiP)		2B	–	–	X
Dibenzo(a,l)pyrene (DlP)		2B	–	X	X
Indeno(1,2,3-cd)pyrene (IcP)		2B	X	X	X
5-methylchrysene (5MC)		2B	–	X	X
7H-benzo-[c]-fluorene (BcL)		no data available	–	–	X
Cyclopenta(cd)pyrene (CPP)		3	–	–	–

* 2A – probably carcinogenic to humans; 2B – possibly carcinogenic to humans; 3 – not classified

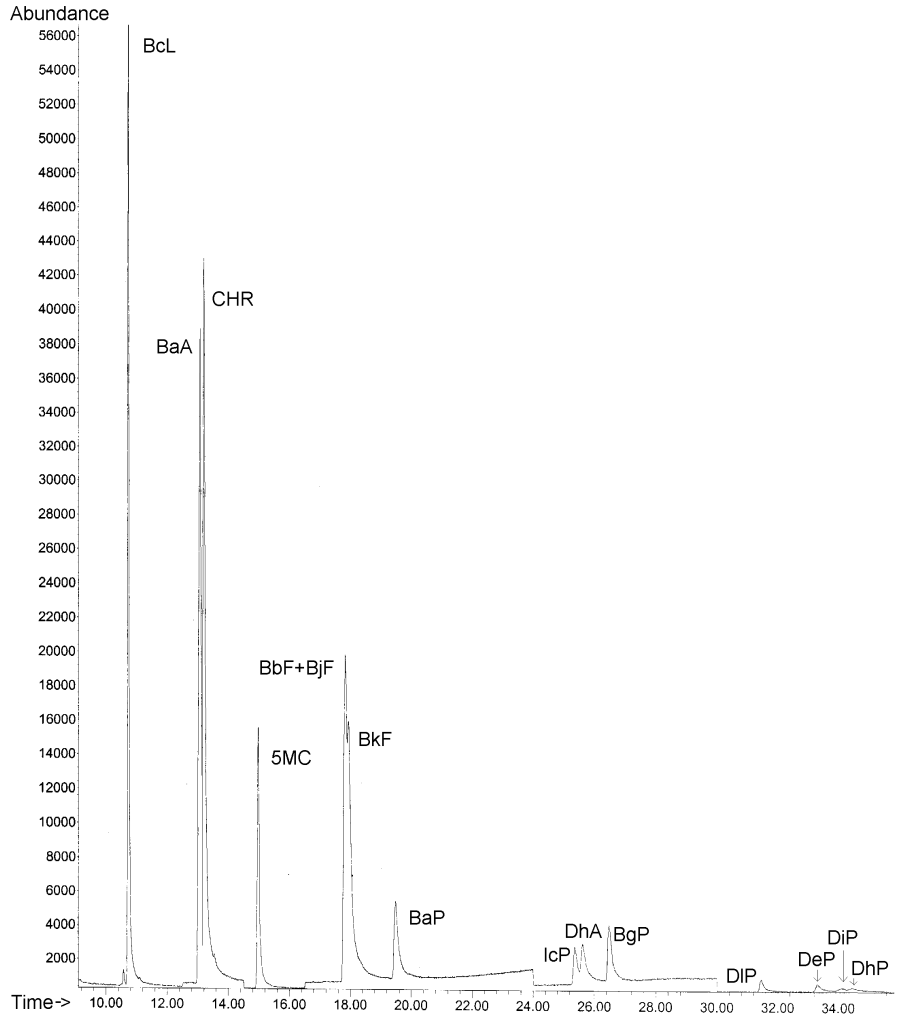


Figure 3. Total ion chromatogram of 15 analysed PAHs

The method has been validated for the analyses of most important matrices, such as meat and fish products and infant food (Tables 6 and 7). The laboratory has successfully participated in intercomaprison test. For FAPAS test round 0621, oil sample the Z-scores were between –1,5 and +1,5 (BaP, BaA, BbF, BgP, IcP). For benzo(a)pyrene the Z-score of –0,5 was achieved. 14 compounds were analysed in another FAPAS test, 13 results (BcL, CPP, CHR, BbF, BjF, BkF, BaP, IcP, BgP, DlP, DeP, DiP) were satisfactory, 1 overestimation (BaA, Z=3,4), for BaP Z=0,7.

For the quality control purposes a sprat paste reference material prepared by the Estonian Institute of Chemical Physics and Biophysics is used in each series

of analyses. The material contains BaA, CHR, BbF, BkF, BaP, IcP, DhA, BgP and several light PAHs which are not analysed by our method.

Table 6. GC/MS method performance characteristics, meat and fish products

PAHs	LOD, µg/kg	LOQ, µg/kg	Recovery, %	Measurement uncertainty U (k=2,norm.) at LOQ level, %
Benzo(a)anthracene	0,1–0,2	0,3–0,6	106–107	39
Σ Benzo(b),(k),(j) fluoranthene	0,2	0,6	93–97	35
Benzo(g,h,i)perylene	0,1–0,2	0,4–0,6	87–96	35
Benzo(a)pyrene	0,1	0,3–0,5	73–90	30
Chrysene	0,1–0,2	0,4–0,5	97–110	40
Dibenzo(a,h)anthracene	0,1–0,2	0,4–0,6	109–121	43
Dibenzo(a,e)pyrene	0,1–0,2	0,4–0,5	96–110	40
Dibenzo(a,h)pyrene	0,1	0,4	101	36
Dibenzo(a,i)pyrene	0,1	0,3–0,4	103–120	43
Dibenzo(a,l)pyrene	0,1	0,4	54–85	31
Indeno(1,2,3-cd)pyrene	0,2	0,5–0,6	92–102	37
5-methylchrysene	0,1–0,2	0,3–0,5	92–107	39
7H-benzo-[c]-fluorene	0,1–0,2	0,3–0,6	88	32

Table 7. GC/MS method performance characteristics, baby food

PAHs	Detection limit (LOD), µg/kg	Quantification limit (LOQ), µg/kg	Recovery, %	Measurement uncertainty U (k=2,norm.) at LOQ level, %
Benzo(a)anthracene	0,1	0,3	101	36
Σ Benzo(b),(k),(j) fluoranthene	0,1	0,3	86	31
Benzo(g,h,i)perylene	0,1	0,3	81	29
Benzo(a)pyrene	0,1	0,3	90	32
Chrysene	0,1	0,3	117	42
Dibenzo(a,h)anthracene	0,1	0,3	95	34
Dibenzo(a,e)pyrene	0,1	0,3	106	38
Dibenzo(a,h)pyrene	0,1	0,3	119	43
Dibenzo(a,i)pyrene	0,1	0,3	120	47
Dibenzo(a,l)pyrene	0,1	0,3	90	33
Indeno(1,2,3-cd)pyrene	0,1	0,3	82	29
5-methylchrysene	0,1	0,3	82	29
7H-benzo-[c]-fluorene	0,1	0,3	91	33

PAHs in drinking water

GC/MS method for the determination of the content of five PAHs – benzo(a)pyrene, indeno(1,2,3-cd)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene and benzo(g,h,i)perylene – in drinking water has been worked out on the basis of EPA method no. 8310. PAHs were concentrated on a Speedisk and eluted with ethyl acetate and dichloromethane. The solvent was evaporated in water bath in an airstream. The residue was dissolved in toluene and taken into chromatographic vial. 2 μl of the sample was injected to gas chromatograph. The temperature of the column oven was programmed from 50°C to 302°C. A 30-metre analytical column HP-5MS was used. PAHs were detected by mass-selective detector. A calibration curve was constructed in the range of 0,25–100 ng/ml corresponding to PAH concentrations 0,25–100 ng/l in water.

Limit of quantification was 0,001 μg/l for BaP, BfF, BkF and BgP, 0,005 μg/l for IcP. Measurement uncertainty U (k=2,norm.) at the limit of quantification was 10–30%. In intercomparison test by Aquacheck the Z-zcores between –2,13 and +0,05 were acchieved, which means that there may be a slight underestimation of PAH contents.

3.2. Samples

Vegetable samples

The results obtained from the analyses of 1468 vegetable and ready-made food samples in 2003 –2006 were used in intake calculations. Samples were collected mainly at the retail level by food inspection authorities within the frames of state official surveillance and monitoring programme, by researchers within activities of the Estonian Science Foundation grant no. 5416, or were brought to the laboratory by the farmers with the aim of self-control of their production. Approximately 80% of the analyzed vegetables had been grown in Estonia. The minimal amount of vegetable sample was 1 kg. Sample collection covered all seasons. In addition to raw vegetables, vegetable-based baby foods and ready-made soups were analyzed. 31 samples of canned infant food and 10 carrot juice samples were analyzed for obtaining data for the calculations of nitrates intake by infants and small children.

Meat products

Altogether 189 meat products of Estonian origin produced by 21 companies were analysed for the nitrate and nitrite content in the first stage of the work in 2000–2001. The second stage included analyses of 126 meat products from 16 companies during the years 2003–2004 (Reinik et al. 2005). Three major groups of cured meat products – cooked sausages, smoked sausages and ham were under investigation and the share of each product group in percentage from the

total sample number was 63%, 19% and 18%, respectively. Samples were taken on retail level.

Altogether 175 meat products in 2001–2004 were analysed for the N-nitrosoamines (NA) content. The results were used for the calculation of NA intake from meat products.

322 samples from the production of 34 Estonian meat manufacturers were analyzed in 2001–2005 for the benzo(a)pyrene content. Samples were collected from the main retail outlets of Tartu. In addition to BaP, concentrations of 11 PAHs, included in the SCF list for which information is required, in 22 industrial and 14 home-grilled meat products were determined in 2005.

Other samples

The number of samples analysed in the frames of official food control or monitoring programmes taken into account in intake estimation for the whole population is listed in Table 8.

Table 8. Samples taken into account for the whole population intake estimation

Food group	Number of samples			
	Nitrates (2003–2006)	Nitrites (2003–2006)	NA (2003–2005)	PAHs (2004–2006)
Vegetables	1468			
Ready-made vegetable-based food	19			
Infant food and food for young children	68	12		15
Cooked sausages	142	142	29	
Smoked sausages	50	50	29	42
Ham, smoked meat	30	30	24	29
Grilled meat			3	14
Preserved meat	30	30	15	
Fresh fish				11
Smoked fish				51
Salted fish			38	
Crustaceans, molluscs				9
Dried fruit				28
Seeds				4
Oil				18
Cheese	34	34	10	
Beer			33	
Dehydrated soups			4	
Pickled vegetables			5	
Drinking water	1815 (2001–2004)	1465 (2001–2004)		176

3.3. Methods of intake estimation

Different methods can be used for the estimation of additive and contaminant intake from food: food supply data, published tables of the mean consumption of dietary items, duplicate portion analysis, dietary survey among the individuals, probabilistic and worse case screening models. Depending on the method used, intake estimates can vary to a large extent (Kroes et al. 2002).

Four different approaches were used in this work to achieve the most representative intake values:

1) as children form the risk group and consumption habits differ a lot between families, for the evaluation of the exposure to contaminants an individual approach, i.e. calculation of intake on the basis of personal dietary records, was used;
2) exposure to nitrates by children was evaluated on the basis of kindergarten menus
3) exposure to nitrates by infants and young children was estimated on the basis of nutritional recommendations for infants;
4) exposure to food additives and contaminants by whole population was calculated using published tables of the mean consumption of food items.

Intake of nitrites, nitrates, N-nitrosamines and PAHs from meat products by children

The questionnaire concerning the consumption of cured meat products was composed by scientists of the Food Hygiene Department of Estonian University of Life Sciences and asked to fill in by the children's parents. The questionnaires were distributed at schools, kindergartens and family doctor centres. The main aim was to estimate the daily and weekly consumption of meat products by children. Data on the number and age of children in the family, consumption of meat products per child, preference of domestic and imported products and attitude towards food additives were included in the questionnaire. Questionnaires for the collection of the consumption data of cured meat products were filled in by parents daily during one week. Data from 346 children at the age of 1 to 16 years were collected in 2000–2003. For the statistical analysis of data the children were grouped by age. According to registered data the maximum and average amounts of consumed meat products in grams by different age groups of children were calculated

Intake of nitrates from vegetables by infants and young children

Intake of nitrates by children was estimated using the consumption data from kindergarten menus collected in year 2005 and nutrition recommendations for infants by Estonian peadiatricians. Consumed amounts of vegetables were reduced, taking into account the effect of peeling, cleaning and removing of

non-edible parts. Nitrate concentrations of the commodities were corrected for cooking loss.

Intake of nitrates, nitrites, N-nitrosamines and PAHs by whole population
Food consumption data from the household consumption survey made by the National Board of Statistics in Estonia were used for the calculation of average intake for the whole population. The tables obtained from the Board of Statistics included monthly average amounts of consumed dietary food items (grouped in detail) for the average person. The consumption data from the years 2001 – 2004 were used in the calculations.

4. SUMMARY OF THE RESULTS AND DISCUSSION

4.1. Concentrations of nitrates and nitrites in food and drinking water

4.1.1. Vegetables and vegetable-based infant foods

Limit values for nitrate concentration in several vegetables including potato, cabbage, carrot, beetroot, onion, cucumber etc. had been valid in Estonia before joining the EU in 2004. Due to the existing legal limits, a lot of samples were analyzed annually within the frames of official food control. Maximum permitted concentrations were exceeded in 17% of the samples, most frequently for cucumber in winter period, spring onion, cabbage, turnip and beetroot. At present time, regulatory limits for nitrates in food have been established in the EU only for spinach, lettuce and baby foods (European Commission 2006). Exceedings of EU permitted concentrations are rare – nitrate concentrations higher than MRL were detected in 4 lettuce samples grown of Estonian origin grown in greenhouse conditions in 2006.

The highest mean values of nitrates were detected in dill, spinach, lettuce and beetroot, the lowest average amounts of nitrates were detected in tomato, onion and potato (Table 9). As in Estonia potatoes are consumed in large amounts, the number of the studied samples is the largest. Statistical difference between nitrate content of early, late and stored potatoes was not observed. Average nitrate concentration in imported potatoes was 200 mg/kg, i.e. significantly higher than that of domestic tubers (Tamme *et al.* 2006). The nitrate concentrations detected in herbs and leafy vegetables have increased during the last years. The reason is probably that more and more green vegetables are grown during winter period in greenhouse conditions when there is lack of light. For example, the average nitrate concentration detected in 2006 in lettuce is 3176 compared to 2167 mg/kg in 2003–2005, in dill 5290 mg/kg compared to 2936 mg/kg in 2003–2005. According to the present study, the nitrate content of lettuce was lower in summer (average concentration 1952 mg/kg) than in winter (average 3024 mg/kg), which is in good agreement with other authors (Escobar-Gutierrez *et al.* 2002). Nitrate concentrations in the same range have also been detected in other countries (Chung *et al.* 2003, Dejonckheere *et al.* 1994, Petersen and Stoltze 1999, Belitz and Grosch 1999, Penttilä 1995). In vegetables of Estonian origin, the content of nitrites was lower than 5 mg/kg – the detection limit of the method.

Table 9. Nitrate contents of Estonian vegetables in 2003–2006

Commodity	No. of samples	Range of nitrate contents, mg/kg	Average nitrate content, mg/kg	MRL, mg/kg
Potato	449	<30–360	94	–
Carrot	202	<30–525	148	–
Cabbage	167	74–1138	437	–
Beetroot	130	214–3556	1446	–
Cucumber	136	<30–1236	160	–
Turnip	58	64–1062	307	–
Chinese cabbage	36	232–2236	1243	–
Tomato	25	<30–100	41	–
Onion	21	30–92	55	–
Spring onion	16	160–920	477	–
Lettuce	34	397–4795	2839	3500–4500
"Iceberg" type lettuce	6	520–1440	1023	2000–2500
Pumpkin	8	<30–445	174	–
Radish	6	670–1500	1309	–
Dill	6	2236–5290	3328	–
Celery	5	256–1113	675	–
Parsley	5	674–3470	1467	–
Spinach	4	340–2508	1456	2000–2500
Rhubarb	4	55–376	201	–
Strawberry	3	<30–111	55	–
Cauliflower	3	104–404	287	–
Zucchini	2	330–511	421	–
Watermelon	1	95	95	–
Fresh basil	1	4040	4040	–
Fennel	1	3060	3060	–
Kale	1	340	340	–
Carrot juice	10	76–251	136	–
Infant purees	38	20–208	77	200
Ready-made salads	14	80–474	259	–
Ready-made vegetable soups	5	588–740	639	–

38 samples of canned infant food and 10 carrot juice samples were analyzed for obtaining the data for intake calculations. Nitrate contents close to the EU limit concentration of 200 mg/kg were detected in two infant puree samples. Nitrate concentration was comparatively higher in the purees containing carrot and pumpkin: 62–148 mg/kg and 124–162 mg/kg, respectively. The highest nitrate content, 251 mg/kg, was detected in fresh carrot juice, which is frequently fed to babies. Similar results have been reported in a Spanish study (Hardisson *et al.*

1996), where nitrate concentrations exceeding the level of 250 mg/kg were found in baby foods in which the main ingredient was carrot. Nitrites were not detected in the samples of infant and ready-made food of Estonian origin.

During the last year, the nitrate concentrations in infant purees have decreased: the average concentration being 83 mg/kg in 2003–2005, but 52 mg/kg in 2006. The tendency may be the result of more thorough selection of raw material by the manufacturers.

4.1.2. Cured meat products and cheese

During the years 2000–2006 the range and average concentrations of $NaNO_2$ and $NaNO_3$ in the studied meat products were examined. The maximum permitted values for residual nitrites and nitrates were not exceeded. Great variations of added amounts of nitrites were found between different manufacturers in earlier stage of the study, in recent years the detected concentrations were more stable.

During the investigated period a tendency of decrease in the content of $NaNO_2$ and $NaNO_3$ has been observed (Table 10). The reason for the decrease in nitrite content is probably that according to the legislation on food additives nitrite can be added to food only in the form of "nitrite salt", i.e. in a mixture with salt, so large or occasional overdosages are practically avoided. Compared to smoked sausages and ham products the mean values of the nitrite concentrations in cooked sausages were higher.

Natural nitrate and nitrite content in cheese is low. In some countries, including Estonia, nitrates can be added as food additives to control the production of gas and undesirable flavours by bacteria in cheese manufacturing. Maximum permitted level for residual nitrate concentration in final product is 50 mg/kg.

34 cheese samples were analysed in 2003–2004. Nitrate concentrations were detected in the range of 10–67 mg/kg, concentration of nitrites remained below the limit of quantification (Table 10).

Table 10. Sodium nitrite and sodium nitrate concentrations in cured meat products and cheese, 2000–2006.

Product group	Year	No. of samples	$NaNO_2$ average concentration, mg/kg	$NaNO_3$ average concentration, mg/kg
Cooked sausage	2000–2001	116	35	59
	2003–2004	81	26	52
	2005	32	32	34
Smoked sausage	2000–2001	36	28	77
	2003–2004	29	16	46
	2006	15	17	46
Ham	2000–2001	44	22	63
	2003–2004	16	20	44
	2006	15	31	18
Salami	2004	3	10	85
Liver paste	2004	3	12	76
Cheese	2003–2004	34	<10	34

4.1.3. Drinking water

Hundreds of drinking water samples are analysed annually at Tartu Laboratory of HPI (Table 11). Most of the samples (ca 75%) are taken by the owners of the water supplies or sampling officer of the laboratory. About 25% of the samples are taken by the inspector of Health Protection Insepctorate in the frames of the official drinking water control program. Almost all the samples have been collected from 6 South-Estonian counties (Tartu, Jõgeva, Põlva, Valga, Viljandi, Võru). Violations of maximum permitted limit concentrations are not frequent: for nitrate the concentration of 50 mg/kg is exceeded in 4,1% of the samples, nitrite content over 0,5 mg/l was detected in 0,96% of the samples.

Table 11. Nitrates and nitrites in drinking water

Year	Nitrate		Nitrite	
	Number of samples	NO_3^- concentration, mg/kg	Number of samples	NO_2^- concentration, mg/kg
2001	790	8,1	655	0,034
2002	580	8,8	408	0,038
2003	191	15	180	0,027
2004	254	7,7	222	0,019
Altogether	1815		1465	
Average		9,0		0,032

4.2. Concentrations of N-nitrosamines in food

In Estonia the maximum permitted values for the content of the sum of NDMA and NDEA in meat products, broth cubes and gelatine (2μg/kg), in fish and fish products and beer (3μg/kg) and in smoked meat products (4 μg/kg) were valid before joining the EU in 2004. Concentration of N-nitrosamines in food is not regulated by the legislation in EU up to now.

Concentrations of NDMA and NDEA in meat products during the years 2001–2004 were examined in the paper by Reinik et al. (2005). The legal limits of NDMA and NDEA were exceeded in 9% of the investigated 175 samples. The average content of nitrosoamines decreased during the years in all investigated groups of meat products namely, for smoked sausages the decrease was from Σ (NDMA, NDEA) 2,9 μg/kg in 2001 to 1,7 μg/kg in 2004 and for cooked sausages from 1,9 μg/kg in 2001 to 0,4 μg/kg in 2004.

The formation of significant amounts of NA in the fish is probably caused by the interaction of nitrites and amines in the fish. The maximum permitted limit concentration of 3 μg/kg was not exceeded in analysed fish samples, although maximum concentrations reached very close to this value. The levels of NA in hot-smoked fish were found to be higher than in cold-smoked fish.

High contents of NPYR (up to 25 μg/kg) and NPIP (up to 7,8 μg/kg) have been detected in some grilled or smoked meat and fish samples (Table 12). The amount of NPYR, formed through decarboxylation of nitrosoproline or some other mechanism, depends on the duration and temperature of frying – much more NPYR is formed at higher temperatures. NPIP almost certainly arises from nitrosation of piperine and other piperidine derivatives in the spices used to flavour meat and fish products (Lijinsky 1999).

Probably one of the reasons for the decreasing of nitrite, nitrate and N-nitrosoamine concentrations in meat products may be related to a positive influence of the systematic food additives and contaminants control programme started in the year 1998. Increase in the knowledge led to public discussions and presentations resulting in manufacturers' higher awareness of the problem.

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Table 12. Results of N-nitrosamine analyses in 2003–2005

Product group	Number of samples	Concentration range of NA, μg/kg					Average conc. of Σ(NDMA +NDEA), μg/kg
		NDMA	NDEA	NPIP	NPYR	NDBA	
Cooked sausages	29	<0,15–1,1	<0,15–0,2	<0,2–6,0	0,7–6,6	<0,2–3,3	0,41
Smoked sausage	29	<0,15–3,3	<0,15–0,5	<0,2–3,8	0,2–16,6	<0,2–3,5	1,70
Smoked meat, ham	19	<0,15–3,1	<0,15–1,5	<0,2–4,5	0,2–19,5	<0,2–4,5	1,69
Canned meat	15	0,5–1,8	<0,15–0,3	0,4–2,1	0,8–5,4	<0,2–1,8	1,50
Grilled meat	3	0,2–3,2	<0,07–0,3	1,0–2,8	0,8–4,9	<0,1–0,3	2,10
Smoked chicken	5	1,2–2,1	<0,07–0,3	0,1–5,3	0,5–22,1	0,1–6,3	1,60
Salted fish	38	0,5–2,1	<0,15–1,4	<0,2–3,9	<0,2–8,5	<0,2–3,9	1,24
Smoked fish	16	<0,15–2,8	<0,15–0,5	<0,2–7,8	0,4–25,4	<0,2–6,0	1,45
Cheese	10	<0,2–0,9	<0,15–0,4	<0,2–0,7	0,3–1,1	<0,2–0,6	0,80
Beer	33	<0,15–0,7	<0,15	<0,2	<0,2	<0,2	0,30
Dehydrated vegetable soups	4	<0,15	<0,15	<0,20	<0,20	<0,20	<0,20
Pickled vegetables	5	<0,15	<0,15	<0,20	<0,20	<0,20	<0,20

4.3. Concentrations of polycyclic aromatic hydrocarbons in food and drinking water

PAHs always occur in food as complex mixtures. The profiles of PAHs found in different matrices vary to some extent, however some certain PAHs like BaP, BaA, CHR and sum of BbF, BkF and BjF are found more frequently (Table 13). Benzo(a)pyrene (BaP) is most studied compound of the PAHs and often used as a marker of PAH levels in environmental samples (Kazerouni *et al*., 2001).
The highest concentrations of PAHs have been detected in smoked and grilled food (Tables 14 and 15, Figure 4). Concentrations of PAHs in other analysed product groups were lower – only in one olive oil sample the maximum permitted limit value of BaP – 2 μg/kg was exceeded.

There are several factors influencing the final PAH content of the products in smoking and grilling process: timber variety, smoke generator type, smoke temperature, duration of smoking or grilling, availability of oxygen, oven dimensions etc. Taking into account beforenamed factors, it is a great challenge for the manufacturers to achieve significant reduction of PAH contents and at the same time retain the characteristic taste and colour of the product.

Table 13. Frequency of detecting PAHs, % or number of analysed 151 samples

Compound	Frequency of detection the PAHs on the basis of the data from 2005–2006, n=151
Benzo(a)anthracene	55%
Σ Benzo(b),(k),(j) fluoranthene	47%
Benzo(g,h,i)perylene	35%
Benzo(a)pyrene	46%
Chrysene	47%
Dibenzo(a,h)anthracene	8,6%
Dibenzo(a,e)pyrene	3 samples
Dibenzo(a,h)pyrene	not detected
Dibenzo(a,i)pyrene	not detected
Dibenzo(a,l)pyrene	4 samples
Indeno(1,2,3-cd)pyrene	23%
5-methylchrysene	4 samples
7H-benzo-[c]-fluorene	30 samples of 44 analysed samples
PAHs not quantified	44%

4.3.1. Fish products

During the years 2001–2006 147 smoked fish product samples have been analysed for BaP content (Table 14). PAH concentrations have been determined in 62 samples of fish products in 2004–2006 (Table 15). The parameters influencing final BaP content in smoked fish or fish preserves were studied in collaboration with two largest Estonian smoked fish producers.

Table 14. BaP concentrations in main analysed food groups in 2001–2006.

Product group	No. of samples	No. of samples in BaP concentration range, µg/kg				Maximum BaP content, µg/kg	Average BaP content, µg/kg
		< 0,3	0,3–1,0	1,1–5,0	> 5		
Smoked fish	107	74	24	6	3	9,7	0,5
Sprats	40	6	12	16	6	13,2	2,4
Ham	140	86	35	9	10	31	1,4
Smoked sausage	132	91	28	9	4	20	0,8
Smoked poultry	27	14	6	6	1	15	1,4
Cooked sausage	16	15	1	0	0	1	0,2
Cheese	7	5	2	0	0	0,5	0,2
Oil	44	24	18	2	0	5	0,5

Table 15. PAHs in analysed food and drinking water samples, 2004–2006

Product group	Number of samples	Maximum BaP content, µg/kg	Mean BaP content, µg/kg	Maximum ΣPAH content, µg/kg	Mean ΣPAH content, µg/kg	MRL, µg/kg
Smoked meat, ham	26	31	1,6	127	11,9	BaP 5,0
Smoked sausage	42	2	0,5	19	5,0	BaP 5,0
Grilled meat	14	1,8	0,9	22	13	–
Smoked poultry	3	0,4	0,3	3,2	1,9	BaP 5,0
Smoked fish	43	9,7	0,80	85,4	7,06	BaP 5,0
Sprats	8	13,2	5,4	118	57,6	BaP 5,0
Fish	11	<0,6	0,2	2,4	1	BaP 2,0
Crustaceans, shrimps	8	<0,3	0,2	1,3	1	BaP 5,0
Bivalve molluscs	1	0,4	0,4	6,7	6,7	BaP 10,0
Dried fruit	28	0,6	0,3	19	1,5	–
Dried seeds	4	2,5	0,7	10	2,9	–
Infant and young children foods	15	<0,3	0,15	2,4	1	BaP 1,0
Edible oil	18	1,1	0,4	14,2	5,7	BaP 2,0
Drinking water	179	0,006	0,00028	0,041	0,0027	BaP 0,01 PAHs 0,1

The highest concentrations of PAHs have been detected in smoked sprat preserves in which average content was 58 µg/kg and maximum reaching up to 118 µg/kg. Germany has established guideline maximum value of 20 µg/kg for "heavy PAHs". According to our results and also the analyses carried out by German laboratories, in most sprat preserves this reference value was exceeded.

Maximum BaP concentration in sprats was found to be 13,2 µg/kg. Exceeding of tolerance limit of 5 µg/kg was detected in the case of 15% of the samples. The reason for such high PAH accumulation is that small smoked fish are used for the preparation of the preserve. As the relative surface area of fish is large, high amounts of PAHs can concentrate on the surface layer of fish during the smoking process. The fish in sprat preserve are eaten with skin, tails and also part of the oil fraction of the preserve, which is especially rich in PAHs. Content of PAHs in final products depends significantly on production technology: benzo(a)pyrene content can be decreased with shortening of smoking time and intensity, frequent changing and cleaning of smoke filters. As PAHs are fat-soluble compounds, they concentrate in oil fraction of sprat preserve – the concentration of BaP in the oil fraction was found to be 5–6 times higher than in homogenated preserve. The concentration of PAHs in the preserve varies significantly between different manufacturers, i.e. different technologies and smoking regimes but also within-lot variations may be 2-fold depending on the position of the fish in smoking oven.

The PAH concentrations in muscle meat of smoked fish are lower compared to sprats. One of the reasons is that skin of the fish is removed, i.e. only edible part of the sample is taken for the analysis. Anyway, in some cases concentrations may rise up to 85 μg/kg of total PAHs. Concentrations of BaP in muscle meat of smoked fish vary to large extent. In 69% of analysed samples, BaP content remains below the limit of quantification. At the same time maximum concentrations rises up to 9,7 μg/kg, exceeding of the maximum permitted limit value was detected in 2,8% of the samples. Raw fish contain low levels of PAHs originating from environmental contamination of water (Table 15).

BaP concentration ratio to overall PAHs is quite constant value – it comprises 9,6% of total analysed PAHs in smoked fish products.

4.3.2. Commercial meat products

During the years 2001–2006 concentrations of BaP in 315 samples of commercial meat products were determined (Table 14). In 64% of the analyzed products BaP concentrations remained below quantification limit. The permitted maximum limit concentration for BaP – 5μg/kg – was exceeded in 11 samples of smoked meat products. Highest contents were found in home-made ham, smoked meat, smoked chicken and smoked sausage samples with values 30, 31, 15 and 20 μg/kg, respectively.

The results are in good agreement with those obtained by the other authors. In 2002 Šimko found concentrations of BaP in the range of 0.03–100 μg/kg in smoked meat products. The highest concentrations were detected in smoked sausages and smoked ham. In Poland 0,11–3,93 μg/kg of BaP was detected in cooked meat products (Janoszka *et al*. 2004).

Concentration of total PAHs reached to 127 μg/kg for smoked meat sample, average amount of PAHs was also highest in smoked meat and ham – 11,9 μg/kg. PAH levels in most analysed samples remain remarkably lower. Our results show higher PAH contents than detected in the study by Phillips (1999) where the concentration range of total PAHs in smoked meat was between 2.6–29.8 μg/kg and concentration of carcinogenic PAHs reached to levels of 16 μg/kg. The difference may be caused because of the fact that some very heavily smoked samples were taken from Tartu market for the analyses in addition to ordinary commercial production. Detailed discussion on PAHs content in commercial meat products is presented in the paper by Reinik *et al.* (2007).

Reports of previous works (Šimko, 2002) indicate that BaP concentrations in meat products are between 1% and 20% of the total carcinogenic PAHs. According to our results BaP comprises 6.1 % of the sum of the total PAHs at the average for commercial smoked meat products (Reinik *et al.* 2007).

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4.3.3. Home-grilled meat products

In present study two different widespread grilling methods were used to compare PAH contents in home-grilled meat products. In traditional wood burning grill the distance between coals and meat was approximately 20 cm and the temperature at the surface of the meat between 180–240 °C. In recent years disposable charcoal grills are frequently used for their convenience and shorter preparation time. Compared to traditional grilling the heat source is much closer to the grilled product in that case. 14 samples of meat products were grilled using both methods and analyzed for the content of 12 PAHs.

Using of disposable grill resulted in higher (maximum for 1,6 times) concentrations of PAHs compared to traditional grilling (Table 16). Total amount of PAHs in grilled pork was higher than in chicken or sausages. This difference occurs probably due to variance in fat content. When fatty meat is grilled, fat drips onto the coals and due to high temperature PAHs are formed. In consequence, smoke carries the PAHs onto the surface of meat products. Lower concentrations of PAHs in sausages can be explained by shorter grilling time. Home-prepared meat products, especially those, which were prepared using disposable grill, contained higher concentrations of BaP and PAHs compared to commercial products. The mean concentration of BaP in industrial and disposable-grill meat was 0,7 µg/kg and 1,0 µg/kg, respectively. The average levels of PAHs in home-grilled meat were higher compared to industrial smoked products, mean contents being correspondingly 12,5 µg/kg and 7,5µg/kg. Details of this experiment are published in a paper by Reinik *et al.* (2007).

In charcoal-broiled beefburgers Elhassaneen (2004) determined 11 PAHs. The range of PAHs was between 0.31–15 µg/kg. BaP concentrations were detected in the range of 0,99–4,8 µg/kg. Contents of sixteen PAHs in 7 different barbequed meat sausages were determined in Swiss study (Mottier *et al.* 2000), BaP contents were between "not detected" and 2,81 µg/kg The highest levels of PAHs reported in meat were detected in food cooked over open flames by Panalaks (1976) – in barbecued meat total PAHs were found to be present at levels up to 164 µg/kg with BaP being present at levels as high as 30 µg/kg.

Table 16. PAH concentrations in home-grilled meat products

Type of grill	Grilled pork		Grilled sausage		Grilled chicken	
	mean BaP, µg/kg	mean Σ12PAHs, µg/kg	mean BaP, µg/kg	mean Σ12PAHs, µg/kg	mean BaP, µg/kg	mean Σ12PAHs, µg/kg
wood burning grill	0,8	13	0,7	10	0,4	8,6
disposable grill	1,4	20	0,5	11	0,9	13

The results for sum of PAHs difficult to compare with the studies published by other authors as the list of the analytes differs to large extent and several non-carcinogenic PAHs have been included in earlier works.

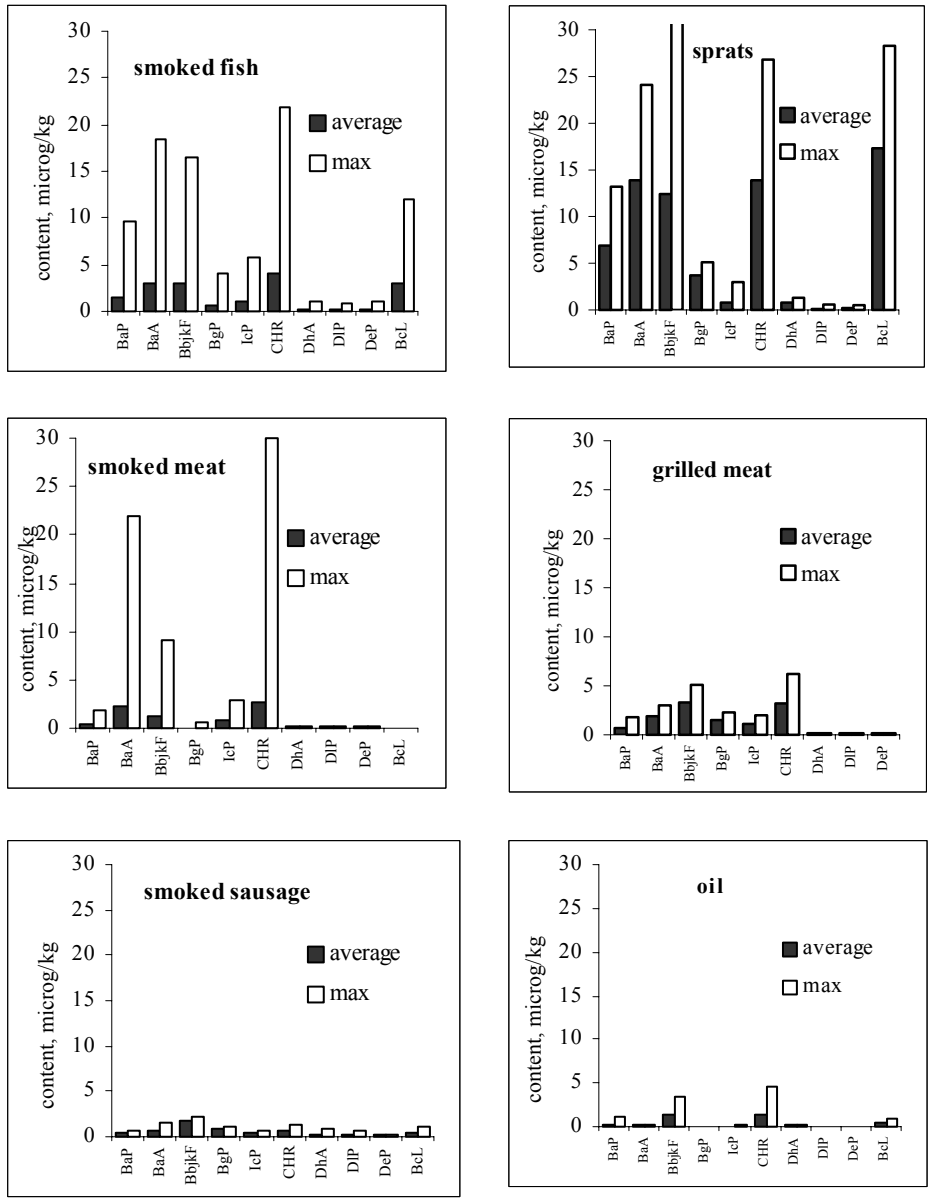

Figure 4. PAH profiles in the main analysed matrices

4.3.4. Drinking water

176 drinking water samples have been analysed for 5 PAHs, including benzo(a)pyrene in 2004–2006. PAHs were detected in 42 samples, in 12 of them PAHs were present in the levels below the limit of quantification. Average detected concentrations of BaP and PAHs were 0,28 ng/l and 2,7 ng/l, maximum contents 6 ng/l and 41 ng/l, respectively. Benzo(a)pyrene was detected in 15 samples, benzo(b)fluoranthene in 31 samples, benzo(k)fluoranthene in 20 samples, benzo(ghi)perylene in 17 samples, and indeno(1,2,3-cd)pyrene in 16 samples. Maximum permitted limit concentrations were not exceeded in any of the analysed drinking water samples.

4.4. Intake of nitrates, nitrites and N-nitrosamines

4.4.1. Nitrate intake from vegetables

Intake of nitrates has been evaluated, using the data from three different consumption surveys in Estonia:

1) data for the whole population – household consumption survey comprised by the National Board of Statistics
2) the consumption data for children – obtained in 2005 from the menu of two kindergartens (n=335).
3) the consumption data for infants and small children – calculated on the basis of the infant menu recommended by Estonian paediatricians.

Details of nitrate intake calculations are presented in the paper by Tamme *et al.* 2006.

The average content of nitrates in each vegetable commodity or vegetable based food has been used in all calculations of the intake. The average body weight used for comparison of the results with ADI (Acceptable Daily Intake) was 60 kg.

The highest intakes of nitrates by Estonian population were obtained from consuming cabbage and potato: 14 and 12 mg/day, respectively followed by cucumber and beetroot. The mean intake of nitrates from vegetables by Estonian population was 58 mg/day, which made up 26% of ADI (Table 17).

Mean daily intake of nitrates by Estonian 1- to 6-year-old children was calculated on the basis of the consumption survey from the menus and amounts of supplied vegetables of two kindergartens. The intake of nitrates by 4- to 6- years-old children was 40% of ADI, the average body weight of 20 kg was used in the calculations. The menu of the age group of 1- to 3-years-old was similar to 4- to 6-years-old children group with the difference of 15% less consumed food amount. According to the calculations, the exposure to nitrates

by this age group was 46% of ADI. For the calculation of intake, the average body weight of 15 kg was used. The obtained results indicated that although the mean nitrate intakes did not exceed ADI, the maximum intakes for the children consuming large amounts of nitrate-rich vegetables could be several times higher.

The mean daily intake of nitrate among Estonian infants aged 6 to 12 months was calculated on the basis of the recommended infant menu. The menu of the age group of 6 to 12 months should contain 50% of vegetable based foods and 50% of fruit based foods in addition to breast-feeding. An infant's average daily intake of nitrates from consumption of vegetable-based infant foods was 7.8 mg (22% of ADI).

Table 17. Exposure to nitrates through the consumption of vegetables and vegetable-based food

Age group	Consumption of vegetable-based food per day	Mean nitrate intake, mg/day	Mean nitrate intake, mg/day/kg body weight	% of ADI
Infants (6–12 months)	50–120	4,4–11	0,56–1,03	15–27
Children (1–3 years)	198	26	1,7	46
Children (4–6 years)	226	30	1,5	40
Whole population	376	58	0,97	26

4.4.2. Intake through the consumption of meat products

According to the data obtained from the questionnaires about the food consumption, the maximum and average amounts of consumed cured meat products in grams by different age groups of children were calculated. In calculations individual approach was applied: using the mean concentrations of $NaNO_2$, $NaNO_3$ and N-nitrosoamines in cured meat products the intake of these compounds by each questionned child was found. According to the answers of questionnaires, most of the children consumed sausage or ham on 2–5 days per week, at the same time there were plenty of those (17%) who did not eat cured meat products at all.

The mean nitrite and nitrate intake was calculated using data from two different periods, namely 2000–2001 and 2003–2004 (Reinik *et al.* 2005).

The mean intake of nitrite compared to ADI rose up to 140% in 2000–2001. Although a positive decrease took place during the study period, the mean values in 2003–2004 still remained considerably high, being 63–105% from the ADI value in the age group 1–12 years. Later on, there has been no further decrease in nitrite content, the concentrations have raised again to approximately 90% of 2000–2001 level (Table 18). On the basis of the results it may be stated that the main risk group is young children. In the 2000–2001 study period

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ADI was exceeded for 40% of the children and in 2003–2004 for 29%. The values of nitrite intake were considerably lower in the cases where the calculations were based on the average consumption of meat products per seven days, taking into account all the children, even those who did not eat meat products during the questionned period. The mean nitrite intake by children was 0,83 (46% of ADI when calculated on body weight of 30 kg), the range of intake being 0,37–1,4 mg/day (20–78% of ADI).

Daily intake of nitrates was calculated to be from 0.75 up to 2.7 mg with the mean value of 1.7 mg/day. The mean nitrate intake was lower than 5% from the ADI for all age groups, the maximum exposure remaining less than 10% of the ADI value.

The daily intake of NDMA+NDEA by children from the consumption of meat products was found to be in the range of 0,032–0,12 μg with the mean value 0,073 μg/day (Reinik *et al*. 2005).

The average nitrite, nitrate and N-nitrosamine concentrations in meat products were used in all intake calculations. Real intake by children who consumed mainly products with high nitrite contents may rise up to 3 times as high as calculated.

Table 18. Mean nitrite and nitrate intakes through the consumption of cured meat products

Age group	Average nitrite intake, % of ADI, only children who concumed cured meat		Average nitrite intake, % of ADI, all children		Average nitrate intake, % of ADI
	2000–2001	2003–2004	2000–2001	2003–2004	2003–2004
1–3	140	105	57	43	4,3
4–6	138	103	68	51	4,3
7–9	97	73	50	38	3,7
10–12	92	63	37	28	3,6
13–16	77	58	34	25	3,0

4.4.3. Intake from all dietary sources

For the calculation of nitrate, nitrite and N-nitrosamine intakes from all sources the food consumption tables from Board of Statistics and experimental average additive/contaminant concentrations have been used. In the case where results of analysis for certain food group (cereal products, dairy products except cheese, fruit, nitrites in vegetables, oil) were not available, the data from the literature were used (Walters 1996, Belitz-Grosch 1999, Ysart *et al.* 1999, Petersen *et al.* 1999).

It can be seen from the Table 19 that intake of nitrates, nitrites by the whole Estonian population from all sources does not exceed ADI values, being 35 and 61% of the ADI, respectively. As maximum concentrations of nitrates and

nitrites in each product group are at least 2–3 times higher than average concentration values and maximum consumption may exceed the average even up to 10 times in the case of some products, exceeding of ADI values certainly occurs among the consumers of high amounts of food rich in nitrates or nitrites.

Our intake values – 84,7 mg nitrate and 2,38 mg nitrite per day are in good agreement with the evaluations carried out in some European countries and USA. The mean total intake of nitrate per person in Europe has been estimated to be between 50–140 mg/day and in the USA about 40–100 mg /day (Mensinga *et al.* 2003; Ysart *et al.* 1999). In similar studies carried out in Finland lower mean exposure to nitrites and nitrates, respectively 1,9 and 55 mg/day, was detected (Penttilä 1995). Lower nitrite and nitrate intakes are probably caused due to the fact that some product groups like cereals have not been taken into account.

Nitrosamine intake has been evaluated only in few studies. Exposure to NDMA+NDEA is 0,083 μg/day according to our calculations. NDMA intake obtained in Finnish study agrees well with our data – the intake was found to be 0.08 μg /day (Penttilä 1995). In a study by Lijnski (1999) it was reported that the average intake of nitrosoamines from food in different countries is of the magnitude of 1 μg/day, being highest for the people consuming large amounts of cured meats and beer.

The main contributors to the nitrate intake are vegetables (78%) and drinking water (11%). Meat products and cereal-based foods give the highest impact to nitrite intake with 42% and 28%, respectively (Table 20).

There are great differences in estimating the share of different product groups in overall intake of nitrates and nitrites. According to different authors meat products supplied from "not significant" up to 98% of dietary nitrites, vegetables and vegetable products provided 40–98% of nitrate intake (WHO 2003, Penttilä 1995, Dich *et al*. 1996, Ximenes *et al*. 2000, Eichholzer and Gutzwiller 2003, Wawrzyniak *et al.* 1999, 2003, Murata *et al.* 2001, 2002). The main reasons for such variances are large differences in dietary habits and the food groups taken into account.

Table 19. Intake of nitrates, nitrites and N-nitrosamines from all dietary sources

	Nitrates, mg/day	Nitrites, mg/day	N-nitrosamines, μg/day
Intake	84,7	2,38	0,08223
Intake per kg of body weight	1,30	0,0366	0,00126
% of ADI	35%	61%	not applicable

Table 20. Share of the different product groups in overall nitrate, nitrite and N-nitrosamine intake

Product group	% of nitrates intake	% of nitrites intake	% of N-nitrosamines intake
Cereal products	3,0%	28%	No concentration data
Meat products	3,6%	42%	61%
Fish products	0,4%	0,9%	13%
Vegetables and fruits	78%	15%	2,3%
Dairy products	3,3%	11%	12%
Oils	0,3%	0,9%	No concentration data
Other foods, including beer	0,4%	0,7%	12%
Drinking water	11%	1,3%	No concentration data

4.5. Intake of polycyclic aromatic hydrocarbons

4.5.1. Intake through the consumption of meat products

As smoking and grilling are predominant meat preparation ways in Estonia, the impact of meat products is supposed to be essential in overall PAH intake.

Using the data obtained in the food consumption questionnaires, the intake obtained from the consumption of cooked sausages, smoked sausages, ham and grilled meat products by each questioned child was found. The mean BaP intake by children was found to be 14 ng/day, maximum reaching to 140 ng/day. Intake per kg of body weight is higher in younger age groups (1–9 years) (Table 21). The average intake of PAHs from meat products was found to be 192 ng/day, maximum intake values reach up to 1575 ng/day. Both for BaP and PAH the highest intake is obtained from smoked meat products followed by cooked sausages and grilled products (Reinik *et al.* 2007).

The average consumption of grilled, smoked and other cured meat products is 65 g/day by Estonian whole population, which results in an intake of 29 ng BaP/day. As food consumption database reflects only average consumption, the real intake values for people who eat meat products frequently and in remarkable quantities are much higher. For example, when 100 g of smoked meat product is consumed per day, the real daily dose of BaP is in the magnitude of 100 ng/day while the same amount of cooked sausage gives only 15 ng of BaP per day.

Consuming 26 g of smoked and grilled meat products per day as average in Estonia, 234 ng of PAHs are digested. The person who eats 100 g of ham products or the same amount of grilled meat, obtains the dose of PAHs is 800 ng and 1600 ng/day, respectively.

The detailed information on PAHs intake from the consumption of meat products is presented in the paper by Reinik *et al.* (2007).

Table 21. Mean BaP and PAH intakes through the consumption of meat products

Age group	Daily intake of BaP, ng/day per kg of body weight		Daily intake of PAH, ng/day per kg of body weight	
	average	maximum	average	maximum
1–3	0,47	4,7	7,0	53
4–6	0,55	3,5	8,2	43
7–9	0,47	5,0	6,5	56
10–12	0,40	3,5	5,1	41
13–16	0,39	1,4	5,0	17
Total population	0,45	–	5,3	–

4.5.2. Intake from all dietary sources

Food consumption data from the Board of Statistics and results of the analyses of food and drinking water samples were used in the calculation of average intake for the whole population. For the food groups for which local data on PAH content were not available (cereal products, fruit and vegetables, dairy products, beer) the concentrations cited in the literature were used (SCF 2002, SCOOP report 2004, Guillén and Sopelana 2003). The intake values are presented in Table 22. The mean intake values obtained in this study were lower compared to the estimates by other countries, however the results showed that for high consumers intake may exceed average value up to 10 times.

In USA and some European countries the estimated BaP intake from food is reported to be between 0,1–1,6 μg/day (Guillen and Sopelana 2003), according to surveys conducted in six EU countries the mean intake of BaP for an adult person was estimated in the range 0,05–0,29 μg/day (SCF 2002), the total 19 PAH intake was estimated to be 8,8 μg/day in Finland (SCOOP report 2004). Dietary intake of BaP by schoolchildren and toddlers estimated in the UK survey of COT (2002) was in the range 1.4–3.8 ng/kg bw day. Similarly to our work the younger age groups were found to be the highest exposed with an intake of both BaP and PAH per kg of body weight about 2,4-fold higher than for adults (SCF 2002).

Large differences in estimated exposure values occur probably due to the variables among different countries such as the food groups considered, differences in analytical methodologies, ways of expression of data below LOQ and different dietary habits.

According to our data, the main contributors to the PAHs intake are meat products covering 36%, cereal products 23% and vegetables and fruits 20% of the total intake (Table 23). The estimated share of different product groups in overall exposure varies a lot between different authors. For example, the contribution of meat products to the overall intake of PAHs is evaluated to be from very low for UK to 21% in USA and 27% in France resulting in the second

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contributing food group after bread and cereals (SCF 2002, SCOOP report 2004).

Table 22. Intake of BaP and PAH from all dietary sources

	BaP, ng/day	PAH, ng/day
Intake	158	1583
Intake per kg of body weight	2,44	24,3

Table 23. Share of the different product groups in overall BaP and PAH intake

Product group	% of BaP intake	% of PAH intake
Cereal products	16%	23%
Meat products	25%	36%
Fish products	4,4%	3,5%
Vegetables and fruits	36%	20%
Dairy products	6,0%	0,5%
Oils	11%	15%
Other foods, including beer	1,4%	1,0%
Drinking water	0,2%	0,2%

5. CONCLUSIONS

The following analytical methods introduced at Tartu Laboratory of Health Protection Inspectorate have been described in the thesis:

1) determination of nitrates and nitrites in food, HPLC method
2) determination of 5 volatile N-nitrosamines in food, GC/MS method
3) determination of benzo(a)pyrene in food, HPLC method
4) determination of 15 PAHs in food, GC/MS method

In addition to beforenamed methods, potentiometric technique was used for the analysis of nitrates in vegetables. Nitrates were determined by ion chromatography, nitrites by spectrophotometry and PAHS by GC/MS method in drinking water samples.

The aim of the thesis was achieved – the introduced methods are sufficiently specific, accurate, reproducible, rugged, rapid and cost-effective and therefore well applicable for routine food and water analysis.

All the methods have been validated and if possible tested in inter-comparison tests. The methods have been granted an accreditation by Estonian Accreditation Centre.

The developed methods were applied for the analysis of official food control and monitoring samples. An overview of the obtained results and comparison with legal limits is given.

According to the EU legislation on food contaminants, exceedings of the permitted limits for nitrate content in infant food were not detected. Nitrate concentrations in vegetables vary significantly depending on vegetable variety, growth period and conditions, tolerance limits were exceeded in some lettuce samples. The nitrate levels detected in Estonian vegetables remain usually lower than in imported products. The possible reason for low levels of nitrates in vegetables and infant foods is probably related to relatively low use of fertilizers in Estonia. Concentrations of nitrates in vegetable-based baby foods have decreased during the last years probably due to more thorough selection of raw material by the producers.

According to the Estonian legislation on food additives, exceedings of the permitted limits of nitrites and nitrates in meat products were not detected. Amounts of residual nitrites in cured meats differed to a large extent between manufacturers in earlier years of the study. Up to now the levels have become more stable, as nitrite can be added to meat products in the ready-made mixture with salt. In connection with using nitrite salt, the concentrations of nitrites have also decreased to some extent.

Levels of nitrites and N-nitrosoamines have decreased from year to year, the possible reason for that being lower amounts of added nitrites and an increase in manufacturers' awareness of the problem. There are no maximum permitted limits established in EU for N-nitrosamine content in food. Taking into account the carcinogenicity of these compounds, the levels in food should be retained as

low as possible. High concentrations of NPYR and NPIP were detected in some fish and spiced meat samples.

The highest concentrations of PAHs have been detected in smoked and grilled food. BaP concentrations exceeding legal limit were detected in 5% of the samples, mainly in sprats and smoked ham. The permitted level was exceeded up to 6 times. Concentrations of 12–15 PAHs were determined in commercial products and home-grilled meat. Traditional home-grilling, especially when disposable grilling unit was used, resulted in higher PAH contents than in commercial meat products. Usually BaP comprises 6–10% of the sum of PAHs, the amounts of summed PAHs reach as high as 120 μg/kg.

The obtained analytical results were used for exposure assessment in dietary intake calculations.

Dietary intake of food contaminants depends on both the nutritional habits of the examined populations groups and the concentrations of contaminants in food. As children form the risk group, their exposure to contaminants was evaluated apart from the whole population.

Average intake of nitrites by whole population was found to be 61% of ADI, for nitrates 35% of ADI. The main contributors to nitrite intake are cured meat products and cereal products. Nitrate intake is obtained predominantly from the consumption of vegetables. The results showed that cabbage and potato were the main sources of nitrate intake. For children, the intake values compared to ADI were much higher – ADI value for nitrites was exceeded at least for 29% 1–6-year –old children only from the consumption of cured meat products; 1–3 year children obtained nitrates from the vegetables in the amount of 46% of ADI. Intake of nitrosamines through food was 0,08 μg per day.

The obtained values indicated that although the mean nitrate and nitrite food intakes did not exceed ADI, the maximum intakes were several times higher. Considering the possibility of nitrates' transformation to nitrite and carcinogenic N-nitrosoamines, the importance of information on daily intake by children is obvious.

Mean BaP intake by whole population from all sources was 158 ng/day and for PAH – 1583 ng/day. The main contributors to the intake were meat products, cereal products and vegetables and fruits.

The mean BaP intake for children from the consumption of meat products was found to be 14 ng/day, maximum intake being more 10 times higher. The PAH intake per kg of body weight was highest among age group of 1–9 years – the mean dose exceeding for 1,5 times, maximum for 10-fold the average intake of the whole population.

Considering the toxicity of PAHs and high consumption of grilled and smoked meat products in Estonia, the importance of the data on the contents of carcinogenic PAHs in food and information on daily intake by children and whole population is clearly indicated.

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18

SUMMARY IN ESTONIAN

Nitraadid, nitritid, N-nitroosamiinid ja polütsüklilised aromaatsed süsivesinikud toidus: analüütilised meetodid, sisaldused ning toidu kaudu saadavate dooside arvutus

Huvi nitritite ja nitraatide sisalduse suhtes toidus ning joogivees on esile kerkinud seoses nende potentsiaalse ohuga inimese tervisele. Nitraat ja nitrit ise ei oma kõrget toksilisust, kuid nii toidus kui ka organismisiseselt võivad neist tekkida kantserogeensed N-nitroosamiinid. Nitraadirikka toidu ja joogivee tarbimine võib imikutel esile kutsuda methemoglobineemiat. Polütsüklilised aromaatsed süsivesinikud (PAH) on osutunud kantserogeenseteks loomkatsetes, mitmed ühendid on tunnistatud võimalikeks inimkantserogeenideks. Tulenevalt nimetatud ainete potentsiaalsest ohust inimese tervisele, on oluline nende ühendite sisaldust toidus ja joogivees kontrollida ning hinnata nende tarbimisega seotud riske.

Töö eesmärgiks oli välja töötada riikliku toidujärelevalve ja –monitooringu raames teostatavate lisa- ja saasteainete uuringute läbiviimiseks sobivad analüüsimeetodid. Rutiinanalüüsil kasutatavad meetodid peavad vastama seadusandluses esitatud nõuetele, seega olema piisavalt täpsed ja usaldusväärsed. Olulist rolli omavad ka tulemuse saamise kiirus ning analüüsi maksumus.

Käesoleva töö raames juurutati Tervisekaitseinspektsiooni Tartu laboris järgmised analüüsimeetodid:

- nitritite ja nitraatide sisalduse määramine toidus, HPLC meetod
- lenduvate N-nitroosamiinide sisalduse määramine toidus, GC/MS meetod
- benso(a)püreeni sisalduse määramine toidus, HPLC meetod
- polütsükliliste aromaatsete süsivesinke (PAH) sisalduse määramine toidus, GC/MS meetod, 15 ühendit

Lisaks mainitud meetoditele kasutati töös potentsiomeetrilist meetodit taimsetes toiduproovides nitraatide sisalduse määramiseks. Joogivee analüüsil kasutati nitraatide määramiseks ioonkromatograafiat, nitritite analüüsil spektrofotomeetrilist meetodit ning PAH sisalduse määramiseks GC/MS meetodit.

Kõik kasutatud meetodid valideeriti olulisemate uuritavate proovimaatriksite abil, võimalusel on osaletud võrdluskatsetes. Kõik nimetatud meetodid on akrediteeritud, seega heaks kiidetud Eesti Akrediteerimiskeskuse poolt.

Töö eesmärk täideti – juurutatud meetodid on piisavalt spetsiifilised, täpsed, reprodutseeritavad, robustsed ja kiired ning seega edukalt kasutatavad vee- ja toiduproovide analüüsil.

Juurutatud meetodeid kasutati toiduohutuse järelevalve ja monitooringuprogrammide raames võetud toiduproovide analüüsiks. Töös on ära toodud saadud tulemused ning nende võrdlus kehtivate maksimaalsete lubatud piirkontsentratsioonidega.

Euroopa Liidus kehtivate nitraatide piirnormide ületamist ei avastatud imiku- ja väikelastetoitudes, samas leiti ülenormatiivseid nitraatide sisaldusi mõningatest aedsalatiproovidest. Saadud andmetest on näha, et aedviljade nitraadisisaldus sõltub oluliselt nii aedvilja liigist kui ka kasvuperioodist ja –tingimustest. Eestis kasvatatud aedviljade nitraadisisaldus jääb tavaliselt madalamaks võrreldes sarnaste importtoodetega. Põhjuseks on ilmselt väetiste tagasihoidlikum kasutamine. Imikutoitude nitraadisisaldused on viimastel aastatel langenud tõenäoliselt tänu tootjatepoolsele hoolikamale toorainevalikule.

Nitriteid ja nitraate kasutatakse lisaainetena lihatoodetes nende värvi ja säilivuse parandamiseks, samuti botulismibakteri arengu pidurdamiseks. Ülenormatiivseid nitritite ja nitraatide sisaldusi uuritud toodetest ei leitud. Lisatud nitritite hulk varieerus oluliselt sõltuvalt tootjast varasematel uuringute läbiviimise aastatel. Viimasel ajal on nitritite sisaldus stabiliseerunud, kuna lubatud on nitritite lisamine ainult nitritsoolana. Samal põhjusel on ilmselt mõningal määral vähenenud nii nitritite kui ka N-nitroosamiinide sisaldused uuritud toodetes.

Euroopa Liidus puuduvad maksimaalsed lubatud piirsisaldused N-nitroosamiinidele toidus. Arvesse võttes nende potentsiaalset kantserogeensust inimesele, tuleb nende sisaldus tarbitavas toidus hoida nii madalal kui võimalik. N-nitroosamiine leitakse eelkõige suitsutatud liha- ja kalatoodetes, soolakalas ning õlles. Kõrgeid N-nitrosopürrolidiini ja N-nitrosopiperidiini sisaldusi detekteeriti mõnedes kala- ja vürtsitatud lihatoodetes.

Kõrgeimad PAH-de sisaldused leiti suitsutatud ja grillitud toitudes. Benso(a)püreenile kehtestatud piirnorm oli ületatud 5% proovides, peamiselt sprottides ja suitsusingis. Töö käigus võrreldi erineval viisil grillitud lihatoodete PAH-de sisaldust – ühekordsel grillil grillitud liha sisaldas PAH-e kõrgemas kontsentratsioonis kui traditsioonilisel grillil valmistatu. Tavaliselt moodustab benso(a)püreeni sisaldus 6–10% 15 PAH summaarsest sisaldusest. Maksimaalsed PAH-de summaarsed sisaldused ulatuvad 120 μg/kg-ni.

Katsetulemusi kasutati nitraatide, nitritite, N-nitroosamiinide ja PAH-de toidust saadavate koguste hindamiseks. Ekspositsioon lisa- ja saasteainetele sõltub nii ainete sisaldusest toidus kui ka toitumisharjumustest. Kuna lapsed kujutavad endast riskigruppi, hinnati nende poolt saadavaid lisa- ja saasteainete doose eraldi.

Keskmine toidust saadav nitritite doos kogu elanikkonnal moodustas 61%, nitraatide doos 35% ADI-arvust (soovitatav maksimaalne päevane tarbitav lisaaine piirkogus kehakaalu kg kohta). Suurema panuse nitritite ekspositsiooni andsid nitititega töödeldud lihatooted ning teraviljatooted. Nitraate saadakse suures osas aedviljadest, kusjuures Eestis on peamisteks nitraadiallikateks kapsas ja kartul. Laste poolt saadavad nitritite kogused on märksa suuremad – vorstitoodete tarbimisest tingituna esineb ADI-arvu ületamist vähemalt 29% 1-6-aastastel lastel. 1–3-aastased lapsed saavad nitraate aedviljapõhistest toitu-

dest 46% ADI-arvust. Toidust saadav N-nitroosamiinide doos on 0,08 µg päevas.

Arvutatud ekspositsiooni väärtused näitavad, et keskmised toidust saadavad nitraatide ja nitritite doosid ei ületa ADI-arvu, kuid suures koguses nitraadi- ja nitritirohkeid toite tarbides on toidust saadud nende ainete kogused mitmekordsed. Arvestades nitraatide ja nitritite N-nitroosamiinideks muundumise võimalust, on andmete kogumine eriti laste poolt saadavate dooside kohta oluline.

Keskmine toidu kaudu saadud benso(a)püreeni doos on 158 ng päevas, PAH-de summaarne kogus 1583 ng päevas. Põhilised PAH-de allikad on lihatooted, teraviljatooted, aed- ja puuviljad. Keskmine laste poolt lihatoodete tarbimisest saadud benso(a)püreeni kogus on 14 ng päevas, maksimumdoosid ulatuvad 10 korda kõrgemale. PAH-de doosid on kõrgeimad vanusegrupis 1–9 aastat.

Arvesse võttes PAH-de toksilisust ja kõrget grillitud ning suitsutatud lihatoodete tarbimist Eestis, on elanikkonna ning eelkõige laste poolt toidust saadavate PAH koguste kindlakstegemine olulise tähtsusega.

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PUBLICATIONS

I

Jurtchenko S., Tenno T., Mölder U. and **Reinik M.** (2002)
Determination of volatile *N*-nitrosamines by gas chromatography–mass spectrometry with positive-ion chemical ionization.
Proceedings of the Estonian Academy of Sciences **51**, 169–184.

II

Reinik M., Tamme T., Roasto M., Juhkam K., Jurtsenko S., Tenno T. and Kiis A. (2005) Nitrites, nitrates and N-nitrosoamines in Estonian cured meat products: intake by Estonian children and adolescents. *Food Additives and Contaminants* **22**(11), 1098–1105.

III

Tamme T., **Reinik M**., Roasto M., Juhkam K., Tenno T. and Kiis A. (2006)
Nitrates and nitrites in vegetables and vegetable-based products and
their intakes by the Estonian population.
Food Additives and Contaminants **23**(4), 355–361.

IV

Reinik M., Tamme T., Roasto M., Juhkam K., Tenno T. and Kiis A. (2007) Polycyclic aromatic hydrocarbons (PAHs) in meat products and estimated PAH intake by children and the general population in Estonia. *Food Additives and Contaminants* **24**(4), 429–437.

V

Reinik M., Tamme T. and Roasto M. Naturally occuring nitrates and nitrites in food, In: *Bioactive Substances in Foods – Natural toxicants and heat processing contaminantss*, (Gilbert J. and Senyuva H. eds), Blackwell Publishers, London, UK. In press.

10. NATURALLY-OCCURING NITRATES AND NITRITES IN FOODS

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CONTENTS

10.1. INTRODUCTION

Nitrates and nitrites can be found in food as naturally occurring compounds. An interest in the dietary intakes of nitrates and nitrites has arisen mainly from the concern about their possible adverse effect on health. The natural occurrence of nitrates in plants is a consequence of the nitrogen cycle whereby mineral nitrogen is assimilated by the plant as nitrates to use them in the synthesis of

plant proteins. Nitrates and nitrites are also used as food additives in cured meats due to their ability to protect products from *Clostridium botulinum* and other *Clostridium* species, and for their red colour fixing properties. Nitrates and nitrites are found in drinking water due to both natural occurrence and contamination of water supplies, mostly from agricultural sources and municipal wastewater.

The concern over nitrates and nitrites in the diet has two aspects: they may create an excess of methaemoglobin possibly leading to toxic effects such as cyanosis and they may cause the endogenous formation of carcinogenic N-nitroso compounds.

Nitrate represents the stable oxidation state (V) of nitrogen and can be reduced to nitrite in the environment by microorganisms and within human tissues. Nitrite represents a less stable oxidation state (III) of nitrogen and therefore can be further reduced to various compounds or oxidized to nitrate. Nitrite may endogenously react with secondary amines to form N-nitrosamines at low pH values, as is the case in the gastric environment of mammals. Nitrosoamines may also be pre-formed in foodstuffs during certain biological, chemical and physical processes in crops, industrial transformation or even at the time of consumption.

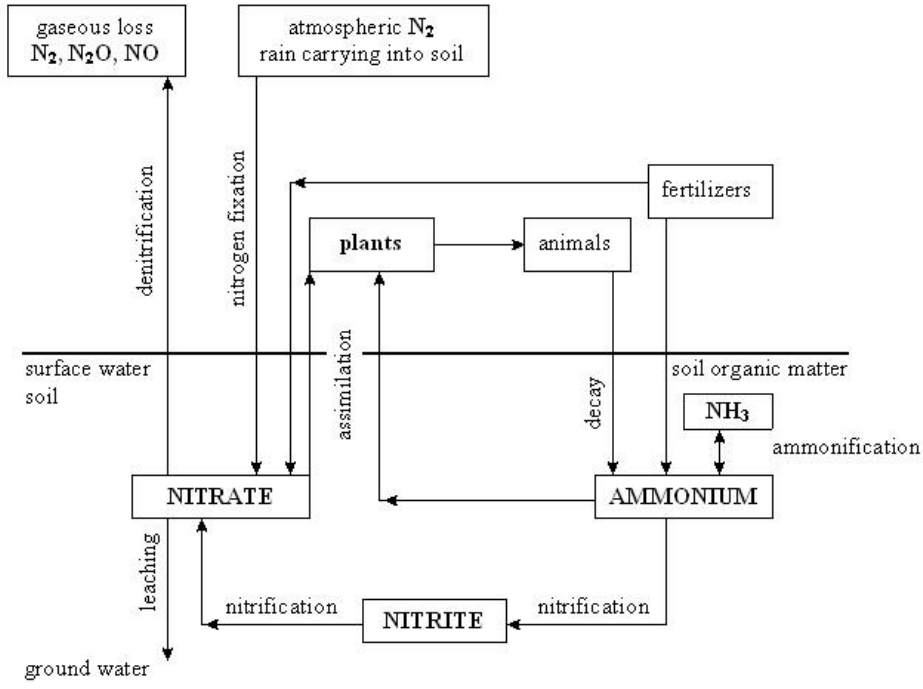

Figure 10.1. The Nitrogen Cycle

The nitrogen cycle as shown in Figure 10.1 is the pathway by which nitrogen is converted from the gaseous atmosphere to various inorganic and organic compounds and back again to the gaseous form (Nelson and Cox, 2000; Luf, 2002). This cycle is one of the most essential endlessly repeating processes in nature. All living organisms need nitrogen since it is an essential constituent of proteins, chlorophyll, nucleic acids and the building blocks of the genetic material as DNA and RNA.

In the biosphere, nitrogen alters continuously between the oxidation states +V (fully oxidized nitrogen – nitrate) and –III (fully reduced nitrogen – ammonia). The nitrogen cycle includes a number of redox reactions used either for assimilatory purposes or in respiratory processes for energy conservation. Prokaryotes play essential role in these reactions since only they have the enzymes carrying out these processes (Cabello *et al*. 2004, González *et al*., 2006).

The primary source of nitrogen is atmospheric air, from which molecular nitrogen makes up 78%. Incorporation of atmospheric nitrogen into terrestrial nitrogenous compounds takes place via number of different pathways, including microorganisms, plants, animals and humans through agricultural and industrial activities. Nitrogen in the air becomes a part of biological matter mostly through the actions of bacteria and algae in a process known as nitrogen fixation. The nitrogen-fixing bacteria take nitrogen from air and convert it into ammonia. The ammonia is further converted into nitrite, and consequently into nitrate by nitrifying bacteria, such as *Nitrosomonas* and *Nitrobacter*. Finally, the nitrate is taken up by plants and incorporated into tissues. Legume plants form nodules on the roots where symbiotic nitrogen fixing bacteria take nitrogen from the air and convert it into ammonia. These plants can assimilate some nitrogen in the form of ammonium ions from the noodles (Lucinski *et al*., 2002).

In plants, much of nitrogen is used in chlorophyll molecules, which are essential for the photosynthesis and further growth. Plant-eating animals use nitrates obtained from food to produce proteins. Carnivores obtain nitrogen compounds through eating herbivores. Humans receive nitrogen both from plants and animals.

Bacteria and plants can take-up and readily reduce nitrate and nitrite to ammonia through the action of nitrate and nitrite reductases. The process requires energy provided by photosynthesis (Nelson and Cox, 2000). Nitrates accumulated in plants form a nitrogen reserve, which is needed for amino acid and protein synthesis (Elliott, 2002).

Nitrate is returned to the environment through microbial degradation of plants and animal remains, as well as in animal faeces. Decomposition of dead organic matter by bacteria is the source of ammonia. Waste material from plants and animals returns nitrogen to the soils in which part of it is recycled and part returned after bacterial denitrification to the atmosphere to complete the nitrogen cycle. The denitrification of nitrate to nitrogen and nitrogen oxides takes

place in the soil by bacteria but also in various natural water sources. Ideally, nitrogen flow into the system and out of it, would be balanced, while any excess import of nitrogen may lead to its accumulation, and/or losses in the form of gaseous nitrogen (N_2), nitrous oxide (N_2O), nitrogen monoxide (NO), and ammonia (NH_3) to the atmosphere, and as nitrate NO_3^- to the hydrosphere (Codispoti *et al*., 2001; Oenema *et al*., 2003). The balance of nitrogen cycle is often destroyed due to increasing use of nitrogen-based fertilizers. As a consequence more nitrogen is transformed to gaseous form (Hardisson and Gonzáez Padrón, 1996). Nitrate and nitrite are readily soluble in water and quite mobile in the environment. They have a high potential for entering surface water when it rains and groundwater through leaching. Due to the increased use of synthetic nitrogen fertilizers and livestock manure in intensive agriculture, vegetables and drinking water in agricultural areas may contain higher concentrations of nitrate than in the past.

10.2. METHODS OF ANALYSIS

A variety of analytical methods, including spectrophotometry, high performance liquid chromatography (HPLC), ion chromatography (IC), gas chromatography (GC), polarography and capillary electrophoresis (CE), for the determination of nitrate and nitrite in food have been developed. Using of HPLC methods has gained more popularity in last decades since they are more rapid than classic methods based on reduction process followed by colorimetry. In a review paper by Moorcroft (2001) the strategies employed to facilitate the determination of nitrate and nitrite, relevant analytical methodologies and various techniques have been presented. Similarly to the analytical determination of other unstable and unevenly distributed compounds in food, systematic bias may be introduced at all stages of the analytical procedure from sampling, storage, extraction to the end-determination step. Tables 10.1 and 10.2 present an overview of the analytical methods used for the determination of nitrates and nitrites in recent years, their performance characteristics, advantages and limitations.

10.2.1. Sampling and storage

Nitrate and nitrite are both potentially unstable, therefore all food and water samples should be analysed as soon as possible after collection. It is well established that nitrate is readily reduced by bacteria and may also be produced or utilized as a result of post-sampling metabolic processes that may occur in vegetables. Nitrite is chemically highly reactive and its concentration may drop rapidly during storage, particularly under mildly acidic conditions. In such circumstances it is essential to perform the analysis as soon as possible after

sampling. If it is unavoidable to store the samples prior to starting analysis, the effects of the storage conditions on the stability of the analyte must be determined in advance.

10.2.2. Extraction

Many analytical methods for nitrite and nitrate determination in foodstuffs employ the same extraction procedure for both anions. In general the sample is extracted into hot water or sodium tetraborate (Borax) and treated with protein precipitation reagents (Carrez reagents) prior to filtration and measurement. Several nitrate extraction methods from plant material have been evaluated by Farrington *et al.* (2006), the hot water extraction method described in European Standard EN 12014–2:1997 (CEN, 1997) was found to give most reliable results. Extraction has to be performed under alkaline conditions, as nitrite may react with other matrix components if the extraction is carried out in even mildly acidic environment (Massey, 1996).

10.2.3. Spectrophotometric methods

Classic methods for the determination of nitrite are based on variations of the Griess diazotisation procedure, in which azo dye is produced in a reaction of diazonium salt with an aromatic amine or phenol. The formed diazonium ions are coupled to another aromatic compound in order to produce an azo dye (Kirk and Sawyer, 1991). This is the basis of many spectrophotometric methods and has also found wide application for the analysis of nitrate after its prior reduction by spongy cadmium to nitrite (Ruiter and Bergwerff, 2005).

The most widely used colorimetric method is based on the reaction of nitrite, sulphanylamide and N-1-naphtylethylenediamine under acidic conditions to form a red azo dye. The red colour produced in the reaction is measured by a spectrophotometer at 538 nm. Nitrite present in the original sample is determined before nitrate reduction in cadmium column and nitrate content is obtained by the difference of the analysis of unreduced and reduced extract (Slack, 1987). Experimental conditions must be carefully controlled since factors such as pH, temperature, nature and concentration of the reagents affect the final colour intensity (Zanardi *et al*., 2002).

Other spectrophotometric methods for nitrite are based on the catalytic effect on oxidation of gallocyanine (Ensafi and Bagherian-Dehaghei, 1999) or pyrogallol red (Kazemzadeh and Ensafi, 2001) by bromate. A novel method for the determination of nitrite and nitrate in food by spectrophotometric detection presented by Andrade *et al*. (2003) is based on the reduction of nitrite and nitrate to nitric oxide, with subsequent reaction with iron(II) and thiocyanate in an acid medium, forming $FeSCNNO^{+}$. The absorbance of the complex measured at 460 nm is proportional to the nitrite and nitrate concentrations.

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Table 10.1. Method performance characteristics in nitrate and nitrite analysis

method	LOQ, mg/kg	Reproducibility RSD	Recovery	Reference
HPIC/HPLC, UV detection	nitrate 2–50 mg/kg; nitrite 0,4–40 mg/kg	nitrate 3,3– 15,2%; nitrite 9,4%	nitrate 96–109%; nitrite 96–108%	Vaessen and Schothorst, 1999; Merino *et al*., 2000; Chou *et al*., 2003; CEN, 2005b
HPIC/HPLC, conductivity detection	20–80 mg/kg; olive oil – LOD 8,4–31μg/kg	2,4–10,2%	nitrate 87–104% nitrite 91–104%	CEN, 1997; De Martin and Restani, 2003; McMullen *et al*., 2005; Farrington *et al*., 2006; Dugo *et al*., 2007
IC/MS	0,1 mg/kg			Saccani *et al*., 2006
Cd reduction, spectrophotometric detection of summed nitrite and nitrate	nitrate – 5 (LOD)–900 mg/kg; nitrite 0,3 mg/kg (LOD)	8,3–21%	80–117%	CEN, 1998; Petersen and Stoltze, 1999; AOAC, 2000
Flow-injection spectrophotometric method, through NO generation	nitrate 20 mg/kg; nitrite 13 mg/kg	2,3–5,5%	nitrate 93–110%; nitrite 88–97%	Andrade *et al*. 2003
Ion chromatographic separation, V(III)post-column reduction, spectrometric detection	25 mg/kg		82–107%	Casanova *et al* 2006
enzymatic reduction of nitrate to nitrite	nitrite10 mg/kg	7,7%		Cruz and Martins Loução, 2002; Zanardi *et al*., 2002; CEN, 2005a
potentiometric methods	nitrate 30 mg/kg	15%		Tamme *et al*., 2006
electrophoresis	nitrite 4 mg/kg (LOD)	nitrate 2,54%; nitrite 4,51%	nitrate 92–106%; nitrite 94–103%	Öztekin *et al*,. 2002
gas chromatography	100–200 μg/kg	3–13%	100%	Ross and Hotchkiss, 1985
polarography	nitrate 39 mg/kg	<7%	85–107%	Ximenes *et al*., 1999

Table 10.2. Advantages and limitations of various analytical methods for the determination of nitrates and nitrites in food

method	**advantages (+), limitations (–)**
HPIC, HPL/UV	+ fast, sensitive, precise – possible positive interference due to co-eluting matrix components, equipment not available in all laboratories
HPIC, HPLC, conductivity detection	+ precise, rapid, multiple anions can be determined simultaneously, – equipment not available in all laboratories
IC/MS	+ robust, sensitive – expensive equipment needed
Cd reduction, spectrophotometric detection of summed nitrite and nitrate	+ traditional method; no need for expensive equipment – carcinogenic Cd used, nitrite has to be determined separately, less precise for complicate matrixes and low concentrations
ion chromatographic separation, V(III)post-column reduction, spectrometric detection	+ no Cd used, simultaneous determination of nitrite and nitrate
enzymatic reduction of nitrate to nitrite	+ no Cd used – nitrite has to be determined separately; overestimation of nitrate values in meat samples
potentiometric methods	+ inexpensive, rapid – suitable only for the determination of nitrates in vegetables, possible positive interference, lack of sensitivity
electrophoresis	+ short analysis time, good alternative to IC methods
gas chromatography	+ high sensitivity – derivatization needed, expensive equipment

The use of cadmium columns to reduce nitrate to nitrite is widely applied in the analysis of cured meats (Oliveira *et al*., 2004), cheese and baby food (AOAC, 2000), fruit and vegetables (CEN, 1998; Petersen and Stoltze 1999).

In the older version of the colorimetric method various steps of analytical procedure are run manually whereas more recent methods apply automated equipment (Zanardi *et al*., 2002; Oliveira *et al*., 2004). Flow-injection spectrophotometric detection for simultaneous analysis of nitrite and nitrate has been used both for food and water samples by Petersen and Stoltze (1999), Monser (2002), Andrade *et al*. (2003) and Ensafi *et al*. (2004). The injected sample is split into two streams. One of the streams is transported through a reductor microcolumn containing copperized cadmium, where nitrate is reduced to nitrite. The total nitrite concentration initially plus that produced in cadmium column is measured spectrophotometrically. The method is quick – according to

the data by different authors 10 to 25 samples can be analysed per hour. The parameters affecting the results of flow injection analysis have been investigated by Gal *et al*. (2004). A continuous flow method for the analysis of vegetables and vegetable products using spectrophotometric detection has also been adopted as a European standard EN 12014–7:1998 (CEN, 1998).

Many attempts have been made to avoid using the toxic and carcinogenic metal cadmium. Other nitrate-reduction techniques include chemical treatment with vanadium(III)chloride for baby food samples (Casanova *et al*., 2006), microbial reduction with E.coli for analysis of plants (Cruz and Martins Loução, 2002) and enzymatic reduction for the analysis of meat samples (CEN, 2005a).

Spectrophotometric assays are potentially vulnerable to interferences from a number of sources, most important of them being incomplete reduction of nitrate and over-reduction of nitrate via nitrite to lower oxides (Norwitz and Keliher, 1985). The close comparability of the results obtained for the nitrite content of cured meats by colorimetry and by HPLC suggests that this is not a widespread problem (Dennis *et al*., 1990). The colorimetric assay may also be adversly affected by the turbidity of the measurement solution, which is likely to occur if the post-extraction precipitation and filtration steps are ineffective. To overcome the latter problem, Fox *et al*. (1982) have recommended charcoal or alkaline extraction.

Detection limits for nitrate and nitrite by reduction methods are generally around 1 mg/kg (Dennis *et al*., 1990). For samples that contain appreciable quantities of ascorbate or other interferants the limit of detection may be an order of magnitude higher (Usher and Telling, 1975). However, at analyte concentrations below 20 mg/kg the agreement with other methods is not very good due to problems of chemical interference or turbidity. As with the exception of cured meats, nitrite is generally present at very low levels, reliable determination of it by colorimetric assay is often a problem. Zanardi *et al*. (2002) reported that 3 analytical methods based on the same principles (Cd manual and automated method, enzymatic method) for the determination of nitrite showed no significant differences. The results concerning nitrate showed a different pattern – the values obtained by the Cd method were considerably lower than those obtained by the chromatographic method (Alonso *et al.*, 1992). The difference has been attributed to a possible decline in efficiency of the Cd column due to possible interferences caused by other anions. Enzymatic test produces an overestimation of the nitrate values.

The AOAC (2000) method for meat samples involves oxidation of nitrite to nitrate with permanganate followed by acidification and treatment with m-xylenol. After nitration the nitroxylenol is removed from the samples by distillation and measured colorimetrically.

10.2.4. Chromatographic methods

The use of high-performance liquid chromatography (HPLC) and ion chromatography (IC) for the analysis of nitrate and nitrite has increased substantially over recent years due to the number of favourable factors compared with colorimetric assays including the speed of the analysis, the fact that both anions can be determined at the same time, toxic reagents such as cadmium are not required and also that in some instances higher sensitivity and accuracy is obtainable.

HPLC/UV methods have been reported for the nitrate and nitrite in vegetables (Cheng and Tsang, 1998; Chou *et al*., 2003; Tamme *et al*., 2006), dairy products (Reece and Hird, 2000; Gapper *et al*., 2004), cured meats (Dennis *et al*., 1990; Reinik *et al*., 2005) and beer (Massey *et al*., 1990).

Reverse-phase (ion interaction/ion pair) HPLC has been used both in the analysis of water and food (Frohlich, 1987; Mullins, 1987; Cheng and Tsang 1998), although ion-exchange HPLC is preferred in foodstuff analysis (Pentchuk *et al*., 1986; Dennis *et al*., 1990; Siu and Henshall 1998; Reece and Hird, 2000; Stalikas *et al*., 2003; Gapper *et al*., 2004). Normal phase ion-pair chromatography was used for water and vegetable samples by Butt *et al*. (2001). UV-detection is not suitable for multi-ion measurement due to the poor UV absorbance of chloride and phosphate. In such cases the analytes can be measured by indirect UV in which a UV-absorbing compound is included in the composition of mobile phase and anions owing a lower absorbance give a signal in the form of a negative peak on elution from the column (Mullins, 1987). Alternatively, the conductivity detector may be applied (Pentchuk *et al*., 1986; De Martin and Restani, 2003; Kissner and Koppenol, 2005; Masson *et al*., 2005; McMullen *et al*., 2005; Dugo *et al*., 2007).

Achieving the stability of chromatographic performance is a problem in the analysis of complex food extracts, as components of the matrix may adsorb onto the analytical column and adversly affect subsequent analyses. Various types of solid phase extraction cartridges have been tested to clean the sample extract prior to HPLC measurement: C18 SPE columns have been used by De Martin and Restani (2003) for analyses of leafy vegetables and by Vaessen and Schothorst (1999) for total diet samples, Dennis *et al*. (1990) have used cyclohexyl Bond Elut cartridges in the analysis of cured meats. Hunt and Seymour (1985) treated vegetable extracts with activated charcoal.

A number of authors have compared HPLC results with those obtained by other methods. In the case of water, good agreement with colorimetric results has been reported (Schroeder, 1987). Pentchuk *et al*.(1986) compared the determination of nitrate by HPLC and colorimetry of a number of types of vegetables and showed good agreement of the results. HPLC was found to give higher results then colorimetry in the analysis of nitrates in carrots, and this was thought likely to be due to incomplete colour development (Schuster and Lee

1987). Dennis *et al.* (1990) have reported good agreement between HPLC and colorimetry for the anaysis of nitrite and nitrate in cured meats. Better agreement between these methods has been achieved using reverse-phase HPLC than ion exchange (Massey, 1996). Three ion chromatographic methods, including two present European Standard methods EN 12014-2 (CEN, 1997) and EN 12014-4 (CEN, 2005b), were tested in a NMKL collaborative study (Merino *et al*., 2000) and compared to spectrophotometric method. In the analysis of nitrite, no statistically significant difference was found between the spectrophotometric method and EN 12014-4. It was concluded that the use of a strong anion exchange column is necessary to ensure the reliability of results. Nitrite determination with the weak anion exchanger column used in standard method EN 12014-2 for the analysis of vegetable samples, is not suitable for the determination of residual nitrate and nitrite in meat products. The method is also not reliable for the analysis of certain vegetables with low concentrations of nitrate.

The detection limits for HPLC-based methods are typically between 0.1 and 10 mg/kg for foodstuffs (Eggers and Cattle, 1986; Dennis *et al*., 1990; Vaessen and Schothorst, 1999; Merino *et al*., 2000; Chou *et al*., 2003). In the validation of an ion chromatographic method for the determination of nitrates in some high-nitrate containing leafy vegetables, de Martin and Restani reported a limit of detection 80 mg/kg.

Gas chromatographic methods for the measurement of nitrate and nitrite in water and foodstuffs involve the formation of a volatile derivative, extraction into organic solvent and measurement by GC using a selective detector. Wu *et al*. (1984) have developed a gas chromatographic electron-capture detector (GC/ECD) method for the pentafluorobenzyl derivative of nitrite. The procedure has a detection limit of 5 μg/l for aqueous samples and showed good agreement with a colorimetric assay for the analysis of cured meat, saliva and water. Similar performance characteristics were acchieved by Funazo *et al*. (1980) in their method for nitrite involving GC/ECD detection of bromo-chlorobenzene formed by reaction of nitrite, bromoaniline and cupric chloride. Ross and Hotchkiss (1985) used the nitrobenzene derivative for the measurement of nitrate in dried foods by GC with thermal energy analyzer, and reported a detection limit of 100 μg/kg.

10.2.5. Other methods

In recent years capillary electrophoretic (CE) methods have been developed for the simultaneous detection of nitrite and nitrate in foodstuffs (Marshall and Trennery, 1996; Öztekin *et al*., 2002). Anions are separated in a capillary coated with polyethyleneimine. Good recovery and reproducibility can be achieved, and the detection limit of the method is sufficiently low for the analysis of meat products and vegetables.

Damiani and Burini (1986) have described a fluorimetric assay for nitrite based on its reaction with 2,3-diaminonaphtalene. Application of the method to milk samples gave results that were approximately 10% lower than a colorimetric assay.

Nitrate-selective electrodes have found little application in the analysis of foodstuffs because of their potential interference from several commonly occuring anions such as chloride, sulpfte and bicarbonate. Pentchuk *et al*. (1986) has reported that nitrate electrodes may give rise to positive interference when compared to other methods in the analysis of vegetables. Other methods that have been reported for the analysis of nitrate and nitrite include ion pair extraction/atomic absorption spectrophotometry (Silva *et al*., 1986), amperometry (Bertotti and Pletcher, 1997), polarography (Ximenes *et al*., 2000) and stripping voltammetry (Van den Berg and Li, 1988).

10.3. INCIDENCE AND LEVELS OF OCCURENCE

10.3.1. Vegetables

Vegetables constitute a major source of human exposure to nitrates contributing approximately 40–92% of the average daily intake (Penttilä, 1995; Dich *et al*. 1996; Ximenes *et al*., 2000; Eichholzer and Gutzwiller, 2003). Nitrate is present as a natural constituent in plants and may accumulate in different tissues. Each plant species have their own unique path of biological photosynthesis in leaves as well the transport mechanism for getting water and nutrients by roots. Those biological mechanisms are most essential factors influencing the nitrate level in plants.

The content of nitrate in different vegetables vary to large extent and according to that the vegetables can be divided into three groups (Tamme *et al.* 2006):

1. Plants with nitrate content higher than 1000 mg/kg – lettuce, spinach, herbs, beetroot, rhubarb, turnip etc.
2. Plants with average content of nitrate (50–1000 mg/kg) – carrot, green beans, cauliflower, onion, pumpkin, eggplant, potato etc.
3. Plants with nitrate content lower than 50 mg/kg – berries, fruits, cereals, pod vegetables.

De Martin and Restani (2003) showed that leafy green vegetables accumulate the highest amounts of nitrates, concentrations reaching up to 6000 mg/kg. According to the data reported in Table 10.3 it can be concluded that the highest mean values of nitrates have been detected in spinach, lettuce and dill. Among root vegetables, nitrate concentration in beetroot is the highest. The lowest mean values of nitrates were detected in tomato, cucumber and onion. Nitrate

Table 10.3. Mean contents of nitrates in vegetables in different countries (Penttilä, 1995; Dejonckheere *et al*., 1994; Petersen and Stoltze, 1999; Belitz and Grosch, 1999; Chung *et al*., 2003; Ysart *et al.,* 1999; Susin *et al.,* 2006; Tamme *et al.,* 2006).

Vegetable commodity	Mean NO_3^- concentration, mg/kg								
	Finland	Great Britain	Belgium	Denmark	Slovenia	Korea	Japan	Germany	Estonia
Potato	82	155	154	182	158	452	713	93	94
Carrot	264	97	278		264	316	193	232	148
Lettuce	1835	1051	2782	2631	1074	2430		1489	2167
Chinese cabbage	1057			1058		1740	1040		1243
Cabbage	607	338		333	881	725		451	437
Turnip	908	118							307
Tomato	170	17	36		< 6			27	41
Sweet pepper	140		93			76	99		
Cucumber	240		344		93	212	384		160
Onion	140	48	59			23			55
Beetroot	1800	1211		1505				1630	1446
Green beans	455		585		298				
Radish			2136			1878	1060	2030	1309
Spinach		1631	2297	1783	965	4259	3560	965	2508
Leek			841	284		56			
Celery leaves			3135						565
Squish pumpkin						639			174
Spring onion						436	145		477
Garlic						124	455		174
Dill/fennel								1541	2936
Rhubarb								986	201
Parsley			2690						966
Endive			1246						
Cauliflower		86							287
Canned vegetables		18							

Table 10.4. Nitrite contents in vegetables in different countries

Country	NO_2^- concentration, mg/kg		Reference
	Range	Mean	
Finland	ND. – 1.5	1.0	Penttilä (1995)
Germany	< 0.1 – 19.6	0.1	Belitz-Grosch (1999)
England	0.3 – 3.8	1.1	Ysart *et al.,* (1999)
Japan	ND – 7.0	2.1	Chung *et al.,* (2003)
Korea	0.3 – 1.1	0.57	Chung *et al.,* (2003)
Denmark	0.15 – 11	2.5	Petersen *et al.,* (1999)
Slovenia	< 0.5 – 1.2		Susin *et al.,* (2006)
Poland	0.2 – 0.83		Nabrzyski *et al*. (1994)

concentrations in the vegetables measured in different countries are in good agreement with some exceptions. According to the data given in Table 10.3 the concentration of nitrates in potatoes differs a lot depending on the region of cultivation. The nitrate content in potatoes in Northern Europe remained below 100 mg/kg, while in the Far-East the concentrations reached over 700 mg/kg (Penttilä, 1995; Chung *et al.,* 2003; Tamme *et al*. 2006).

Nitrate concentrations in some salad crops of different varieties during the summer and winter seasons were screened by Escobar-Gutierrez *et al.* (2002): great variability between the cultivars and also varieties within one cultivar were detected. The exceeding of maximum limit concentrations were more frequent in the summer season than in winter. This may be explained by lower maximum limit values valid for summer period. Tamme *et al.* (2006) found in their study that the content of nitrates in lettuce was lower in summer time (average 1952 mg/kg) than in winter (average 3024 mg/kg), and exceedings of limit concentrations were not detected. Szymczak and Prescha (1999) and Järvan (1993) reported that nitrate concentration in the greenhouse vegetables, lettuce, cucumber and radish, was greater than in the field-grown analogues.

Concerning organically farmed vegetables, controversial results have been achieved by different authors. Significantly higher nitrate content was found in Italian organically grown green salad and rocket compared with the same conventionally produced products (De Martin and Restani 2003). On the contrary, US investigation (Worthington 2001) stated that organic crops contained significantly less nitrates than conventionally grown analogues. The review of literature conducted by Heaton (2001) found 14 studies showing 50% lower nitrate content in organically grown crops and two studies showing insignificant differences.

For nitrite, the main source of exogenous human exposure is also food.. The nitrite content of most fresh, frozen or canned vegetables is relatively low and usually of the order of 0–2 mg/kg (Siciliano *et al*. 1975; Corré and Breimer, 1979). Comparison of data regarding the nitrite contents of vegetables obtained by different authors is shown in Table 10.4.

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The studies have proved that ascorbic acid is very efficient at preventing the conversion of nitrate to nitrite in plant tissue and within the human body. Fresh vegetables that are rich in ascorbic acid, such as kale, green pepper and broccoli, may contain enough vitamin C to avoid significant nitrate reduction to nitrite and subsequent formation of nitrosoamines **(**Mackerness, *et al*., 1989; Kolb and Haug, 1997; Naidu, 2003).

10.3.1.1. Factors affecting nitrate and nitrite levels in plants

Nitrate concentrations in vegetables can vary from 1 to 10,000 mg/kg depending on biological properties of cultivars, light intensity, soil composition, air temperature, growth density, moisture, maturity of plant, duration of growth period, harvesting time, size of the vegetable, storage time, edible plant portion and nitrogen source (Tivo and Saskevic, 1990; Walker 1990; WHO, 1995; Fytianos and Zarogiannis, 1999; Laslo *et al,* 2000).

Plant portion. Accumulation of nitrate in plants differs largely between the parts of the crop (Fytianos and Zarogiannis, 1999). The root of the cabbage contains higher levels of nitrates compared to the leaves. On the contrary, root vegetables such as carrots and beetroots contain lower levels of nitrates than do their leaves. Experiments have shown that some potato breeds accumulate lower amounts of nitrates than others. Studies with carrots have indicated that the core of the carrot contains higher levels of nitrates than the outer layers do (Järvan 1993). In the outer layers of cucumbers and radish, the nitrate concentration is 2–3 times higher than in the pulp (Golaszewska and Zalewski, 2001; Mozolewski and Smoczynski, 2004). The nitrate content is decreased by 40% by removing the stem and midrib of the spinach before stewing as the nitrate content in these plant portions was 2–3 times higher than in leaf tissue (Sokolov, 1987).

Lighting.Via the disturbance of nitrate reductase the decreased lighting conditions cause disorders in the formation of organic compounds and the concentrations of nitrates in plant stay high. Extended periods of cloudy weather increase nitrate content and dangerously high levels can occur when wet days follow a severe drought (Vulsteke and Biston, 1978).

Soil composition. In similar fertilization and growth conditions, the lowest nitrate concentrations are detected in vegetables grown in light sandy soils. Higher accumulation of nitrates is reported in clay- and humus-rich soils and the highest concentrations from low-lying swamps. At the same time, soils with higher concentrations of organic compounds are able to supply plants with nitrogen more equally compared with sandier soils, which have higher water filtration ability (Rückauf *et al.* 2004).

Temperature is an important factor influencing the residual nitrate content in vegetables. Low temperatures in spring or autumn slow down photosynthesis and favour nitrate accumulation. Too high temperatures reduce nitrate reductase activity resulting in higher nitrate concentrations in plants. In optimal growth

temperature conditions the stress in plants is avoided and no temperature related nitrate accumulation is observed (Tivo and Saskevic, 1990; Järvan, 1993).

Moisture. Low moisture conditions favour accumulation of nitrates into the plants. Rückauf *et al.* (2004) reported that higher soil moisture resulted in more efficient plant nitrogen uptake. Moderate soil moisture conditions conduce the reasonable plant nitrogen nutrition while wet conditions increase the nitrate concentrations in plants.

Growth density. The connection between nitrate content and growth density has been described from two aspects. Firstly, the growth density influences the lighting conditions on leaves. Higher density will shadow the plants and causes decreased growth via the enzyme inhibitions. The same type of reductase inhibition has been reported as well for weedy fields. Secondly, there is a mutual relationship between growth density, soil fertility and final nitrate content of the plants. In the conditions of soil rich in plant nutrients and low growth density, overconsumption of nitrogen by plants may occur (Vulsteke and Biston, 1978; Järvan 1993).

Plant maturity. Growth period. Nitrate content generally is highest in early stages of plant growth and decreases with maturity. Stems of vegetables contain higher amounts of nitrates than leaves. The growth period is generally species-specific, but sometimes it may be shortened due to leaf damages caused by night frost, hail, plant diseases, pests, herbicide drift etc. Longer and lighter growth periods favour the reduction of nitrates in plants for the time of harvesting (Järvan, 1993; Sheehy *et al.* 2004).

Harvesting time. All season growing cultivars have more nitrates in early crops. Some vegetable species are harvested in different growing stage, which results in variable nitrate concentrations in plants harvested from the same field. According to the data by Järvan (1993) the nitrate content in radishes harvested in early growth stage nitrate concentration was 1,4-fold higher compared with the mature roots, for carrots the nitrate content in early plants was 2-fold higher.

Fertilization. Plants grown without excessive nitrogen fertilizer contain far less nitrate. Nitrate fertilizer applied shortly before harvest causes the greatest increase in nitrate levels and should be avoided. Use of slower nitrogen-releasing natural fertilizers such as animal and green manures enables vegetables to be produced with significantly lower nitrates. Low availability of phosphorus and potassium from soil can contribute to nitrate accumulation. Plant species, stress factors and plant growing conditions have been reported to have more influence to the nitrate levels in plants than amount of nitrogen fertilizer applied (Järvan, 1993; Hlusek *et al.*, 2000).

Storage conditions. Studies have shown that during storage, the nitrate content in vegetables decreases 15–20% (Sokolov, 1989). This is related with transformation of nitrates to nitrites. Nitrate concentration decreases and nitrite concentration increases. In optimal storage conditions, optimal temperature and moisture, the concentration of nitrates decreases slowly in all vegetables. Nitrite

concentrations in vegetables may increase to elevated levels due to bacterial nitrification of nitrate to nitrite when vegetables are stored in rooms with high humidity and poor sanitation (Sokolov, 1987). Vegetable cuts, salads and raw juices have to be prepared preferebly shortly prior to the consumption. Storage at room temperatures increases the nitrite concentrations to potentially hazardous level (Sokolov, 1987). Chung *et al.* (2004) found that during storage of leafy vegetables at ambient temperature, nitrate levels in the vegetables dropped significantly from the third day while nitrite levels increased dramatically from the fourth day of storage. Over 7 days refrigerated storage did not lead to changes in nitrate and nitrite levels in the vegetables.

Food handling. High nitrate concentrations initially present in vegetables can be decreased during the treatment of food by utilization of the ability of nitrates and nitrites to dissolve in water (Buck, 1973). Dejonkheere *et al*. (1994) measured the nitrate loss in a number of vegetables after normal culinary practice such as washing, peeling, cooking and stewing. Washing of leafy vegetables with tap water reduced the nitrate concentration with 10–15% and for lettuce the elimination of the thick midrib resulted in a decrease of the nitrate content of 30–35%. Peeling and washing of vegetables can decrease the nitrate content 20–30% (Laslo *et al,* 2000). Studies have shown 25–30% decrease in nitrate content of potato, carrot, beetroot, turnip and cabbage after at least one hour of soaking (Mozolewski and Smoczynski, 2004). A slightly lower decrease of 20% was achieved for spinach, celery, dill and spring onion. Longer soaking reduces nitrate concentrations even more but the loss of beneficial food components is higher as well (Sokolov, 1987). Boiling of vegetables can decrease the nitrate concentrations by almost 80%. The reduction is related to dissolution of nitrates in the boiling water. Nitrate concentration decreases more when an abundant amount of boiling water is used and after cooking the vegetables are drained carefully. Adding sodium chloride is recommended at the end of boiling, as when this is done too early, nitrate solubility in water is reduced (Sokolov, 1987).

10.3.2. Fruits

The levels of nitrates in fruit are low compared with the vegetables. White (1976) reported the nitrate contents in fruits to be 10 mg/kg. Another earlier study by Herrmann (1972) showed that strawberries may contain nitrates over 100 mg/kg, grapes reached the level of 17 mg/kg. Nitrate concentrations in fruits and fruit products reported by different authors are represented in Table 10.5. In a recent Slovenian study (Susin *et al.* 2006) nitrate and nitrite contents were generally low: less than 6 mg/kg of nitrate was found in grapes, peaches, apples and pears. The highest average nitrate content, 94 mg/kg, was found in strawberries. In apples and pears, the average nitrite contents were 1.5 mg/kg and 1.0 mg/kg, respectively. The content of nitrites did not exceed 0.5 mg/kg in

other fruits. Nabrzyski (1994) determined concentrations of nitrates and nitrites in Polish fruit and berries during 1989–1992. The highest levels of nitrates were detected also in strawberry samples (maximum to 322 mg KNO_3/kg), mean level was found to be 59 mg KNO_3/kg. Other berries, such as currants, gooseberries, raspberries and cherries contained from "not detected" to 36 mg KNO_3/kg. Very low level of nitrates was found in seven species of apples (from 1.3 to 9.7 mg KNO_3/kg). The concentration of nitrites in all samples remained from "not detected" to fractions of mg/kg (Nabrzyski *et al*. 1994). The results are in good agreement with the earlier work by Gajewska *et al.* (1989) who reported that content of nitrates in frozen fruits (strawberries, black and red currants and plums) ranged from 2.5 to 57 mg KNO_3/kg, with the highest concentrations detected in garden strawberries. In cherry, strawberry, black and red currant jams the concentrations were detected from 6.3 to 97 mg KNO_3/kg. The nitrite content in all these products was low, not exceeding 1 mg $NaNO_2$/kg, with the exception of plum jam where the maximal value of 1.6 mg $NaNO_2$/kg was found. The ranges of nitrate and nitrite concentrations in fruit juices have been reported to be 9.7–21 mg/l and 3.1–9.7 mg/l, respectively (Okafor *et al*., 2003).

Table 10.5. Nitrate contents mg/kg in fruits and fruit products

Fruits and products	Mean NO_3^- concentration, mg/kg	Reference
Apples	11	Dejonckheere *et al.* 1994
Apples	6	Susin *et al*. 2006
Apples	19	Belitz and Grosch, 1999
Oranges	13	Dejonckheere *et al.* 1994
Bananas	402	Dejonckheere *et al.* 1994
Grapes	8,2	Belitz and Grosch, 1999
Grapes	46	Dejonckheere *et al.* 1994
Fresh fruits	27	Ysart *et al.* 1999
Fruit products	13	Ysart *et al.* 1999
Melons	375	Dejonckheere *et al.* 1994
Pears	14	Dejonckheere *et al.* 1994
Pears	1,0	Susin *et al*. 2006
Peaches	10	Dejonckheere *et al.* 1994
Strawberries	156	Dejonckheere *et al.* 1994
Strawberries	94	Susin *et al*. 2006
Strawberries	139	Belitz and Grosch, 1999
Strawberries	34	Nabrzyski *et al*. 1994

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10.3.3. Milk and dairy products

Increased nitrate concentrations in milk are not only dangerous to human health as milk is used for production of baby and infant food, but may also cause many technological problems in milk processing (Baranova *et al.* 1993). Contamination of milk with nitrate may occur during and/or after secretion. Due to a very low carry-over rate of nitrate from forage to milk, the main route of contamination is post-secretory. Contamination of milk and dairy products with nitrites during and/or after secretion is lower compared with nitrates (Blüthgen *et al.,* 1997). Residues of nitric acid used as a cleaning reagent combined with inadequate rinsing, addition of water with high nitrate content or the use of nitrate as a food additive in cheese manufacturing are the reasons for increased nitrate contents in dairy products (Luf, 2002).

The investigation of Kammerer *et al.* (1989) showed that drinking water has no significant effect on milk nitrate content, which remains very low and does not constitute a health risk to consumers. A study of transport of nitrates and nitrites into the milk of dairy cows through the digestive system (Baranova *et al*. 1993) showed that following the per oral application of KNO_3 to dairy cows, a marked increase in nitrate content in milk appeared being dependant on applied KNO_3. Increased levels of residual nitrate in milk were found also 38 hours after KNO_3 administration. In a study by Bouchard *et al.* (1999) the effect of endotoxin-induced mastitis leading to increased nitrite and nitrate concentrations in milk was reported. Results of Bouchard *et al*. (1999) suggest that nitric oxide production during endotoxin-induced mastitis resulted from the activity of the inducible form of nitric oxide synthase.

The nitrate/nitrite content in milk and dairy products is generally lower than in other foods such as vegetables, cured meat and drinking water. Nitrate and nitrite contents in milk and dairy products are presented in Table 10.6.

The content of nitrates and nitrites was determined in raw milk in a study by Przybylowski *et al*. (1989). The results of this investigation showed that most samples contained nitrates in amounts not exceeding 2 mg/kg, while nitrites were present in trace amounts or were not detected at all. Studies in Denmark estimated average nitrate concentration of 8 mg/l in milk. Danish cheeses contain comparable levels of nitrate and nitrite, approximately 10 mg/kg and 0.2 mg/kg respectively, whether or not nitrate had been used in processing (Statens Levnetsmiddelinstitut, 1981). According to the data of Estonian Health Protection Inspectorate in 2004 the nitrate content of different Estonian cheeses on retail sale was within the range <7–49 mg/kg and nitrite content in all samples remained below 5 mg/kg. Edam cheeses on retail sale in the UK in 1980–1981 had nitrite contents within the range 3,1–20 mg/kg whilst 15 samples of each of three English-type cheeses (Cheddar, Cheshire and Leicester) were relatively low in nitrate levels, 1.0–6.1 mg/kg (MAFF 1987).

A survey of nitrates and nitrites in French dairy products was carried out by Amariglio and Imbert (1980). It was shown that 93% of dried milk samples contained nitrates at less than 30 mg/kg, and 82% of cheese samples at less than 5 mg/kg. The nitrate/nitrite content in milk and dairy products from Austrian dairies was determined in 1986. The mean value of nitrate and nitrite content in pasteurized milk was 0.31 mg/kg and 0.006 mg/kg, respectively. In fresh cheese, the mean nitrate and nitrite concentrations were 4.58 mg/kg and 0.013 mg/kg, respectively (Luf, 2002). In Greece where the use of nitrates in cheese production is prohibited, contamination of cheese products was related to using of nitrate containing fertilizers, animal feeds and drinking water at primary production level (Nikolas *et al*. 1997).

The contribution of milk and dairy products to overall nitrate/nitrite ingestion is very low (Blüthgen *et al.* 1997). In Austria an intake from milk and dairy products was estimated to be 0.096% of ADI for nitrate and 0.069% of ADI for nitrite (Luf, 2002).

Table 10.6. Nitrate and nitrite contents in milk and dairy products

Product	Mean NO_3^- concentration, mg/kg	Mean NO_2^- concentration, mg/kg	Reference
Milk	4.8	–	Wright and Davison, 1964
Milk	8	–	Statens Levnetsmiddelinstitut, 1981
Milk	5.3	–	Ysart *et al*, 1999
Milk	0.31	0.006	Luf and Brandl, 1986
Milk	1.4	–	Belitz-Grosch, 1999
Dairy products	27	–	Ysart *et al*, 1999
Soft cheese	1.5	0.17	Luf and Brandl, 1986
Edam cheeses	7.5	0.4	MAFF, 1978
Cheese	–	0.3	Belitz and Grosch, 1999
Cheese	16	0.9	Penttilä, 1995
Cheese	4	0.5	Nikolas *et al*, 1997
Cottage cheese	12.1	0.008	Luf and Brandl, 1986
English-type cheeses	2.8	0.4	MAFF, 1987
Danish cheeses	10	0.2	Statens LevnetsMiddelinstitut, 1981
Milk powder	5.4	0.011	Luf and Brandl, 1986
Yoghurt	0.72	0.002	Luf and Brandl, 1986

10.3.4. Cereals and bread

Only few data is available on the content of nitrate and nitrite in cereals, bread, flour and various bakery products. This is probably related to the fact that the nitrate and nitrite contents in cereals and various bakery products are generally low. Early studies by Wu and McDonald (1976) reported nitrate content in

grains to be far lower than in stems or leaves of the plants. The nitrate content of winter wheat seeds depends on growth conditions and varies in the range of 0.4–11 mg/kg (McNamara *et al*. 1971). Wu and McDonald (1971) reported that the nitrate concentration in white flour was 4–14 mg/kg. The data by Nabrzyski *et al*. (1990) showed that the content of nitrates in various bakery products varied from 0.96 in wheat rolls and baguettes to 44 mg KNO_3/kg in pumpernickel bread. The mean content of nitrites in bread varieties was 1.8 mg $NaNO_2$/kg. The same study reported that in white wheat flours the content of nitrates stayed in the range of 1.1–19 mg KNO_3/kg, and in the dishes produced from them under household conditions ranged from 0.5–16 mg KNO_3/kg. The content of nitrites in flour was found to be from "not detected" (ND) to 4.2 mg $NaNO_2$/kg, and in corresponding bakery products from ND-1.6 mg $NaNO_2$/kg. Eleven types of popular biscuits, wafers, gingerbread and hard cakes were tested in which the content of nitrates was found to be 3.7–17 KNO_3 mg/kg, and that of nitrites ND-8.8 mg $NaNO_2$/kg (Nabrzyski *et al*. 1990). Belitz and Grosch (1999) reported the nitrate and nitrite contents in cereals within the range of 0.3–19 mg/kg and 0.3–1.0 mg/kg, respectively. Ysart *et al.* (1999) detected the nitrate concentrations in bread and miscellaneous cereals in the range of <4–20 mg/kg.

10.3.5. Fresh meat

Mostly the levels of naturally-occuring nitrate determined in meat are low and only few data is reported regarding to nitrate and nitrite contents in fresh meat and fresh meat products. This chapter does not deal with cured meat products, to which nitrite and/or nitrate are added as food additives. Wright and Davison (1964) reported nitrate content of 0.9 mg/kg in fresh meat. Usher and Telling (1975) concluded in a series of studies that the nitrate concentration in fresh meat ranged from ND to 49 mg/kg. Ysart *et al.* (1999) reported the nitrate concentration in carcass meat and offal as a mean of 5.1 mg/kg and 5.3 mg/kg, respectively. Dry-cured hams treated only with sodium chloride and sugar contained nitrite 5 mg/kg in average (Kemp *et al.* 1975). Fresh meat products may contain <2.7–9.5 mg NO_3^-/kg and <0.2–1.7 mg NO_2^-/kg (ECETOC, 1998).

10.3.6. Drinking water

Drinking water is regarded to be the second-largest source of nitrate in the diet after vegetables (Belitz and Grosch, 1999; Fytianos and Zarogiannis, 1999; Knobeloch *et al.* 2002; Caballero Mesa and Rubio Armendáriz, 2003). According to the results of several studies 20% of the total nitrate intake comes from the consumption of drinking water (White, 1983). Nitrate and nitrite can occur in drinking water mainly as a result of intensive agricultural activities. Contamination of soil with nitrogen-containing fertilizers, including anhydrous ammonia as well as animal or human natural organic wastes can raise the

concentration of nitrate in water. Nitrate-containing compounds present in the soil are generally soluble and readily migrate into groundwater. As nitrite is easily oxidized to nitrate, nitrite levels in water are usually low, and nitrate is the compound predominantly found in groundwater and surface waters. Water in highly polluted wells may also contain nitrites at elevated levels. To guaranteei drinking water safety, maximum allowable concentrations have been established for nitrate and nitrite, being 50 mg/l and 0,5 mg/l, respectively (EC, 1998). Contamination of drinking water with nitrates is a global problem. Studies have showed that in China, Botswana, Turkey, Senegal, and Mexico, private well water nitrate levels exceeded the WHO guideline value of 50 mg/l, in some cases the levels of nitrate-N were over 68 mg/l (WHO 2004). At the same time, all bottled water samples complied with legislative requirements, being therefore usable for the preparation of infant foods. The problem of high nitrates in water can be solved by using artesian wells as a substitute for draw wells as in artesian wells the water is taken from deep water layers in which the contamination is generally low.

A Finnish study in 1984 reported that only 0.3% of analyzed samples exceeded nitrate levels of 30 mg/l (Lahermo, 1988). In Denmark and in Great Britain the reported concentrations of nitrates in drinking water were 13 and 14 mg/l, respectively (Kampmann, 1983, Kinght *et al.* 1987).

Short-term exposure to drinking water with a nitrate level at or just above the health standard of 10 mg/l nitrate-N is a potential health problem primarily for infants. Infants and small children consume large quantities of water relative to their body weight, especially if water is used to mix powdered or concentrated milk formulae or juices. Also, the immature digestive system of infants is more vulnerable to the reduction of nitrate to nitrite (Spalding and Exner 1993). During many years studies in different countries have reported thousands of cases of children with nitrate-nitric methaemoglobinemia and more than hundred children have died (Hayes, 2001). Mild toxicoses were reported when the nitrate concentration was 80–100 mg/l in water used for infant food preparation.

Nitrate can be removed from drinking water by three methods: distillation, reverse osmosis, and ion exchange. Heating or boiling are not applicable for reducing nitrate contents, and the concentration of nitrate in water even increases during boiling due to evaporation of 15–25% of water. Mechanical filters or chemical disinfection do not remove nitrate from water. The distillation process involves heating the water to boiling temperature and following collecting and condensing the steam by means of a metal coil. Nearly 100% of the nitrate-N can be removed in this process. In the reverse osmosis process, pressure is applied to water to force it through a semi-permeable membrane. The membrane filters out most of the impurities as the water passes through. It is known that 85 to 95% of the nitrate can be removed with reverse osmosis. Actual removal rates may vary, depending on the initial quality of the

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water, the system pressure, and water temperature. For the nitrate removal process, special anion exchange resins are used that exchange chloride ions for nitrate and sulfate ions in the water as it passes through the resin. Since most anion exchange resins have a higher selectivity for sulfate than nitrate, the level of sulfate in the water is an important factor in the efficiency of an ion exchange system for removing nitrates (Jasa *et al.* 2006).

10.4. TOXICITY OF NITRATE AND NITRITE

The health aspects can be divided into acute toxicity and the effects of chronic exposure.

The nitrate ion has a low level of acute toxicity, but when transformed into nitrite in food or human organism, it may constitute a health problem. The reduction of nitrate to nitrite may take place in the presence of bacteria or enzyme nitrate reductase, and in contact with metals. Nitrite is unstable at acidic pH values at which it can disproportionate into nitrate and nitrogen oxide or react with food components including amines, phenols and thiols (Hill, 1996). It has been estimated that 5 to 8% of the nitrate from the diet may be reduced to nitrite by the microflora in the oral cavity (Mensinga *et al*., 2003). It has only recently been discovered that nitrate is manufactured endogenously in mammals by the oxidation of nitric oxide and that the nitrate formed has the potential for disinfecting the food we eat (Benjamin, 2000; Archer, 2002).

Nitrite has higher acute toxicity than nitrate. As an unstable ion it undergoes series of reactions when added to food. In an acidic environment, nitrite is converted into nitrous acid, which decomposes to nitric oxide. Nitric oxide, being an important product from the standpoint of colour fixation in cured meat, reacts with myoglobin to produce a red pigment – nitrosomyoglobin. The intake of nitrite is normally low compared to the dose that is acutely toxic, but nitrite in food is considered primarily causing health problems because its presence both in food and in the body may lead to the formation of carcinogenic nitrosoamines (JECFA, 1996; Vermeer *et al*., 1998) and the clinical symptom of methemoglobinemia (WHO, 1995; Sanchez-Echaniz and Benito-Fernández, 2001).

The principal mechanism of nitrite acute toxicity is the oxidation of ferrous II ion (Fe^{2+}) in oxyhaemoglobin (Hb) to ferric III ion (Fe^{3+}) to produce methaemoglobin (Met-Hb). Methaemoglobin is unable to reversibly bind and transport oxygen. Over 10% Met-Hb of the total Hb causes cyanosis – the lips and skin becomes bluish-grey and the blood is chocolate brown in colour. The generally accepted lethal level of Met-Hb is 60% of the total Hb. Infants fed on infant formula, which is mixed with high-nitrate well water are particularly susceptible to methaemoglobinemia because of their high fluid intake per kg of body weight. In infant organism the upper gastrointestinal tract is heavily

colonized by bacteria able to reduce nitrate to nitrite due to the lack of gastric acidity (Hill, 1996). Consumption of vegetables containing high level of nitrates and incorrect storage of homemade vegetable purees have also been found to be potential causes of infant methaemoglobinemia (Sanchez-Echaniz and Benito-Fernández, 2001).

N-nitroso compounds have been shown to be carcinogenic to multiple organs in several animal species, including higher primates (Eichholzer and Gutzwiller, 2003). Although carcinogenicity of N-nitrosoamines in humans cannot be tested, epidemiological studies have suggested a possible link to the incidence of various cancers in humans (Knekt *et al*., 1999; Pegg and Shahidi, 2000). On the basis of the available data, a relationship between dietary N-nitroso compounds, nitrite and nitrate cannot be concluded or excluded. It is possible that other factors such as intake of vegetables, fruit and nitrosation inhibitors, or some other constituent of cured meat and salted fish could partly be responsible for the observed associations (Eichholzer and Gutzwiller, 2003).

The European Commission's Scientific Committee for Food (SCF) considered the possible influence of nitrate and nitrite on human health and set acceptable daily intake (ADI) values for nitrate and nitrite in 1990. The ADIs were reviewed in 1995, which resulted in present value for nitrate of 0–3.7 mg nitrate per kg of body weight and for nitrite 0–0.06 mg nitrite per kg of body weight established in 1995 (EU Scientific Committee for Food, 1995).

10.5. DIETARY EXPOSURE

Many assessments of nitrate and nitrite intakes have been reported in the literature, but most of them are difficult to interpret or compare, as all details of how they were conducted are not available. Ideally, all sources of nitrate and nitrite should be included in an intake assessment; however, in many cases only food and drinking water, known to be the major contributors to the overall exposure, have been included.

Dietary exposure to nitrate is very variable between individuals, regions and countries. The intakes of nitrate and nitrite from food were calculated at a global level on the basis of mean food consumption data and the mean concentrations in foods by WHO (2003). Intake from drinking water was added to the exposure obtained from food.

The estimated intakes of nitrate and nitrite from sources other than food additives are below their respective ADIs. The intakes of nitrates range from 70% of the ADI for the European diet and between 10–25% for other diets. The nitrite intakes represented 50% of the ADI for the Middle Eastern and Far Eastern diets and 40% of the ADI for the African, Latin American and European diets. Vegetables, including potatoes, were the main contributors to nitrate intake, accounting for 30–90% of total estimated value. Drinking water

was the second highest contributor to the exposure of nitrates making up 5–40% of the intake (WHO, 2003).

The data for nitrate exposure calculated by WHO are in good agreement with the works by other authors. The mean total intake of nitrate per person estimated by different authors in Europe ranges between 50 and 140 mg per day and in the USA about 40 to 100 mg per day (Ysart *et al.,* 1999; Mensinga *et al*., 2003). According to British intake estimations dietary exposure to nitrates for 1997 was 52 mg/day compared with 68 mg/day in 1994. The decrease can be partly explained by lower nitrate concentrations in green vegetables (Harrison *et al*., 2005). The estimated intakes of nitrate show that vegetables are the major contributors to total dietary intake, followed by water.

Several authors have tried to estimate nitrate intake by children, as infants are more susceptible to health implications possibly caused by nitrites and nitrates. The daily nitrate intake by Finnish adolescents and children was 48 mg (Penttilä, 1995). In a Polish study (Wawrzyniak *et al*., 2003), nitrates intake for 1- to 6-years-old children exceeded ADI twice. Nitrate intake by Estonian 1- to 6-years-old children was found to be 28 mg/day (46% of ADI), the mean nitrate intake from infant food by children aged 6 to 12 months was 7.8 mg/day (22% of ADI) (Tamme *et al*., 2006).

According to the WHO data (2003) the major contributors to nitrite intake are also sources other than food additives, including cereals, beverages and water. Cereals were the main contributor to nitrite intake, accounting for 35–60% of the estimated intake. Drinking water was the second highest contributor to the estimated intakes of 20–40% of nitrite (WHO, 2003). The mean nitrite exposure for whole population in 1997 was 1.3 mg/day, as compared with 1,7 mg/day in 1994 in UK (Harrison *et al*., 2005). Estimation of the impact of consumption of meat products, including cured meat, to overall nitrite exposure varies a lot among different authors. In the countries where consumption of cured meat is high, large part of nitrite intake has been reported to come from nitrite as food additive. In Polish study meat products supplied 98% of dietary nitrites, nitrite intake being less than 88% of ADI for 1–6-year-old children (Wawrzyniak *et al*., 1999; Wawrzyniak *et al*., 2003). According to Japanese studies (Murata *et al*., 2001; Murata *et al*., 2002) meat products provided 98% of nitrite intake. A Finnish study reported 5.3 mg/day for nitrite (150% of the ADI), and 95% of nitrite was derived from meat products (Dich *et al*., 1996). Especially for children ADI can easily be exceeded in result of frequent consumption of nitrite-treated sausages (Reinik *et al*., 2005).

The results of studies of the intake of nitrate and nitrite from all dietary sources showed mean consumptions of both nitrate and nitrite below the ADIs, although some consumers at high percentiles exceeded the ADI for both compounds (WHO, 2003).

10.6. REGULATIONS

In order to protect human health and taking into account the possible association of nitrates and nitrites in food with the formation of carcinogenic N-nitrosoamines, the level of these compounds should be reduced to as low as reasonably achievable (ALARA principle). At present time, regulatory limits for nitrates in food have been established in the EU only for spinach, lettuce and baby foods. The maximum level of nitrates in baby foods and processed cereal-based baby foods for infants and young children should not excced 200 mg/kg . The content of nitrates in spinach is limited to 2000–3000 mg/kg, lettuce 2500–4500 mg/kg and "iceberg" type lettuce 2000–2500 mg/kg (European Commission, 2006). The regulatory limit depends on the harvesting season and place of growth of the vegetables – highest concentrations are permitted in plants grown in winter period and/or greenhouse conditions.

The nitrate content of drinking water is limited to 50 mg/l by the current regulatory standard on the quality of water intended for human consumption 98/83/EEC (European Commission, 1998). The EU standard is based on the World Health Organisation's guideline value for drinking water, which is also 50 mg/l. Limit value for nitrite is set to 0.5 mg/l.

10.7. CONCLUSIONS

Nitrate and nitrite can be found in food as naturally occurring compounds, vegetables and drinking water being substantial sources of nitrate intake. They are also used as food additives in the processing of meat products due to their ability to inhibit the growth of *Clostridium* species and to give meat characteristic pink colour, texture and flavour. Although the permitted levels of added nitrates and nitrites have been decreased during last years, the other factors such as growing emissions or nitrogen oxides from fuel combustion, increased sewage recycling and use of nitrate-based fertilizers have led to net increase in exposure to nitrate in several countries.

When nitrate is transformed into nitrite in food or human organism, it may constitute a health problem because the presence of nitrite both in food and in the body may lead to the formation of carcinogenic nitrosoamines. Ingestion of high quantities of nitrates or nitrites by babies may result in methaemoglobinemia.

Nitrate levels, present in vegetables naturally via the nitrogen cycle, are affected by factors such as plant species, climatic and light conditions, soil characteristics and fertilization regime. The concentrations of nitrates in vegetables can vary enormously ranging from below 10 and up to 10 000 mg/kg. It has been estimated that vegetables constitute a major source of human exposure to nitrates contributing up to 92% of the average daily intake. The naturally

occurring nitrate concentrations in other food commodities, such as milk and milk products, cereal products, fresh meat and fruits are generally much lower. Drinking water may be an essential source of nitrates for some consumers. Natural levels of nitrites in food are low, usually remaining under the limit of detection. ADI values established for nitrates and nitrites are not exceeded for the majority of consumers. Exceedings of ADI may occur more easily among the risk groups – small children and vegetarians in the case of nitrates and people consuming large quantities of products containing added nitrites.

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2. L. Saarinen, K. Ilmoja, L. Margna, M. Reinik. (1995) Methods of Monitoring Work Environment. *Publications on Chemistry, University of Tartu* 23, 127–132.
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4. S. Jurtchenko, T. Tenno, U. Mölder and M. Reinik. (2002) Determination of volatile N-nitrosamines by gas chromatography–mass spectrometry with positive-ion chemical ionization. *Proceedings of the Estonian Academy of Sciences* 51, 169–184.

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Olulisemad publikatsioonid

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2. L. Saarinen, K. Ilmoja, L. Margna, M. Reinik. (1995) Methods of Monitoring Work Environment. *Publications on Chemistry, University of Tartu* 23, 127–132.
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