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Tesis Doctoral Internacional

Analysis of Nitrite and Nitrate in Foodstuffs

Method development, occurrence, regulation, metrological aspects and exposure

Análisis de Nitrito y Nitrato en Alimentos

Desarrollo de métodos, contenido, reglamentación, aspectos metrológicos y exposición

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Department of Preventive Medicine and Public Health, Food Science, Toxicology and Forensic Medicine - University of Valencia

Doctoral Thesis

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DECLARE: that the work entitled Analysis of Nitrite and Nitrate in Foodstuffs. Method development, occurrence, regulation, metrological aspects and exposure presented by Leonardo Merino has been developed under our direction/supervision and accomplishes the requisites for opting to the Doctor degree.

and sign this declaration in Uppsala and Valencia on the 10th of April of 2017.

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Analysis of Nitrite and Nitrate in Foodstuffs. Method Development, Occurrence, Regulation, Metrological Aspects and Exposure.

Abstract

Nitrite and nitrate occurrence in food and suitability as food additive are a controversial issue. Nitrite is an approved additive considered a foremost curing ingredient for the preservation of meat products. Nitrate is a natural constituent of the human diet that, however, raises concern for its suggested potential harmfulness related to cyanosis condition, carcinogenesis and environmental contamination. Therefore, reliable nitrite/nitrate determination is necessary for three main reasons: a) to assess compliance with current regulations on additives and contaminants, b) to determine the content of nitrite/nitrate in individual foodstuffs, diets and water and c) to study the fate of nitrite/nitrate in biological fluids.

This thesis summarizes the work on analytical research, regulations enforcement and exposure estimate on nitrite/nitrate in vegetables and meat products carried out at the Swedish National Food Agency.

The work undertook a series of studies: First, select and standardize an HPLC-Ion Chromatography method for the determination of nitrite/nitrate in foodstuffs. This method was used to generate data of the occurrence of nitrate in Swedish-produced vegetables contributing to the discussion on t he European regulation setting maximum levels for nitrate in lettuce and spinach. Second, an alternative environmental friendly spectrophotometric method was developed to gather data on the content of nitrite/nitrate in meat products in the Swedish market. Actually, these analytical methods are European (CEN) and Nordic (NMKL) official methods, respectively.

The collected data plus data from consumption of vegetables, fruit and drinking water was integrated with a nationwide food consumption survey (4-day food diary) to estimate the total intake of nitrate and nitrite in Swedish children and adults. The Acceptable Dairy Intake (ADI) approach applied, which included the nitrite intake from all dietary sources and the endogenous nitrate-nitrite conversion, suggest that the international approach currently used to estimate the ADI causes an underestimation of the real nitrite intake with potential health-relevant risk.

Análisis de Nitrito y Nitrato en Alimentos. Desarrollo de Métodos, Contenido, Aspectos Metrológicos, Reglamentación y Exposición

Resumen

La presencia y la inocuidad de los nitritos y nitratos son un tema controvertido. El nitrito es un aditivo autorizado considerado como un ingrediente importante para la conservación de productos cárnicos. El nitrato es un componente natural de la dieta humana que, sin embargo, despierta preocupación por su supuesta potencial nocividad relacionada con la cianosis, carcinogénesis y contaminación ambiental. Por lo tanto, determinaciones veraces de nitrito/nitrato son necesarias por tres razones principales: a) vigilar el cumplimiento de los reglamentos actuales sobre aditivos y contaminantes, b) determinar el contenido de nitrito/nitrato en alimentos, dietas y agua potable y c) estudiar el comportamiento de nitrito/nitrato en fluidos biológicos.

Esta tesis resume el trabajo de investigación analítica, control oficial del reglamento y estimación de la exposición de nitrito/nitrato en alimentos realizado en la Agencia Nacional de Alimentos de Suecia.

Este trabajo comprende una serie de estudios para: primero, seleccionar y normalizar un método ion cromatográfico para la determinación de nitrito/nitrato en alimentos. El método fue utilizado para obtener datos sobre la presencia de nitratos en vegetales cultivados en Suecia. Esta información contribuyó a la discusión en la Comunidad Europea para establecer máximos límites permitidos de nitratos en lechugas y espinacas. Segundo, desarrollar un método espectrofotométrico alternativo, ambientalmente inocuo, para determinar el contenido de nitrito/nitrato in productos cárnicos en el mercado sueco. Actualmente, ambos métodos han sido adoptados como métodos oficiales Europeo (CEN) y Nórdico (NMKL), respectivamente.

La información obtenida del contenido de nitrito/nitrato en productos cárnicos junto con información del consumo de vegetales, frutas y agua potable obtenido de una encuesta nacional de consumo diario de alimentos (4 días) fueron integrados calculándose la ingesta total de nitrito/nitrato en la población infantil sueca. El procedimiento utilizado para el cálculo de la Ingesta Diaria Aceptable (IDA), el cual incluye la ingesta de nitrito de todas las fuentes dietéticas y la conversión endógena de nitrato a nitrito, sugiere que el procedimiento internacional usado actualmente para estimar el IDA determina una subestimación de la ingesta real de nitrito con sus consecuentes riesgos potenciales.

Anàlisi de nitrit i nitrat en Aliments. Desenvolupament de Mètodes, Contingut, aspectes Metrològics, Reglamentació i Exposició

Resum

La presència i la innocuïtat dels nitrits i els nitrats és un controvertit tema de les Nacions Unides. El nitrit és un additiu autoritzat i considerat com un important ingredient per la conservació dels productes carnis. El nitrat és un component natural de la dieta humana que, però, desperta preocupació per la seva suposada nocivitat potencial rel.lacionada amb cianosi, carcinogènesi i contaminació ambiental. Per tant, les determinacions exactes de nitrit/nitrat són necessàries per tres raons principals: a) Complir amb els reglaments actuals sobre additius i contaminants, b) Determinar el contingut de nitrit/nitrat en aliments, dietes i aigua potables i c) estudiar el comportament de nitrit/nitrat en fluids biològics.

Aquesta tesi repren el treball de recerca analítica, control oficial del Reglament i estimació de l'exposició de nitrit/nitrat en aliments realitzat a l'Agència Nacional d'Aliments de Suècia.

El treball va dur a terme una sèrie d'estudis: En primer lloc, seleccionar i estandarditzar un mètode HPLC de cromatografia iònica per a la determinació dels nitrits / nitrats en els productes alimentaris. Aquest mètode va ser utilitzat per generar les dades de l'ocurrència de nitrats en les hortalisses produïdes a Suècia i contribueixen a la discussió sobre l'establiment de nivells màxims de nitrats dels enciams i els espinacs regulació europea. En segon lloc, un mètode espectrofotomètric respectuós del medi ambient és va desenvolupar per tal de recopilar dades sobre el contingut de nitrit / nitrat en els productes carnis al mercat suec. En realitat, aquests mètodes analítics són mètodes oficials europeus (CEN) i dels països nòrdics (NMKL), respectivament.

Les dades recollides, a més de les dades de consum de verdures, fruites i aigua potable és va integrar amb una enquesta de consum d'aliments a tot el país (aliment diari de 4 dies) per tal de calcular la ingesta total de nitrat i nitrit en nens suecs i adults. El càlcul de l´ingesta diària admissible (IDA), que incloïa la ingesta de nitrit de totes les fonts dietètiques i la conversió de nitrat-nitrit endògena, suggereix que l'enfocament internacional que s'utilitza actualment per tal d´ estimar l'IDA provoca una subestimació de la ingesta real de nitrit amb el conseqüent risc potencial per a la salut.

A mi hijo mayor ausente Rodolfo Leonardo

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Contents

List of publications

The present thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. Merino, L., Örnemark, U., Toldrá, F. (2017). Analysis of Nitrite and Nitrate in Foods: Overview of Chemical, Regulatory and Analytical Aspects. In Fidel Toldrá, editor: Advances in Food and Nutrition Research, Vol. 81, AFNR, UK: Academic Press, pp. 65-107

II. Merino, L., Edberg, U., Fuchs, G., Åman, P. (2000). Liquid Chromatographic Determination of Residual Nitrite/Nitrate in Foodstuffs: NMKL Collaborative Study. Journal of AOAC. Vol. 83, No. 2, 356-375

III. Merino, L., Darnerud, PO., Edberg, U., Åman, P., Castillo, MdP. (2006). Levels of nitrate in Swedish lettuce and spinach over the past ten years. Food Additives and Contaminants 23(12): 1283-1289

IV. Merino, L. (2009). Development and Validation of a Method for Determination of Residual Nitrite/Nitrate in Foodstuffs and Water after Zinc Reduction. Food Analytical Methods Vol. 2, 212–220

V. Larsson K., Darnerud PO., Ilbäck N.G., Merino L. (2011). Estimated dietary intake of nitrite and nitrate in Swedish children. Food Additives and Contaminants: Part A Vol. 28, No. 5, 659-666

VI. Merino, L., Darnerud PO., Toldrá, F., Ilbäck, N.G. (2016). Time-dependent depletion of nitrite in pork-beef and chicken meat products affects nitrite intake estimation. Food Additives and Contaminants: Part A, Vol. 33, No. 2, 186-192

Key to abbreviations

ADI	Acceptable Diary Intake
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
CEN	European Committee for Standardisation
EC	European Commission
EU	European Union
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
GAP	Good Agricultural Practice
GLP	Good Laboratory Practice
GUM	Guide to the Expression of Uncertainty in Measurement
Hb	Haemoglobin
HPLC	High Performance Liquid Chromatography
IUPAC	International Union of Pure and Applied Chemistry
IP	Integrated Production
IS	International system of units
ISO	International Standardization Organization
JCGM	Join Committee for Guides in Metrology
JRC	Join Research Centre
LOD	Limit of Detection
ML	Maximum Level
MU	Measurement uncertainty
MetHb	Methaehemoglobin
NMKL	Nordic Committee of Food Analysis
NFA	Swedish National Food Agency
OECD	Organization for Economic Co-operation and Development
PT	Proficiency testing
RM	Reference material
RSD_R	Reproducibility Standard Deviation
SD	Standard Deviation
WHO	World Health Organization

1. Introduction

1. Introduction

Accurate nitrite and nitrate determination is important to a) assess compliance with current regulations on additives and contaminants, b) to determine the content of nitrite/nitrate in individual foodstuffs, water and diets and c) to study the fate of nitrite/nitrate in biological fluids. The various applications are characterized by well-defined requirements that the analyst has to take into consideration to demonstrate that the method is suitable for the purpose of the analysis

Nitrite and nitrate are key intermediates of various biochemical reactions in the nitrogen cycle. Nitrate is the most fully oxidised nitrogen compound and therefore stable to oxidation, but potentially a strong oxidising agent. In hypoxic conditions, nitrate can be reduced and then acts as an oxidising agent, with release of energy. Nitrite can be oxidised to nitrate by strong chemical oxidants or by nitrifying bacteria or reduced to nitrogen oxides through several enzymatic and non-enzymatic pathways, producing energy. Because of their high bioavailability, nitrite/nitrate are capable of playing complicated and contradictory roles as food additive *(substance added intentionally to foodstuffs to perform certain technological functions, (European Union, 1988)),* natural contaminants *(chemical present in the environment that is either a part of a food's natural growing conditions or that cannot be controlled by intervention (NZFSA, 2009)* and/or contaminant *(substances that have not been intentionally added to food (European Union, 2008)*. Emerging studies strengthen the new understanding of the role of nitrate and nitrite in the human body, motivating revision of the long-held view that these ions pose a health risk. Research has shown that there are indisputable benefits of nitrite and nitrate in promoting human health, suggesting that these ions could be considered indispensable dietary components and even used as possible therapeutic agents (Bryan et al., 2015, Weitzberg et al., 2013). The scientific debate is ongoing.

During the past two decades, the analytical community has devoted considerable efforts to improving concepts and practices of quality assurance and quality control, as part of the work to develop the science of chemical measurement. The general aim is to devise a conceptual and practical scheme for quality. An important milestone has been the introduction of the uncertainty concept (GUM, 2008), a new approach aimed at expressing the quality of the measurement result rather than that of the method. To avoid confusion with previous concepts and ensure that the same concepts are understood in the same way by all parties concerned, efforts to harmonise terminology relating to fundamental metrological concepts have been undertaken by various patronising organisations (JCGM, 2012). This introduction presents a background describing a number of conceptual and practical aspects relating to a single-laboratory validation approach. Validation is unanimously recognised as an important tool to demonstrate that the performance of a particular analytical system (method, equipment, analyst etc.) is satisfactory. The ultimate goal of validation work is to ensure that the results produced by the analytical system under study are sufficiently reliable.

The specific aim of this background is to present critical information on some principles supporting a pragmatic approach to choosing an analytical method for the determination of nitrite/nitrate. The complex matrix of foodstuffs, diets and biological samples commonly limits the utility of various methods for nitrite/nitrate determination. There are generally many potentially applicable methods available from standards bodies, e.g. CEN (EU), AOAC (USA), ISO, Codex (international) and NMKL (Nordic). Standardised methods and a large number of other methods published in the scientific literature can be considered by analysts when selecting the most potentially appropriate method for their requirements. Many factors are involved in deciding the suitability of an analytical method for analysis of nitrite/nitrate. After getting a clear understanding of why the analytical data are required, the available analytical resources and the quality of the data needed, a return to considering the background chemical reactivity of nitrite/nitrate puts the analyst on the right path to building a solid foundation for the selection or development of the most appropriate method. In this section, a strategy based on assessing advantages and disadvantages of five derivatisation reactions that could be utilized in the selection and /or developing of analytical methods is presented.

a. Occurrence of nitrate and nitrite. Current EU regulations setting maximum limits for nitrate and nitrite in meat products, vegetables and water

1.1.1. Nitrate/nitrite in the environment

The natural occurrence of nitrite and nitrate in the ecosystem is influenced by their interactions with other molecules and ions included in the nitrogen cycle. Bacteria play a dominant role in biological reactions, because they are equipped with enzymes for catalysing the various reactions. The cycle includes six major processes (Figure 1):

(1) Assimilation of inorganic forms (primarily ammonia and nitrate) by plants and micro-organisms to form organic nitrogen, *e.g.* amino acids, proteins and nucleic acids.
(2) Heterotrophic conversion of organic nitrogen from one organism (food or prey) to another organism (consumer or predator).
(3) Ammonification, the decomposition of organic nitrogen to ammonia.
(4) Nitrification, the oxidation of ammonia to nitrite and nitrate.
(5) Denitrification, the bacterial reduction of nitrate to nitrogen oxides and molecular nitrogen under anoxic conditions.
(6) Nitrogen fixation, the reduction of nitrogen gas to ammonia and organic nitrogen by various organisms.

As a broad generalisation, nitrogen (N_2) is reduced to ammonia (NH_3), a process partly governed by specific bacteria, and then quickly incorporated into DNA, RNA, proteins and other organic nitrogen compounds. Nitrification is a two-step process. Ammonium (NH_4^+) is initially oxidised to nitrite (NO_2^-), which is then converted to nitrate (NO_3^-). The cycle is completed by the denitrification process, where nitrate is sequentially reduced to nitrite, nitric oxide (NO), nitrous oxide

(N_2O) and finally nitrogen gas (N_2), which is released back into the atmosphere (NAS, 1978, Weitzberg et al., 2013).

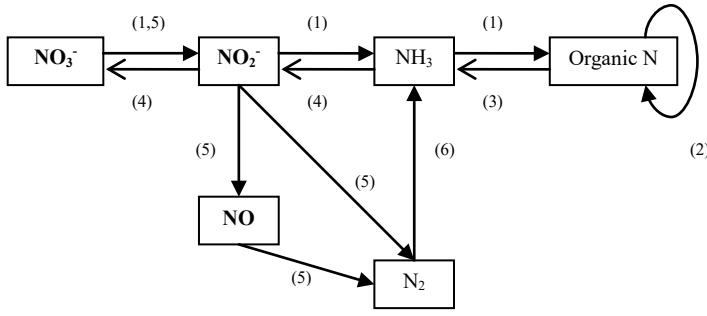

Fig. 1 Schematic representation of the biological nitrogen cycle, showing major molecular transformations: (1) Assimilation, (2) Heterotrophic conversion, (3) Ammonification, (4) Nitrification (5) Denitrification and (6) Nitrogen fixation. Slightly modified from National Research Council (1978).

1.1.2 Sources of nitrite/nitrate in the human body

There are two sources of nitrate and nitrite in the body: exogenous (external) and endogenous (internal). Human exposure to nitrate is mainly from the exogenous source, while exposure to nitrite is mainly endogenous, through nitrate metabolism. The intake of exogenous nitrates and nitrites is mainly via food, particularly vegetables, meat products and drinking water (EFSA, 2008).

1.1.2.1 Exogenous sources

1.1.2.1.1 Nitrite/nitrate as additives to meat products and other food

Nitrite and nitrate are used as food additives in cured meat to stabilise the colour of red meat, inhibit spoilage and growth of food poisoning organisms and contribute to flavour development. Nitrate is also used in the preservation of fish products and the production of cheese. Since 1995, nitrate and nitrite are listed as officially accepted preservatives in European Union (EU) legislation (European Union, 1995).

Following recommendations by the European Food Safety Authority (EFSA), the level of nitrate and nitrite in meat products is set in amended legislation as 'added amount' rather than 'residual amount', since it is the added amount that contributes to the inhibitory activity against *Clostridium botulinum* (European Union, 2006). The rationale for this change can be found in studies by Sebraneck et al. (1973, 1978), who used N-labelled nitrite to study inhibition of *C. botulinum* toxin by the water-soluble fraction of cured meat, i.e. the low molecular weight fraction representing conversion of a substantial amount of nitrite to bound-nitrite compounds. They found that incubation of this fraction with *C. botulinum* type A

and type B did not inhibit spore growth or production of toxin, which proved lethal to mice.

The limit for addition of nitrate (E 251 = $NaNO_3$, E 252 = KNO_3; all additive products expressed as $NaNO_2$) during processing ranges from 100 to 150 mg/kg for processed meat and cheese products. In addition, the current legislation allows certain traditional products to be produced based on residual amounts, with the maximum residual amount ranging from 10 to 300 mg/kg for traditionally cured meat products (European Union, 20011b).

The statutory limit on the use of nitrite (E 249 = KNO_2, E 250 = $NaNO_2$; expressed as $NaNO_2$) in meat products is 150 mg/kg and in heat-treated processed meat 100-150 mg/kg. Maximum accepted residual amount for various traditional products ranges from 50 to 180 mg/kg (European Union, 2011).

Table 1. Maximum levels for nitrites and nitrates (expressed as $NaNO_2$) in processed meat and cheese and cheese products. Adapted from Commission Regulation (EU) No 1129/2011. N.A. = Not applicable.

Name/product	Maximum permitted level (mg/kg)	Maximum residual level (mg/kg)
Non-heat-treated processed meat		
KNO_2, $NaNO_2$, KNO_3, $NaNO_3$	150	N.A.
Heat-treated processed meat		
KNO_2, $NaNO_2$, KNO_3, $NaNO_3$	100 - 150	N.A.
Traditional immersion-cured products (Meat products cured by immersion in a curing solution containing nitrites and/or nitrates, salt and other components)		
KNO_2, $NaNO_2$ KNO_3, $NaNO_3$	N.A.	50 - 175 10 - 300
Traditional dry-cured meat products (Dry curing involves dry application of a curing mixture containing nitrites and/or nitrates, salt and other components to the surface of meat, followed by a period of stabilisation/maturation)		
KNO_2, $NaNO_2$ KNO_3, $NaNO_3$	N.A.	50 - 175 250
Other traditionally cured meat products (Immersion and dry curing processes used in combination or where nitrite and/or nitrate are included in a compound product or where the curing solution is injected into the product prior to cooking)		
KNO_2, $NaNO_2$ KNO_3, $NaNO_3$	N.A.	50 - 180 10 - 300

Cheese and cheese products		
KNO_3, $NaNO_3$	150	N.A.
Dairy analogues (only dairy-based cheese analogue)		
KNO_3, $NaNO_3$	150	N.A.

During the 1970s, the debate on carcinogenic nitrosamines resulted in strong pressure to decrease the use of nitrite as a food additive, in order to reduce the risk of nitrosamine formation and thereby the potential health risks (Sindelar et al., 2012). However, opinion differs within the EU regarding the need to use nitrite in meat processing. For example, Denmark maintains national legislation specifying a maximum level of 60 mg/kg, instead of 150 mg/kg according to EU legislation. Danish authorities state that the necessary preservative effect and microbiological safety can be achieved at the lower maximum level in the Danish regulations, while at the same time reducing the risk of nitrosamine formation (European Union, 2010; Hermann, 2014).

The World Cancer Research Fund (WCRF) recommendation on avoiding consumption of processed meat, which is the main source on nitrite intake, is again putting the subject on the agenda for the scientific community and the regulatory authorities (WCRF, 2007).

1.1.2.1.2 Nitrate/nitrite as natural contaminant (vegetables)

The amount of nitrate in vegetables depends on genetic factors, environmental variables (season, light, temperature etc.) and agricultural practices (Maynard et al., 1976). However, most vegetables usually have low levels of nitrate, with leafy vegetables clearly having the highest levels (EFSA, 2008). In contrast, only trace levels of nitrite are present in vegetables (at mass fractions well below 10 mg/kg). Exceptions to this are poorly stored vegetables or vegetables stored for extended periods, probably for bacterial reduction of nitrate to nitrite.

In order to protect public health, reduce possible presence of contaminants and ensure market unity, the European Commission has established maximum levels for nitrate in vegetables (European Union, 1997). So far, the current legislation has been amended several times to take into account differences between crop varieties, seasons, growing conditions and processing methods. The current maximum levels were laid down in Commission Regulation (EC) No. 1258/2011 setting maximum levels for nitrate in the following five food commodities: fresh spinach, preserved, deep-frozen or frozen spinach, fresh lettuce (protected and open-grown lettuce), iceberg-type lettuce, rocket and processed cereal-based foods and baby foods for infants and young children (European Union, 2011a). All maximum levels fall within the interval 200-7000 mg nitrate/kg fresh weight.

Table 2. Maximum levels for nitrate in lettuce and spinach. Adapted from Commission Regulation (EU) No. 1258/2011 (European Union, 2011)

Foodstuffs		Maximum level (mg NO_3/kg)	
1.1	Fresh spinach (*Spinacia oleracea*)		3500
1.2	Preserved, deep-frozen or frozen spinach		2000
1.3	Fresh lettuce (*Lactuca sativa* L.) protected and open-grown lettuce, excluding lettuce listed in point 1.4	Harvested 1 October to 31 March: lettuce grown under cover lettuce grown in the open air	 5000 4000
		Harvested 1 April to 30 September: lettuce grown under cover lettuce grown in the open air	 4000 3000
1.4	'Iceberg' type lettuce	Lettuce grown under cover Lettuce grown in the open air	2500 2000
1.5	Rucola (*Eruca sativa*), *Diplotaxis* spp., *Brassica tenuifolia*, *Sisymbrium tenuifolium*)	Harvested 1 October to 31 March: Harvested 1 April to 30 September:	7000 6000
1.6	Processed cereal-based foods and baby foods for infants and young children		200

1.1.2.1.3 Nitrate/nitrite as contaminant (drinking water)

The nitrate concentration in surface water and groundwater is normally low (1-50 mg/L), but increasing levels have been detected in many European countries since the 1950s (Dudley, 1990). Water pollution by nitrates has worsened since the introduction of intensive farming methods (including excess application of chemical fertilizer and manures) and livestock production. Nitrate contamination of aquifers (eutrophication) occurs as a result of leaching or runoff from agricultural land and contamination from sewage discharge (human and animal wastes) (European Union, 1991).

In the case of drinking water, the primary health concern in legislation regarding nitrate and nitrite is protection against methaemoglobinaemia in infants. This disorder is characterized by reduced ability of the blood to carry oxygen because of reduced levels of haemoglobin (L'hirondel J.L. 2001). Affected infants show signs of blueness around the mouth, hands and feet, hence the common name 'blue baby syndrome' (World Health Organization, 2009). This acute condition is potentially life-treating. Intake of high levels of nitrate with drinking water has also been associated with cancer and adverse reproductive outcomes such as spontaneous abortion and premature birth (Ward et al., 2005).

In 1998, the EU laid down its Drinking Water Directive (European Union, 1998) setting a maximum level of 50 mg/L for nitrate and 0.50 mg/L for nitrite. This

directive was later amended (European Union, 2009) with the establishment of technical specifications to ensure the quality and comparability of analytical results. Hence, implementation of the practices set out in ISO/IEC 17025 using validated methods, participation in proficiency testing (PT), use of reference materials (RM), statistical process control etc. is promoted. The current Commission Directive 2015/1787 (European Union, 2015) establishes that the limit of quantification (LOQ) of analytical methods used must, as a minimum, be capable of measuring concentrations equal to or lower than 30 % of the relevant maximum value, with a measurement uncertainty of not more than 15 and 20 % of the parametric value for nitrate and nitrite, respectively.

The European Council Directive concerning the protection of water against pollution by nitrates (European Union, 1991) encourages good agricultural practice (GAP) in order to reduce and prevent pollution from nitrogen compounds emitted by agricultural sources. Nitrogen is often the rate-limiting factor controlling plant growth. This means that, within limits, the more fertilizer added to the soil, the greater the crop yield. However, a sizeable proportion of the fertilizer used to ensure the rapid growth of crops is lost from the system, leading to water pollution (Addiscott, 2005). This relationship has been responsible for many current problems associated with nitrates and modern chemical farming. Encouraging the application of GAP is expected to avoid excessive use of fertilizer and thus reduce nitrate levels in certain vegetables and in surface waters and groundwater.

1.1.2.2 Endogenous sources of nitrite/nitrate

The endogenous sources of nitrite/nitrate in mammals are derived from oxidation of endogenous NO and the diet. Nitrate formation in human body was first mentioned in the early 1900s, but this synthesis was only confirmed in the mid-1980s. The non-

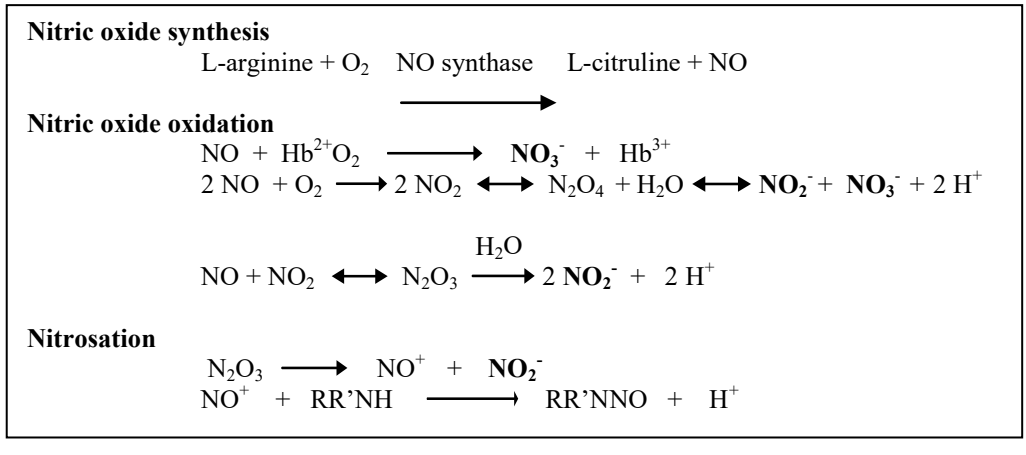

Fig. 2. L-arginine-NO pathway. Some reactions involving nitrate/nitrite as products

essential amino acid L-arginine is oxidised by molecular oxygen in the presence of NO synthase to L-citruline and nitric oxide. The nitric oxide formed participates in numerous reactions involving proteins and enzymes radicals, enzymes, oxyhaemoglobin, myoglobin, auto-oxidation etc. It has been demonstrated that nitrate and nitrite are the by-products of the L-arginine-nitric oxide pathway (Leaft et al*.,* 1989). See further (Figure 2).

It has been estimated that about 25 % of ingested nitrate is secreted in human saliva, of which about 20 % is reduced to nitrite by bacterial nitrate reductase, i.e. about 5 % of the overall dose of nitrate, clearly establishing saliva as a major site of nitrite production in the body (Walker, 1990). See Fig. 3 and Fig. 4.

After swallowing, the nitrite is reduced to NO and other nitrogen oxides via nitrous acid by a number of proteins and enzymes in in blood and tissue. These include xanthine oxidoreductase, deoxyhemoglobin and other globins, cytochrome P450, mitochondrial proteins, carbonic anhydrase, aldehyde oxidase, endothelial NO synthase, protons, polyphenols and vitamins E and C (Weitzberg et al., 2013).

For the average population, the dominant source of nitrate dietary exposure is vegetables (60-80 %). Other sources are drinking water (15-20 %) and cured meat (10-15 %), whereas the primary sources of nitrite intake are cured meat (39 %), baked goods and cereals (34 %) and vegetables (16 %). However, these exogenous sources of nitrite are of minor relevance in comparison with the endogenous formation of nitrite mainly occurring via reduction of dietary nitrate through the microbial action of saliva in the mouth (Figure 3). Approximately 83-85 % of human exposure depends on endogenous conversion of nitrate to nitrite (EFSA, 2008).

Due to exogenous sources of nitrite being overshadowed by endogenous reduction of secreted salivary nitrate to nitrite, it is of the utmost importance to determine the total exposure to nitrite from all food sources. While meat products are the single most important nitrite-containing food for the average consumer, nitrite formation in the body from dietary nitrate (vegetables and water) must also be considered. It has been suggested that any approach to estimate the Acceptable Daily Intake (ADI) that does not account for conversion of dietary nitrate results in underestimation of the true nitrite intake (Thomson et al., 2007; Leth et al., 2008; Menard et al., 2008).

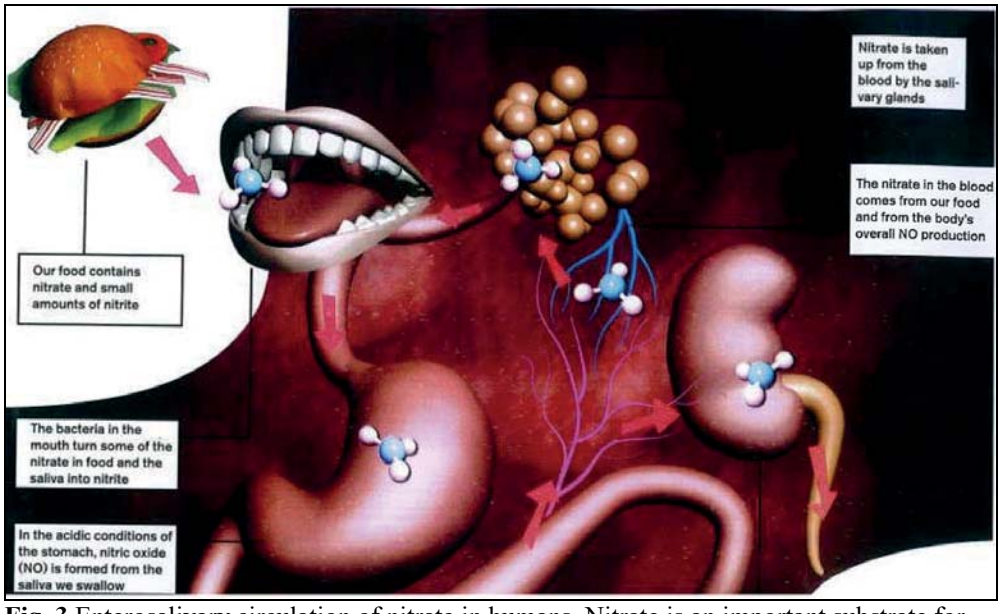

Fig. 3 Enterosalivary circulation of nitrate in humans. Nitrate is an important substrate for nitrite and nitric oxide (NO) production. Illustration by G. Elam (Dock, 2005). Reproduced with permission from Karolinska Institutet.

The discovery of the physiological role of nitric oxide as a regulator of many body functions (signalling agent in the cardiovascular system, marker for inflammation, host defence against numerous micro-organisms, gastric mucosal protection etc.) may have broad implications for the treatment of many disorders and is allowing the bad reputation of nitrate/nitrite to be reappraised (Moncada & Higgs, 1993; Lundberg et al., 2004). Moreover, new research over the past decade has provided evidence that nitrate and nitrite are not just inert metabolic end-products, but also enable an alternative and complementary pathway for formation of nitric oxide during physiological hypoxia (a pathological condition in which the body as a whole or a region of the body is deprived of adequate oxygen supply). This alternative source of nitric oxide, known as the nitrate-nitrite-nitric oxide pathway (Figure 4), ensures sufficient nitric oxide generation, which helps kill pathogenic bacteria, regulate the blood pressure by dilating the blood vessels and stimulate mucosal generation, thereby enhancing gastric protection (Lundberg et al., 2004, 2008).

Nitrite oxidation *(Oxyhaemoglobin)*

$$\mathbf{4NO_2^-} + 4Hb(Fe^{2+})O_2 + 4\,H^+ \longrightarrow 4\mathbf{NO_3^-} + 4MetHb(Fe^{3+}) + 2H_2O + O_2$$

Nitrate reduction *(bacterial nitrate reductase)*

$$\mathbf{NO_3^-} + e^- + 2\,H^+ \xrightarrow{\text{Nitrate reductase}} \mathbf{NO_2^-} + H_2O$$

Nitrite reduction *(Deoxyhaemoglobin)*

$$\mathbf{NO_2^-} + Fe^{2+} + H^+ \longrightarrow \cdot NO + Fe^{3+} + OH^-$$

Nitrite acidification

$$\mathbf{2NO_2^-} + 2H^+ \longrightarrow 2HNO_2 \longrightarrow N_2O_3 + H_2O$$

$$N_2O_3 \longrightarrow NO + NO_2$$

$$N_2O_3 \longrightarrow NO_2 + NO$$

Fig. 4 Nitrate-nitrite-NO pathway. Examples of nitrate/nitrite as precursors.

1.2 General considerations on quality in food analysis

1.2.1 Quality infrastructure system: Tools and mechanisms to ensure reliable and comparable chemical measurements

The globalisation of trade has produced a need for the metrological comparability of analytical results. To achieve this, the analytical community is building up a quality infrastructure system consisting of three pillars: Metrology, standardisation and accreditation.

Legislation and laboratory infrastructure (networking) are the major driving forces in developing this quality infrastructure, as they provide the appropriate regulatory framework and activities to ensure reliable data for informed decision making (Papadakis, 2016).

1.2.1.1 Metrology in chemistry

Metrology is the science of measurement and its applications (Eurachem, 2011). Metrology in chemistry is concerned with the development of a structured support system based on measurement standards and measurement procedures through which the metrological traceability of measurement results can be demonstrated. The birth and early development of metrology in chemistry was marked by its special relationship to metrology in physics. During the period of classical analytical chemistry (volumetry, gravimetry), a l earning period regarding a w ell-structured physical metrological system was necessary and fruitful. However, progress in understanding the particularities of chemical measurements, as a result of a transition to modern analytical chemistry of trace analysis, made evident the advantages of developing a n ew and different conceptual model to establish traceability of chemical measurement results (King, 1997; Horwitz, 1998; Thompson, 2014). It is now recognised that, even though challenges still remain, the quality of chemical measurements has improved remarkably during the past decade. The main

metrological principles have now been established (validation, uncertainty estimation and metrological traceability) and a set of mechanisms, procedures and tools are available in increasing diversity (Leito, 2015).

1.2.1.2 Standardisation

Important benefits of standardisation are improvement of the suitability of products, processes and services, prevention of trade barriers and facilitation of technological cooperation.

A standard describes "what to do", providing a set of general or precise requirements. In practice, there are a number of standards that can be considered by an analytical laboratory, depending on its size, the range of its activities and the type of analysis.

ISO/IEC 17025:2005 — based partly on ISO 9001:2000 and intended for use by laboratories in developing their quality management system and administrative and technical operations — specifies the general requirements for the competence to carry out tests and/or calibrations, including sampling. It covers testing and calibration performed using standard methods, non-standard methods and in-house laboratory methods. It is applicable to all organisations performing tests and/or calibrations.

It should also be borne in mind that for the food industry, ensuring the safety of food to be put on the market and intended for human consumption is an obligation under Regulation (EC) 852/2004 (European Union, 2004) "*On the hygiene of foodstuffs*". The requirement includes mandatory self-control for all operators, at any level in the food production chain, in compliance with application of the Hazard Analysis and Critical Control Points (HACCP) principles. This means that the analytical laboratories used by industry for self-control, although not official and in general commercial, must operate and be assessed and accredited in accordance with ISO/IEC 17025 if they want to stay in business (Di Domenico A., pers. comm. 2016).

ISO 15189:2012, based upon ISO/IEC 17025 and ISO 9001, specifies requirements for quality and competence that are particular to medical laboratories. This standard underpins the accreditation of many medical laboratories.

The provision of proficiency testing schemes (ISO/IEC 17043, 2010) and the production of reference materials (ISO 17034, 2016) are two other laboratory-related activities for which competence requirements are set out in international standards.

Good Laboratory Practice (GLP) (OECD, 1997) contains a set of principles that provides a framework within which laboratory studies are planned, performed, monitored, recorded, reported and archived. These studies are undertaken to generate data by which the hazards and risks to users, consumers and third parties, including the environment, can be assessed for pharmaceuticals, agrochemicals, cosmetics, food and feed additives and contaminants, novel foods, biocides, detergents etc.

Standardized procedures for the determination of nitrite and nitrate in food are discussed in Section 1.4.2.

1.2.1.3 Accreditation

Accreditation is the "procedure by which an authoritative body gives formal recognition that a body or person is competent to carry out specific tasks" (ISO/IEC Guide 2:2004).

Conformity assessment is a set of processes used worldwide to check whether a product, service or system meets the requirements of a standard, regulation or other set of specifications. The international standards mentioned above are used by laboratories, reference material producers and providers of proficiency testing schemes to demonstrate their competence, whether in self-declaration (first party), at the request of a customer (second party) or at the request of an external body (third party).

1.2.1.4 Legislation and laboratory infrastructure

There is an international regulatory framework establishing laws, regulations and administrative provisions governing food and food safety for the protection of human life and health and the protection of consumer interests. Examples of national, regional and global level regulations are the FDA Food Safety Modernization Act (FSMA, 2011), the General Food Law in the European Union (European Union, 2002) and the Codex Alimentarius (Codex, 2016), respectively. The latter is a collection of standards, codes of practice, guidelines and other recommendations for food safety and consumer protection.

In order to support authorisation and efficient checks on compliance of food and feed with the European legislation, the European Union (EU) has established a laboratory network composed of national reference laboratories (NRLs) and European Union Reference laboratories (EURLs) to contribute, among other things, to formulating a common language for measurement, improvement of measurement capabilities, improvement of standardisation and accreditation, production of reference materials, organisation of comparative testing and training of laboratory staff (Ulberth, 2011). The European food-testing infrastructure work boosts implementation of EU legislation, contributing to harmonisation of compliance testing, reducing the need to repeat testing and saving cost. As a result, consumers benefit from safe food products, while the EU's single market is strengthened (JRC, 2015).

1.2.2 Fitness for purpose

The service to society supplied by analyst chemists is condensed in the analytical data they produce. These data summarise the information obtained about the identity and composition of the materials and resources that society uses. Production of analytical data is a service of a techno-scientific nature in which the accuracy of measurement (closeness to the true or accepted value), timeliness, cost and satisfaction of customer needs are of particular relevance in characterizing its quality (Dux, 1990). Although many conventional quality definitions are still in use (Westgard, 2010), the trend in recent years has been towards identifying the quality

of analytical results using the concept of fitness for purpose, which is defined as "*the property of data produced by a measurement process that enables a user of the data to make technically correct decisions for a stated purpose*" (Thompson & Fearn, 1996). It follows from this essential concept that the analytical data should be sufficiently accurate and obtained at a reasonable total cost, i.e. analytical cost plus the losses incurred by the customer in using the result (Fearn et al., 2002).

1.2.3 Metrological principles to ensure measurement quality in analytical laboratories: Validation, measurement uncertainty and metrological traceability

The pillars of achieving measurement quality in any analytical laboratory are three metrological principles: Validation, measurement uncertainty and metrological traceability. The analytical system (refers in this chapter to the use of a method/procedure, test sample, analyst under constant laboratory conditions) used to provide the analytical result is a complex entity. Since the ultimate aim of applying the above metrological principles is to ensure that every single result in routine analysis is sufficiently accurate, an internal and external quality control (QC) programme is required. This QC programme should demonstrate that the measuring system remains under statistical control at the time of analysis, ensuring the measurement results obtained are of the same quality as at the time of validation.

1.2.3.1 Validation: A simple and practical experimental design

Many guidelines have been written, most describing interesting aspects of some elements of the validation procedure, but the experimental designs required to estimate them are generally omitted or framed as an issue involving several or many replicate analyses of different samples. In general, a good experimental design would enable the analyst to estimate most of the target performance characteristics with a minimum of analyses.

The experimental design presented in Table 3 is of a general nature and can be used in principle for all analytical methods (Cheeseman & Wilson, 1978). The design permits estimation of analytical sensitivity, working interval, detection capability (limit of detection (LOD) and limit of quantitation (LOQ)), precision (repeatability and intermediate precision) and bias/recovery. Moreover, the design specifies that analyses should be performed in several batches over a period of time (1-6 months). Each batch consists of replicated analyses of blanks, calibration solutions, test samples, spiked samples and available reference material.

Table 3. Performance characteristics for each type of sample in a single batch in a single-laboratory validation process

Sample description	Performance characteristic
5-6 calibration solutions (or matrix matched calibration) + sample blank	Analytical sensitivity, limit of detection/quantitation, working interval including linearity
Test sample (level 1)	Repeatability, intermediate precision, uncertainty
Spiked test sample (level 2)	Repeatability, intermediate precision, recovery, uncertainty
Spiked test sample (level 3)	Repeatability, intermediate precision, recovery, uncertainty
Reference material	Repeatability, intermediate precision, bias, uncertainty

1.2.3.1.1 Bias

Bias is the difference between the mean value of a large number of test results and an accepted reference value ($\bar{x} - x_{ref}$). Bias is often expressed as a relative number in per cent $(\bar{x} - x_{ref}) / x_{ref}) \times 100$ or as relative 'apparent recovery' in per cent, i.e. $(\bar{x} / x_{ref}) \times 100$ (IUPAC, 2002).

In line with recommendations (IUPAC, 2002), the term 'recovery' is used to depict the yield observed in a spiking experiment. Analyses of a routine test sample both before and after the addition of a known quantity of the analyte (a spike) are used to estimate the recovery at two (or more) levels. In the absence of appropriate reference materials and/or reference methods, the recovery from a well-designed spiking experiment is used to estimate the bias. The relationship between recovery and bias is: %Recovery = 100 + %Bias (Prichard, 2007).

Having estimated the bias, it should be decided whether it is statistically significant. According to IUPAC (1999), a significance test involves comparing the bias with its expanded uncertainty. If the bias is larger than its expanded uncertainty, a systematic effect is revealed. Note that a statistically significant bias may still be ignored if it has no practical importance. However, regardless of the significance of bias and whether corrected or not, its uncertainty should be included as a component when calculating the combined uncertainty (see section 3.3.2).

The measurements included in the bias/recovery test are subject to shortcomings involving different assumptions and corresponding different uncertainties (Kirchmer, 1983). In general, if a representative certified reference material (CRM) is available it should be used, because its property values and associated uncertainty and metrological traceability are documented in detail. In a critical review of published approaches to estimate bias and its uncertainty, Magnusson & Ellison (2008) suggest that, assuming an associated uncertainty, a correction for recovery may be treated as a correction for bias.

1.2.3.1.2 Precision

The precision is generally dependent on analyte level and matrix and therefore at least two concentration (or other relevant quantity) levels should be included in the validation study. Estimates of both the repeatability and the intermediate precision are normally carried out during single-laboratory validation, and this can be achieved by applying a simple one-way ANOVA. Practical examples are available in the literature cited (e.g. Cheeseman & Wilson, 1978; Eurachem, 2014).

The validation procedure should consist of analysis of n replicates in m independent batches. The experimental design recommended for general use is to make $n = 2$ and $m = 8$-10. Such a design provides estimates of repeatability and intermediate precision with approximately equal numbers of degrees of freedom. For example, 10 batches of two replicates lead to 9 and 10 degrees of freedom in estimates of intermediate precision and repeatability, respectively (Cheeseman & Wilson, 1978). The total number of experiments may vary depending on the requirements on statistical representativeness or regulatory constraints.

1.2.3.1.3 Selectivity

Selectivity is the extent to which a method can be used to determine particular analyte(s) in a mixture or matrix without interferences from other components with similar behaviour (Codex, 2009). Data on the selectivity, as with robustness, are usually obtained during method development and should be included in the validation report.

1.2.3.1.4 Working interval

The working interval is the interval over which the method provides results with acceptable uncertainty. The lower end is bounded by the limit of quantification (LOQ) and the upper end by the concentration (similar) at which significant anomalies in the analytical sensitivity are observed (Eurachem, 2014).

1.2.3.1.5 Detection capability

The limit of detection (LOD) is the smallest concentration of analyte that can be reliably detected by the measuring system (Thompson & Lowthian, 2011). The rationale behind this definition is based on several interpretations of statistical hypothesis testing (chosen probability levels for type I error- false positive error, α, and type II error- false negative error, β) and diverse measurement conditions (instrument signal-to radio, measuring blank sample or low level spiked sample, number of replicates etc.). This makes the magnitude of the LOD estimates very dependent on the definition used. Hence, great caution is necessary when drawing conclusions on the suitability of a particular analytical method to determine low concentrations of nitrite/nitrate based on incomplete information on the LOD. Indeed only an estimate of LOD including the matrix effect provides reliable information on the performance of the method at low concentrations.

The limit of quantification (LOQ) is not based on statistical concepts. It establishes the lowest level at which the performance of a method is acceptable, where acceptability is a subjective matter that depends on the application. By convention, LOQ is a number of standard deviations (often 6 or 10 of repeated determinations on a blank sample or a low-level spiked sample. A LOQ = 10 s is equivalent to a relative standard deviation (RSD) of 10 %, as

$$\frac{s}{10s} \times 100 = 10\ \%$$

It is often suggested that the method selected should have an LOD of less than one-tenth and an LOQ of less than one-fifth of the level of interest (e.g. parametric value, maximum level) (Codex 2015, European Union, 2015).

1.2.3.2 Uncertainty

A number of guides from various organisations describe different approaches for estimating measurement uncertainty in chemical analysis. One report (Eurolab, 2007) outlines three empirical approaches using data from single-laboratory validation, interlaboratory validation and proficiency testing (PT) respectively. These three “empirical approaches”, or a combination thereof, are now accepted alternatives to the “modelling approach” described in “Evaluation of measurement data — Guide to the expression of uncertainty in measurement" (GUM, 2008), which is acknowledged as the reference text on uncertainty throughout the scientific community. It is clear that the different approaches have pros and cons and that the estimated uncertainty values will never be exactly the same. However, consistent similarity is expected between the different approaches when the main sources of uncertainty in a particular measuring system are being examined. As an example, a comparison of the measurement uncertainty data obtained applying the three empirical approaches have been carried out at the Swedish National Food Agency. The relative expanded uncertainty (coverage factor k=2) for determination of the nitrate mass fraction in vegetables (CEN, 2005) for single-laboratory validation, interlaboratory validation and PT was 9 % , 11 % and 12 %, respectively (unpublished data). It is worth noting that a more harmonised estimate of measurement uncertainty will improve the comparability of measurement results between laboratories (Näyki et al., 2014).

A well-designed, single-laboratory validation study allows the main sources of uncertainty in routine conditions to be calculated from precision and bias data. The uncertainty is estimated from a combination of the intermediate precision and the uncertainty involved in the bias measurement. The bias contribution is obtained by e.g. combining the standard uncertainty of the reference material with the standard deviation of the mean for replicate measurements of that reference material. In the absence of a suitable reference material, the bias can be estimated through the recovery obtained in a spiking experiment or by comparison with a reference method. The uncertainty calculation for recovery contribution using spiking studies is complex, because the mathematical model contains a mixture of operations.

However, King (2003) proposed a simplified approach to estimate uncertainty from spiking studies by applying appropriate approximations.

The combined standard uncertainty (u_c^2) is given by:

$$u_c^2 = u^2_{\text{precision}} + u^2_{\text{bias}}$$

This equation assumes that the bias is estimated and reduced, eliminated or, if deemed insignificant, disregarded. Note, however, that the uncertainty of the bias should be included in the combined uncertainty, irrespective of its significance (Eurachem, 2012).

1.2.3.2.1 Individual results and measurement process performance

As pointed out in ISO 21748 (2010), measurement uncertainty relates to a single result. Precision and bias estimates in the validation study, by contrast, relate to the performance of a measuring system. Nevertheless, it is common practice to use validation data to estimate the uncertainty of a subsequent single result. In this situation, the question that arises is how to estimate the uncertainty associated with a single analytical result when the population standard deviation is not known. The approach universally adopted by analysts of extrapolating the uncertainty of the measuring system to subsequent individual analyses of test materials is not theoretically valid (Hunt & Wilson, 1986). However, this unavoidable practical approach is widely accepted on the condition that an internal and external quality control, properly devised and executed, demonstrates that the performance of the measuring system during the validation study is maintained (Thompson & Magnusson, 2013).

1.2.3.3 Metrological Traceability

In order to compare analytical results, it is necessary to link individual measurement results to some common, stable reference, ideally a unit in the international system of units (SI), or an agreed reference measurement standard or reference measurement procedure. Any analytical measurement consists of several combined determinations to give a result under specific conditions. As pointed out by Thompson (2014), the main sources of uncertainty in analysis are in recovery (loss or gain of analyte) or interferences (loss or gain of the net analytical signal). These uncertainty sources are the cornerstone of poor metrological traceability in chemical analysis, leading to poor quality of results and breaks in the traceability chain. Consequently, Thompson suggests rejecting the common idea that incomplete metrological traceability in the SI system is related to shortcomings in chemical analysis, and instead scrutinising the main sources of error, i.e. recovery and interferences, in analytical chemistry.

Wolff (1997) described the preparation of isotopic reference materials for nitrate and developed a reference measurement procedure based on isotope dilution mass spectrometry. The potential of this procedure, in terms of obtaining SI-traceable results with a small combined uncertainty, was demonstrated for various water samples. Wolff et al. (1998) outlined a similar approach for nitrite.

1.3 Important factors in selecting analytical methods for nitrite and nitrate

1.3.1 Setting analytical requirements

In general, four performance characteristics can be considered the main factors in deciding on the most suitable analytical method for nitrite/nitrate. These are: selectivity, limit of detection, precision and bias. Other factors such as speed, cost and safety, which are not directly related to the accuracy of analytical results, should also be considered by the analyst in final selection of a method.

1.3.1 Selectivity

Nitrite and nitrate may be present in a variety of compounds within foodstuffs due to their high reactivity (Cassens et al., 1979). The diversity of unidentified compounds formed, e.g. protein-bound nitrite in meat products, requires an unambiguous definition of the analyte of interest. As a rule, in the analysis of nitrite/nitrate in individual foodstuffs and diets, the analyte of interest is the free nitrite/nitrate ions present in the test sample, i.e. the residual nitrite/nitrate.

The combination of efficient extraction, separation, clean-up and detection in a system adapted to the properties of the target analytes determines the selectivity of analytical methods. For instance, the derivatisation reaction is one of the most exploited strategies to improve the instrumental discrimination of the analytical signal (Lavilla et al., 2014). There is a lack of specific requirements on selectivity in the analytical literature. In this situation, instead of using their experience and intuition, the analyst can consider the criterion proposed in the OECD document (OECD, 2007), which provides guidance on typical validation characteristics for residue analysis methods. According to this criterion, a method can be judged as sufficiently selective if interfering substances never exceed 30 % of the analytical limit of quantification (LOQ).

1.3.2 Limit of detection

The three above-mentioned nitrite/nitrate applications involve different levels of interest (see Table 5), e.g. for law enforcement (100 to 1000 mg/kg), for analysis of diets (1 to 10 mg/kg) and for analysis of body fluids (1 to 100 µg/kg). Hence, the general recommendation suggesting that the LOD should be one-tenth of the level of interest and the LOQ should be one-fifth can be used to set numerical values to these performance characteristics.

1.3.3 Precision, bias and uncertainty

Numerical values for precision and bias data during single-laboratory validation should be compared against available quality criteria requirements, to judge the potential suitability of analytical methods. At present, regulatory bodies and organisations follow a variety of practices for setting analytical requirements (European Union, 2006b, 2006c, 2015).

Magnusson & Koch (2011) pointed out that these differences reflect the change in terminology used to describe the measurement quality from accuracy

(trueness and precision) to measurement uncertainty. As part of a laudable effort to converge the conventional approach for evaluating the performance of a method based on the concept of 'total error'[1] and the modern uncertainty approach (Rozet, 2011; Theodorsson et al., 2014), Magnusson & Koch (2011) proposed converting the required data on p recision and bias to a maximum standard uncertainty. This conversion is made using internationally accepted rules (GUM, 2008). As an example, this suggested approach is applied below for the requirements set in Regulation (EC) No. 1882/2006, which lays down methods of sampling and analysis for official monitoring of the levels of nitrates in certain foodstuffs (Table 4).

Table 4. Performance criteria for methods of analysis used in official monitoring of nitrate levels according to Regulation (EC) No. 1882/2006 (European Union, 2006c)

Characteristic	Level (mass fraction)	Recommended value	Maximum permitted value
Recovery	< 500 mg/kg	60-120 %	
	≥ 500 mg/kg	90-110 %	
Reproducibility RSD_R	All	As derived from the Horwitz Equation	2 × value derived from the Horwitz Equation

Repeatability RSD_r may be calculated as 0.66 × RSD_R at the level of interest.

Note 1. Concentration ranges are not stated, as the precision values are calculated at the concentrations of interest.

Note 2. The precision values are calculated from the Horwitz equation, i.e. $RSD_R = 2^{(1 - 0.5\log C)}$, where RSD_R is the relative standard deviation in % calculated from results generated under reproducibility conditions ($(s_R/x) \times 100$) and C is the concentration ratio (i.e. 1 = 100 g/100 g, 0.001 = 10 mg/kg).

In order to illustrate the conversion, the following is the calculation for a level (mass fraction) of 500 mg/kg (C = 0. 05; see Table 4, Note 2):

- Precision as derived from the Horwitz equation, i.e. $\%RSD_R = 5.3$ %.
  The standard uncertainty arising from precision of a single result is equivalent to the standard deviation = $u_1 = u_{precision} = 5.3$ %.
- Recovery 90-110 %.
  To convert the stated recovery interval to standard uncertainty, a rectangular distribution is assumed, i.e. values are equally likely across the range. Dividing the half-width of the interval by √3, $u_2 = u_{recovery} = 10/\sqrt{3} = 5.8$ %.

These two uncertainties are added together to obtain a combined standard uncertainty (u_c):

[1]Total error, TE, represents the overall error that may occur in a test result owing to both the random error and systematic error of the measurement procedure. TE is an analytical requirement that sets an upper limit for both the random error and bias in a single measurement or test result (Westgard, 2007)

$$u^2_{\text{c}} = u^2_{\text{precision}} + u^2_{\text{recovery}}$$
$$u_{\text{c}} = (5.3^2 + 5.8^2)^{1/2} = 7.9$$

The combined standard uncertainty is multiplied by a coverage factor k=2 to provide an interval containing approximately 95 % of the distribution of results:

$$U = 2 \times u_{\text{c}} = 2 \times 7.9 = 15.8 \approx 16\ \%$$

Based on this reasoning, the laboratory can set the target relative expanded uncertainty to ≤16 %.

1.3.3.1 The maximum standard uncertainty to expect (target uncertainty)
A more generalised procedure for calculating an upper limit for the uncertainty ('target uncertainty') is performed using the numerical values proposed by the Codex Alimentarius (2015) as criteria for precision and recovery for analytical methods. Laboratories may deem suitable any method that meets these numerical values (Table 5). The Horwitz-Thompson values are a formal international requirement for

Table 5. Examples of calculation of maximum relative combined standard uncertainty $u_{c,\text{Rel}}$ and relative expanded uncertainty U_{Rel} using the numerical values of requirements for precision, $u_{\text{precision}}$ (Horwitz-Thompson equation) and recovery (u_{bias}) proposed by the Codex

Level (mass fraction) [(1)]	$u_{\text{precision}}$ (%)	Recovery (%)	u_{bias} (%)	$u_{c,\text{Rel}}$ (%)	U_{Rel}, k=2 (%)
100 % (100 g/100 g)	2	98 – 102	1.2	2.3	4.6
≥ 10 % (10 g/100 g)	3	98 – 102	1.2	3.2	6.4
≥ 1 % (1 g/100 g)	4	97 – 103	1.7	4.4	8.8
≥ 0.1 % (1 mg/g)	6	95 – 105	2.9	6.7	13
100 mg/kg	8	90 – 107	4.9	9.4	19
10 mg/kg	11	80 – 110	8.7	14	28
1 mg/kg	16	80 – 110	8.7	18	36
100 µg/kg	22	80 – 110	8.7	24	48[(2)]
10 µg/kg	22	60 – 115	16	27	52[(2)]
1 µg/kg	22	40 – 120	23	32	64[(2)]

Note 1. The levels have been grouped according to the purpose of application of determination of nitrite/nitrate, i.e. a) law enforcement (100 to 1000 mg/kg), b) analysis of individual food water and diet (1 to 10 mg/kg) and c) analysis of body fluids (1 to 100 µg/kg).
Note 2. For levels close to the LOD, the absolute measured uncertainty should be used, as absolute uncertainties tend to be constant (Rocke et al., 1995; Eurachem, 2012).

the acceptability of the precision of any particular method. These values are supported by examination of almost 10 000 individual datasets from method precision studies. On the other hand, the recovery data could be used to estimate the standard uncertainty of the method bias. Both sources of uncertainty are combined using the above approach (section 4.1.3).

There is a trend towards selecting the appropriate method based on a concept developed by Thompson (2011) to define a characteristic function, which is an uncertainty function that specifies maximum levels of uncertainty regarded as fit for purpose. This "fitness-for-purpose" approach has now been adopted by the EU, particularly in respect of contaminants in food (European Union, 2006b, 2007). However, while metrologists are developing the idea and tools to standardise the characteristic function approach, the maximum expanded uncertainties given in Table 5 could certainly be used to select a method for the analysis of nitrite/nitrate. Other inputs to set the target uncertainty for a range of quantity values are given in a Eurachem/CITAC Guide (2015).

1.4 Strategies based on chemical reactions for method selection and optimisation

The fate of nitrate and nitrite is complex. Nitrate (NO_3^-) is the most fully oxidised compound of nitrogen and is therefore stable to oxidation. Nitrate is chemically stable owing to its chemical structure, in which the nitrogen atom and the three oxygen atoms lie in the same plane in a symmetrical trigonal resonance hybrid with bond angles of 120° (Addiscott, 2005). Nevertheless, it is able to accept electrons and thereby act as an oxidising agent, being itself reduced to nitrite (NO_2^-) in the process. Nitrate and nitrite are ions which co-exist in most foodstuffs and actually analysts rarely find one without the other. For this reason, methods for the analysis of nitrite/nitrate are considered together here.

1.4.1 Fundamental considerations

The selection and optimisation of an analytical method to analyse nitrite/nitrate in foodstuffs and water pose difficulties largely related to the reactivity, stability, concentration levels and complexity of the matrix to be analysed. We briefly discuss these critical factors below.

1.4.1.1 Nitrite and nitrous acid reactivity

From an analytical point of view, the nitrite ion does not have reactivity attributed to it. Indeed, the chemistry of nitrite is ultimately the chemistry of nitrous acid (HNO_2) (Fox, 1985). Thus by controlling the formation and elimination of nitrous acid, analysts can steer the reactivity and instability of nitrite. Because the pK_a of nitrous acid is 3.37 at pH 5.5, the anion (NO_2^-) comprises >99 % of the total acid concentration. As a result, the kinetic factor also plays a role, because this small quantity of associated HNO_2 is in dynamic equilibrium with its anhydride, the powerful nitrosating species dinitrogen trioxide (N_2O_3), and other potential

nitrosating compounds such as nitric oxide (NO) and nitrogen dioxide (NO_2). Furthermore, the NO_2 in backward reactions reacts with NO and H_2O regenerating previous reductants, such as N_2O_3 and HNO_2, maintaining the nitrosating potential of the system (see Figure 2). Note that the strongest nitrosation species is the positively charged nitrogen oxide, either in its simplest form, the nitrosonium ion (NO^+), or as nitrous acidium ($H_2NO_2^+$). Both forms exist only in strongly acid conditions and therefore at the pH of meat (5.5-6.5) they are not found in appreciable quantities (Sebranek & Fox, 1985; Pegg & Honikel, 2015). In sum, all nitrosating species can be used by the analyst, forming suitable chromophores that will later permit quantification of the nitrite ion and indirectly also the nitrate ion.

1.4.1.2 Nitrite stability

The instability of nitrite is one of the main difficulties encountered in analysis of nitrite in all areas of interest. For example, in analysis of biological fluids, MacArthur et al. (2007) developed an ingenious procedure to stabilise nitrite in whole blood until the time of measurement. The solution used consists of potassium ferricyanide ($K_3Fe(CN)_6$) to oxidise ferrous heme to ferric heme, Nonidet (a non-denaturing detergent) to solubilise red cell membranes and N-ethylmaleimide to block free thiols. Samples must be stored at -80 °C with the preservation solution. In a later study, Schwarts (2011) observed that nitrite is stable in blood for several hours if samples are stored in an ice bath, and thus considered cooling of blood in an ice bath a promising approach in investigations of nitrite losses. In a study Merino (2009) showed that adjusting the pH to 10.5 (in which the anion (NO_2^-) comprised 99.99 %) and storing samples at -18 °C can stabilise the nitrite/nitrate ions in diverse foodstuffs (vegetables, meat products, baby food, surface water and dairy products (milk)) for at least 6 months. Wolff et al. (1998) demonstrated that 1 mg/L solutions of nitrite, preserved with NaOH (pH 9), were stable for almost 1.5 years when stored in fluorinated ethylene propylene bottles at room temperature and daylight. A similar degree of stability was observed for solutions in polyethylene bottles (pH 10.5) which also contained 0.02 mL/L of chloroform, and for solutions at pH 12.

Depending on the area of application, the analyst may be interested in differentiating between residual free nitrite and protein-bound nitrite, e.g. the residual nitrite/nitrate is commonly determined in the analysis of foodstuffs, water and diets because the analyst wants to know the amount of these ions in the samples at the time of sampling. In contrast, the analysis of nitrite/nitrate in human biological fluids within the framework of clinical studies of toxicity and bioavailability could require quantifying even the protein-bound nitrite (Tsikas, 2005).

1.4.1.3 Nitrite level and matrix complexity

As noted previously (Table 5), the target measurement uncertainty, used to quantify the expectations on the performance of an analytical method, varies inversely with the concentration of the analyte to be determined. Obviously, the higher the concentration of nitrite/nitrate to be determined, the lower the acceptable magnitude

of the target uncertainty demanded for the candidate method. This basic model is described with reasonable certainty by the empirical Horwitz function, irrespective of the kind of analyte, method or matrix (Horwitz, 1982). Note that the complexity of the matrix would have a minor impact on the performance of the method if proper sample preparation and detection, to decrease the influence of interferences, are used. Accordingly, the quality criteria for selection of methods discussed below are grouped in a way that agrees with the three analytical purposes to determine nitrite/nitrate: Law enforcement, exposure assessment and studying the fate of nitrite/nitrate in biological fluids.

Purpose 1: Law enforcement. In order to decide on the acceptance or rejection of a product as a result of monitoring and surveillance activities, the method used should have acceptable uncertainties at the level of the maximum values for concentrations of nitrite/nitrate established in the legislation. These levels are of the order of 50 to 7000 mg/kg. For example, the maximum permitted level for nitrate is 150 mg/kg for meat products, 50 mg/L for drinking water and 7000 mg/kg for green leafy vegetables (rocket). Actually, the available standardised methods are sufficiently reliable for the determination of these ions at this relatively high content level. However, modifications of such methods may be necessary depending on the matrix (CEN, 2005, 1998a, 1998b; ISO, 2004a, 2004b, 2004c; AOAC, 2005).

Purpose 2: Exposure assessment. The increased need to develop health diets that fulfil the Acceptable Daily Intake (ADI) for nitrite and nitrate has directed interest toward obtaining best suited food composition data to calculate population dietary exposure and assess potential impacts on public health (Kroes et al., 2002; EFSA, 2011). In practical terms, analysis of individual foodstuffs or diets using standardised methods may not provide reliable results because these methods may have a limit of detection or quantification higher than the background level of nitrite/nitrate expected in these foodstuffs and diets. The concentration levels in these kinds of samples lie in the interval 1 to 10 mg/kg. As a result, substantial modifications to such methods, together with new analytical methods, are continually being reported in the scientific literature. These new methods combine state-of-the-art techniques of clean-up (e.g. solid-phase extraction devices), separation (e.g. capillary electrophoresis) and detection (e.g. mass spectrometry) aimed to ensure satisfactory performance of the methods.

Purpose 3: Fate of nitrite/nitrate in biological fluids. Since the discovery of the new role of nitrite/nitrate in the physiology of the human body, interest among analysts has shifted toward developing analytical methods capable of producing reliable results in concentration levels as low as 1 ng/kg to 1 µg/kg. The concentration intervals of circulating nitrate and nitrite are 20-40 µmol/L and 50-100 nmol/L respectively (Weitzberg et al., 2013). The need for more sensitive methods has increased in direct correlation with the discovery of new functions attributed to the nitrate-nitrite NO pathway. Thus, they have changed from being regarded as inert products of the metabolism of nitric oxide to being recognised as essential precursors in its formation. Furthermore, more recent discoveries are demonstrating that nitrite

can perform many actions previously attributable to NO acting as an important molecule in its own right (Bryan, 2006). Hence, the extremely low levels of nitrite/nitrate and the complexity of the composition of biological fluids, coupled to the brevity of their half-life, has opened the way for new innovations at each stage of the analytical process.

1.4.2 Derivatisation reaction pathways

The various detection strategies for the determination of nitrite/nitrate found in the literature are based on one of five derivatisation reaction pathways: nitration, ammonification, nitrosation, diazotization-coupling and chemiluminescence reaction. The last three pathways are specific for nitrite (Figure 5).

Due to the higher specificity of nitrite derivatives, analysts have given preference to the nitrite derivatives reaction pathways instead of nitrate reactions. However, applying these options requires prior reduction of nitrate to nitrite, which in turn poses severe analytical problems. The great difference in efficiency of different reduction reactions has been treated with varying degrees of attention, although this step can be regarded as one of the weakest links in the analytical process. For example, some methods propose reductions and/or sample preparation (extraction and clean up) carried out in acid conditions, ignoring the formation of HNO_2, which can react with various compounds present in the sample (e.g. thiols) before they are removed (Tsikas, 1997). Unless such reactions are carefully controlled, losses of nitrite can be expected.

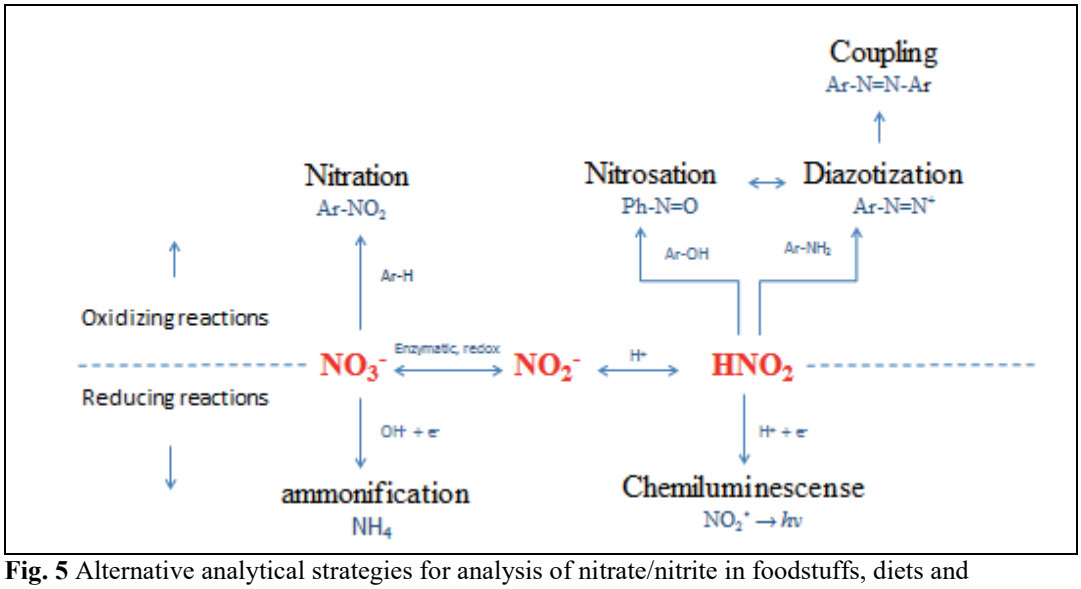

Fig. 5 Alternative analytical strategies for analysis of nitrate/nitrite in foodstuffs, diets and biological samples (oxidising and reducing reactions). Adapted from Moorcroft et al. (2001), with permission from Elsevier Science B.V.

1.4.2.1 Nitration

Nitration is a chemical reaction for the introduction of a nitro functional group ($-NO_2$) into an organic chemical compound. Both aromatic and aliphatic compounds can be nitrated by various methods such as heterolytic (electrophilic and nucleophilic) and radical nitration. Aromatic nitration is most frequently electrophilic, whereas aliphatic nitration proceeds via a free-radical mechanism (Olah, 1982).

In the early decades of the 20th century, the need to monitor nitrate as an indicator of environmental contamination of the surroundings, such as water and soil, and the amount of nitrate in certain industrial products (fertilisers) resulted in a number of analytical strategies for its determination based on the nitration pathway. At that time, the importance and role of nitrite were not fully understood, so analytical interest in nitrite was minor. Accordingly, one of the most widespread methods for the determination of nitrate uses the colorimetric properties of the nitration of phenolic-type compounds (e.g. phenol-disulphonic acid, chromotropic acid, 2,4-xilenol and salicylic acid). Actually, these techniques are of little value in practice, as they have been shown to have poor selectivity, precision and trueness, as well as being tedious and using strongly acid solutions (Usher, 1975; Zolfigol, 2001). This renders them unsuitable according to current good quality practice. Nevertheless, an official method for determination of nitrate/nitrite in meat and cured meat is available and has been adopted as a Codex reference method (Type II). In this method, the nitrate ions react with 2,4-xilenol in sulphuric acid, steam-distilled and measured to 450 nm . Nitrite is oxidised to nitrate with potassium permanganate and determined by difference (AOAC, 2005).

More modern techniques such as gas chromatography, also using the nitration reaction, have been applied to a wide range of analyses of nitrite/nitrate in biological fluids. Gutzki (1992) devised a GC-MS-MS method for the quantification of nitrite/nitrate after conversion to trimethoxybenzene under strongly acid conditions to catalyse the nitration reaction. Hydrogen peroxide is then used to convert nitrite to nitrate. To circumvent the problems associated with the separate analysis of nitrite and nitrate, Tsikas (2000) developed a GC-MS method for simultaneous quantification of nitrite/nitrate in various biological fluids. The method uses pentafluorobenzyl (PFB) bromide, which reacts with nitrite and nitrate, leading to the formation of the nitro PFB derivative (PFB-NO_2) and nitric acid ester PFB derivative (PFB-ONO_2), respectively. Tsikas (2005) reported that the major shortcoming of the nitration reaction is that nitrite cannot be accurately determined in the presence of high excess of nitrate over nitrite. In order to investigate the distribution and metabolism of circulating nitrite in blood, Schwarz et al. (2011) optimised the method above by essential modifications to stabilise nitrite, remove interference and improve the derivatisation reaction.

1.4.2.2 Ammonification

Nitrate/nitrite has also been measured by a procedure involving reduction to NH_3 under hot alkaline conditions in the presence of a reducing agent, Devarda's alloy (containing 50 % Cu, 45 % Al and 5 % Zn), zinc, copperised zinc or titanous chloride. The reduction is carried out in a Kjeldahl distillation apparatus. The distilled ammonium is determined by acidimetry, spectrophotometry, fluorometry, conductimetry, potentiometry, selective electrode or ultraviolet. The method is slow, tedious and has several drawbacks, such as incomplete reduction of nitrate and interference from organic nitrogen compounds in the analysis of wastewater samples (APHA, 1995; Nollet et al., 2014).

1.4.2.3 Nitrosation

Nitrosation is the chemical term for the process of replacing a hydrogen atom in a molecule with the nitroso group (-N=O) (Austin, 1961). The general pattern of nitrosation reactions results in the formation of C-; N-, S-, O-nitrosation. Several reported analytical methods based on C-nitrosation and N-nitrosation are discussed below.

C-nitrosation reactions involve attack on a carbon atom by nitrosating species. This includes the nitrosation of reactive aromatic compounds. The most familiar reaction is the nitrosation of phenol and naphthols. Both compounds undergo ready nitrosation by nitrous acid in dilute aqueous mineral acid (Williams, 2004). The analytical applications of C-nitrosation reactions were investigated by Davis et al. (1999), who explored their suitability for nitrite determination of various phenolic compounds (phenol, hydroquinone, catechol, resorcinol, pyrogallol, phloroglucinol and gallic acid). They evaluated response interferences and colour stability under induced alkaline conditions for the nitroso derivatives of phenol, resorcinol and phloroglucinol. They found that electrochemical measurement of phloroglucinol possesses several beneficial qualities compared with spectrometric alternatives for nitrite detection in biological fluids (saliva and urine) and water.

1.4.2.4 Diazotization

Diazotization is a reaction that converts an $-NH_2$ group connected to a phenyl ring to a diazonium salt. When a solution of a diazonium salt is heated, the diazo group is replaced with a hydroxyl group. This reaction was used by Jain et al. (1997) to develop a method based on the diazotization of 2-aminobiphenyl in acid medium and thermal hydrolysis of the diazo compounds to form 2-phenylphenol, which is extracted into hexane and analysed by GC-flame ionisation detection (FID). The hydrolysis of the intermediate diazonium ion, instead of the coupling reaction to an azo dye, avoids interferences encountered in conventional determination of nitrite by diazotization-coupling followed by spectrophotometry (see below). The method has been applied to the determination of nitrite in tap water and pond water.

A number of fluorometric methods using the diazotization pathway has been studied. Stalikas et al. (2003) used the property of fluorescence quenching of

tryptophan in the presence of nitrite and nitrate. The fluorescence quenching of tryptophan is enhanced by the presence of phosphate ions, which are utilised as buffer of the solution (pH 9.5) in the flow stream for the post-column reaction. The quenched fluorescence intensity exhibits concentration dependence in the interval 1-25 mg/L and 3-65 mg/L for nitrite and nitrate respectively. This ion chromatographic method with post-column indirect fluorescence detection determines simultaneously nitrite and nitrate concentrations in foodstuffs, fertiliser and soil.

More recently, Gu et al. (2016) investigated the analytical application of a diazotization reaction and subsequent cyclisation. The derivative 2-(1H-phenanthro(9.10-d)imidazol-2-yl)aniline (PA) has very strong fluorescence. When it reacts with NO_2^-, this generates the corresponding benzotriazine derivative, which quenches the fluorescence of PA. The analytical application demonstrates that PA can easily be applied to quantitative analysis of nitrite in environmental samples and food products. The average recovery of nitrite is within the interval 98.6-102 % and there is a low relative standard deviation (0.97-3.2 %) for spiked samples of pork, sausage, river water and tap water.

1.4.2.5 Diazotization-coupling

Since 1879, when Griess first proposed the diazo coupling reaction for the identification of nitrite, it has been the most widely used method to measure nitrite. Nitrate may also be measured after conversion to nitrite with metallic (NMKL 2013) or enzymatic reduction (CEN 1998a). Nitrite is reacted with sulphanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride to produce an azo dye. The Griess reaction is regarded as absolutely specific for nitrite, because no other compound will form diazo pigments (Fox, 1985).

However, it is recognised that the Griess reaction is prone to numerous redox and other interferences from substances present in environmental and food samples, but these shortcomings are considered minor in view of the relatively high regulatory limits for both contaminants. Hence, this reaction forms the basis for the many international standards method approved by US, European and international organisations. For example, the European Committee for Standardisation has described a method for the determination of nitrite in meat products. Nitrite/nitrate is extracted from a homogenised sample under alkaline conditions (pH 8-8.5). Nitrate is previously converted into nitrite by nitrate reductase. The extract is clarified with Carrez solutions (potassium hexacyanoferrate (II) and zinc acetate solutions), followed by Griess reagents (sulphanilamide and *N*-(1-naphthyl)-ethylenediamine dihydrochloride). The content of nitrite/nitrate is determined spectrophotometrically at a wavelength of 540 nm (CEN, 1998a). The nitrate content is calculated from the difference between the spectrophotometric measurements. An automated version of the manual procedure of cadmium reduction for nitrate determinations in leafy vegetables is presented in CEN (1998b).

Furthermore, three analytical methods for the determination of nitrate and nitrite in milk and milk products have been described (ISO, 2004a, 2004b, 2004c).

All three employ the diazotization-coupling Griess reaction for determination of nitrite. A reduction step involving cadmium is required to determine nitrate, which is calculated by difference.

The Nordic Committee on Analysis of Food specifies a sp ectrophotometric method for the determination of nitrate/nitrite content in foodstuffs and water after zinc reduction and Griess reaction (NMKL, 2013). The method has been validated for vegetables (lettuce), meat products, baby food, dairy products (milk) and surface water. The nitrite LOD for surface water is 0.05 mg/L, while for other matrices it ranges from 2 to 5 mg/kg.

1.4.2.6 Chemiluminescence reaction

Another way to determine nitrate/nitrite at nmol/L concentrations is to use methods able to minimise the matrix effect, offering superior sensitivity in comparison with spectrometry, fluorescence and electrochemical approaches. In recent years, determination of free radical NO has been used to measure the precursor nitrite/nitrate of the formation of NO. Equations for the respective half-reaction are:

$$NO_3^- + 4H^+ + 3e = NO + 2H_20 \qquad E° = +0.96 \text{ V}$$
$$NO_2^- + 2H^+ + 2e = NO + H_20 \qquad E° = +1.00 \text{ V}$$

The basis of chemiluminescent NO detection is the rapid reaction of NO in the gas phase with ozone (O_3). This reaction yields nitrogen dioxide (NO_2*) in an excited state. A photon is emitted as the ion returns to its original stable "ground" state and is detected as chemiluminescence ($h\times\nu$) (Grau et al., 2007).

$$NO + O_3 = NO_2* + O_2$$
$$NO_2* \rightarrow NO_2 + h\nu$$

Nitrite is selectively reduced under mild conditions and the total nitrate/nitrite content is determined by stronger reducing conditions. Cox (1980) found that Fe(II) and Mo(VI), and Ti(III) are efficient for the conversion of NO_3 to NO and that for the nitrite reaction, iodine ion is the most efficient reducing agent. Other researchers have found NO detection from nitrite by tri-iodide (I_3^-) chemiluminescence to be linear over a broad range of nitrite concentration (10 nmol/L to 1 m mol/) with intermediate precision of 0.5 % (MacArthur et al., 2007).

1.4.2.7 Direct measurement without reaction: Ultraviolet spectrophotometric and electrochemical detection

Direct and simultaneous methods for the determination of nitrate/nitrite have been developed. They are applied after separation by HPLC or capillarity electrophoresis and detection by UV or electrochemical detection, such as amperometric (Zhou et al., 2013), voltametric (Thomas, 2012), coulometric (He et al., 2000) or ion-selective

(He M-M et al., 2012). The European Committee for Standardisation (CEN, 2005) describes a HPLC-UV method in which nitrate and nitrite are extracted from the sample in hot water. Interfering substances are removed from the extract with acetonitrile. The ions are separated by ion-exchange chromatography and quantified using ultraviolet detection at 205 nm. Validation data obtained from interlaboratory studies show that the method is applied for the determination of nitrite and nitrate in meat products, baby food and vegetables. The limit of detection of nitrite and nitrate is 1 and 10 mg/kg, respectively.

1.5 Remarks and perspectives

Measurement of nitrite and, to a lesser extent, nitrate in foodstuffs and water continue to represent a challenge to the analyst. For a century, the Griess reaction has been widely used for quantification of nitrite (and nitrate after its reduction to nitrite). In addition, a combination of the Griess reaction with flow injection analysis, coupled with HPLC techniques, has shown that both batch and automated assays are "fit for purpose" in monitoring, surveys and control of nitrite/nitrate in foodstuffs and water. The working interval in this area falls within the interval 100-7000 mg/kg. Complete reviews of nitrite/nitrate 'classical' methodologies are provided by Fox (1985).

On the other hand, the analysis of microgram levels of nitrite/nitrate in individual foodstuffs and diets demands more sensitive methods (i.e. with a low limit of detection), which has stimulated the development of modern analytical techniques based on chromatographic and electrophoretic separations in combination with detection techniques such as absorption/emission spectrometry and electrochemistry. However, these advances in instrumentation and automation do not preclude analytical problems associated with reduction and elimination of bias. On the contrary, due to the low levels studied in these analyses, the magnitude of the uncertainty from bias is comparable to the uncertainty from precision. Thus these modern analytical tools require even more careful sample preparation, elimination of interferences and chemical derivatisation to reduce, eliminate or, if deemed insignificant, disregard bias. The LOQ for suitable methods should be typically within the interval 1-10 mg/kg. Strategies employing diverse methods in a variety of samples matrices are explored by Moorcroft et al. (2011).

Nitrite and nitrate are essential products and precursors of two complementary pathways in mammals: the L-arginine-NO and the nitrate-nitrite-NO pathway, respectively. Considering the analytical problems arising from the short half-life of NO *in vivo* in the human circulatory system ($\approx$ 0.1 s), nitrite/nitrate plays an important role as a biomarker of NO, which is the main compound of both pathways. Analysis of traces of nitrite/nitrate in research on their physiological and therapeutic properties requires analytical methods with LOQ in the interval 0.001-0.100 mg/kg. The review articles by Tsikas (2005, 2006, 2007) provide a good deal of evidence on the importance of pre-analytical factors (e.g. contamination from laboratory

chemicals, materials) and analytical factors (e.g. poor selectivity and incomplete reactions) for reliable quantification of nitrite and nitrate in analysis of body fluids.

Food preferences are not innate. Over the years, the focus of food consumers has changed from a primary and elementary interest in meeting survival needs or a hedonistic lifestyle (depending on the social class to which the consumer belongs) to other essential nutritional aspects such as quality and safety. Furthermore, the latest emerging trend is the increasing consumer concern for more sophisticated nutritional aspects, such as the health promoting effects of certain substances and diets (Bryan 2015; Trichopoulou et al., 2014). With respect to nitrite/nitrate, as we described, the question of quantification of the relatively large amounts of nitrite/nitrate as additive or contaminant has been answered using 20th century laboratory techniques. This is depicted in the available standard methods used by the control authorities in their work on ensuring certain aspects of the quality and safety of foodstuffs and water. However, the internationally accepted method performance-based approach that establish a set of criteria to which a particular methods should comply (e.g. target uncertainty and recovery), gives also the analyst the freedom to developed more environmental friendly alternative methods. An important aspect here is that together with preserving the analytical objectives, due consideration should be given to the replacement of toxic compounds and reducing the amount and toxicity of solvents to avoid or minimize the volume of waste (De la Guardia et al. 2012). Furthermore, the fact that nutritionists, epidemiologists, toxicologists and physicians are now examining possible relationships between their respective findings and the occurrence of nitrite/nitrate poses new questions to be answered by analytical chemists, metrologists and scientific instrumentation manufacturers. Actually, much of the stimulus to develop modern analytical, metrological, risk-benefit assessment and instrumentation tools is coming from the field of nutritional biochemistry and clinical analysis.

It is worth pointing out that from an analytical perspective, the well-directed effort to develop a sound metrological infrastructure of chemical measurements in the last decades has allowed a remarkable improvement in the quality and reliability of the chemical measurement. The increasingly harmonized guides in different fields of chemical measurement give a good account of what has been achieved (Eurachem, 2011, 2012, 2014, 2015). However, the origin and mechanisms of the matrix effect are still not fully understood, as well as those involved in the sample preparation and representatives of the sampling (Thompson, 2014). Hence, new theoretical and practical contributions addressing these and other issues relating to the traceability of chemical measurement of nitrite and nitrate are expected.

Based on chapter of book (Paper I): Merino, L., Örnemark, U., Toldrá, F. (2017). Analysis of Nitrite and Nitrate in Foods: Overview of Chemical, Regulatory and Analytical Aspects. In Fidel Toldrá, editor: Advances in Food and Nutrition Research, Vol. 81, AFNR, UK: Academic Press, pp. 65-107

2. Objectives

2. Objectives

The main aim of this thesis was to select, standardize and develop analytical methods that meet the fitness for purpose criteria to carry out the monitoring, control and exposure estimate of nitrite/nitrate in foodstuffs. This was achieved by the following specific objectives:

1. Identify and interpret conceptual and practical principles of the modern metrology in chemistry, which support the selection and develop of analytical methods for the determination of nitrite/nitrate in foodstuffs (Paper I-Chapter of book).

2. Selection and in-house validation of a method for the determination of nitrite/nitrate in foodstuffs (vegetables). Standardization of the HPLC-ion chromatographic method in an international collaborative study (Paper II).

3. Monitoring and control of nitrate in Swedish lettuce and spinach using the standardised HPLC-ion chromatographic method (Paper III).

4. Develop an environmental friendly and cost-effective method based on zinc reduction and Griess reaction to determine nitrite/nitrate in foodstuffs (meat products) (Paper IV).

5. Control of nitrite/nitrate in meat products using the spectrophotometric method and estimate dietary intake of nitrite/nitrate in Swedish children (Paper V).

6. Investigate the time-depletion of nitrite in meats products and its effect on nitrite intake estimation (Paper VI).

3. Results

3.1 Work related to the standardization of an analytical method: Monitoring and control of nitrate in vegetables (Papers II-III)

3.1.1 Paper II - Liquid chromatographic determination of residual Nitrite/Nitrate in foods: NMKL Collaborative study.

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FOOD COMPOSITION AND ADDITIVES

Liquid Chromatographic Determination of Residual Nitrite/Nitrate in Foods: NMKL[1] Collaborative Study

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Nitrite and nitrate are used as additives in the food industry to provide color and taste and to control undesirable gas and flavor production by anaerobic bacteria by virtue of their antimicrobial properties. The analytical method that has been widely used to determine nitrite and nitrate involves the use of toxic cadmium. In response to a request from the Nordic Committee on Food Analysis, a study was performed to obtain an alternative chromatographic method to determine residual nitrite and nitrate in meat products. The study was done in 3 stages: (*1*) comparative evaluation of the performance of 3 liquid chromatographic methods, (*2*) internal validation of the selected ion chromatographic method, and (*3*) a collaborative study in which 17 laboratories from European countries participated. Furthermore, the applicability of the method to matrixes other than meat and meat products was demonstrated. The results of the collaborative study show that the European Prestandard prENV 12014-4 is well suited for the determination of nitrite and nitrate in different foods (e.g., meat products, vegetables, baby food, and cheese). The limits of detection for nitrite and nitrate ions are 1 and 10 mg/kg, respectively. Recoveries of residual nitrite/nitrate ranged from 96 to 108%. Repeatability and reproducibility were satisfactory.

Nitrite either alone or in combination with the nitrate salt is used as a curing agent for meat products. The role of nitrite is to produce the characteristic pink color, texture, and flavor and, especially, to provide protection against poisonous microorganisms, such as *Clostridium botulinum*, which is of particular interest. Because of toxicological considerations, nitrite and nitrate are the concern of agencies responsible for food safety. Nitrite (and nitrate as its reservoir) is a precursor of the carcinogenic *N*-nitroso compounds.

Nitrite is an unstable ion and undergoes a series of reactions as soon as it is added to food products. In an acid environment, the nitrite ion ionizes to yield nitrous acid, which further decomposes to yield nitric oxide (NO), an important product from the standpoint of color fixation in cured meat. Nitric oxide reacts with myoglobin to produce the red pigment nitrosomyoglobin.

Time, temperature, pH, and additives have an important effect on the depletion of nitrite in cured meat. Nordin (1) reported an equation that relates the rate of nitrite depletion to time, temperature, and pH. Sen et al. (2) had considerable losses of nitrite during analysis of acidic samples. Fujimaki et al. (3) studied the fate of nitrite in meat-curing model systems that showed the effect of sodium ascorbate in the decomposition of nitrite. These factors, besides the fact that the analytical procedure itself promotes certain chemical reactions, explain why determination of nitrite in meat products is difficult.

On the other hand, Olsman and Cees (4) differentiated between residual free nitrite and protein-bound nitrite, and they proposed a method to estimate the protein-bound and the residual free nitrite content of meat. The principle on which the method is based is the release of the bound nitrite, as described by the following reaction:

$$RS - NO + HgCl_2 + H_2O \rightarrow RS - HgCl + HNO_2 + HCl$$

Acording to our analytical objective, we were not interested in releasing nitrite from any reaction products (even though they are reservoirs for nitrosamine formation). On the contrary, the choice of the conditions and chemicals used in the methods we evaluated was guided by the desire to minimize the losses of or increase the numbers of free nitrite/nitrate ions present during the analytical procedures. Therefore, the analytical methods determine the residual nitrite/nitrate, i.e., the free nitrite/nitrate ions that are present when the chemical analysis is initiated.

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[1] Nordic Committee on Food Analysis (Secretariat General, c/o Veterinærinstituttet, Pb 8156 Dep., N-0033 Oslo, Norway).

Experimental

Analytical Methods Assessed

Three ion chromatographic methods were assessed: (*1*) Weak anionic exchanger (aminopropyl bonded-phase column). This liquid chromatographic (LC) method is based on the European Standard prEN 12014-Part 2 (5). (*2*) Strong anionic exchanger (quaternary ammonium column). This method was proposed to the Nordic Committee on Food Analysis (NMKL) some years ago. Earlier collaborative studies showed low recoveries of nitrites in meat, and in general the performance of the method was variable. (*3*) Strong anionic exchanger (quaternary ammonium column). This method was proposed to the European Committee for Standardisation (CEN). This method is actually a European Prestandard (6). The methods are summarized in Table 1.

Weak Anionic Exchanger Method (Aminopropyl Bonded-Phase Column)

The method has been validated for determination of nitrate in vegetables. The liquid chromatogram of a standard solution containing nitrite and nitrate, each at 5 mg/L, is shown in Figure 1. The separation of the peaks was inadequate, but this is not a shortcoming in the analysis of vegetables, which usually do not contain nitrite. Although the compounds were added at the same concentration, the nitrite peak is smaller than the nitrate peak. Because the mobile phase is pH 3, the NO_2^- forms HNO_2 (pKa = 3.4), allowing the possibility of nitrite losses by the following reaction in acidic solution:

$$3HNO_2 \rightarrow HNO_3 + H_2O + 2NO$$

To avoid this reaction, the pH of the mobile phase was increased from 3 to 9. At this higher pH, the mobile phase showed a high molar absorptivity at 205 nm that interfered with the measurement of the analytes. The mobile phase was changed to 5 mM K_2HPO_4 in 15% acetonitrile, pH 8.6 (the mobile phase from the NMKL method). No improvement was achieved; just 1 peak appeared. No further evaluations of this method were made because of its poor performance.

Strong Anionic Exchanger Methods; Comparison of NMKL and CEN Methods

The NMKL and the CEN methods are based on the same ion chromatographic principle, but they use different mobile phases and preparation procedures. Assays were performed to assess which mobile phases and sample preparations give better performance.

Evaluation of Mobile Phase

The extract of a meat product (sausage) was used for the analyses. The extract was separated on a strong anionic exchanger column (IC-PAK A 4.6 × 50 mm) by using 2 different mobile phases: 5 mM K_2HPO_4 in 15% acetonitrile, pH 8.6 (NMKL mobile phase), and buffer (boric acid, gluconic acid, lithium hydroxide, and glycerol) in 12.5% acetonitrile, pH 6.5 (CEN mobile phase). Figure 2 shows the

Table 1. Summarized description of the analytical methods assessed

Method	Sample preparation		Chromatographic conditions				
	Extraction	Clarification	Column	Mobile phase	Flow	Detection	Injection volume
Aminopropyl	10 g sample homogenized in hot water (50°–80°C), placed in a boiling water bath 15 min	Filter through filter paper and membrane filter	Aminopropyl bonded phase LiChrosorb-NH_2 (Hichrom)	57 mM K_2HPO_4 in 5% acetonitrile. Adjust to pH 3 with H_3PO_4	0.7 mL/min	205 nm	20 µL
NMKL	10 g sample + 0.5 g activated charcoal + 5 mL saturated borax + 50 mL water (80°C), placed in a boiling water bath 15 min	2 mL Carrez I and Carrez II solutions + 5 mL borax solution. Filter through filter paper and membrane filter	IC-PAK A4.6 × 50 mm (Waters)	5 mM K_2HPO_4 in 15% acetonitrile, pH 8.6	0.6 mL/min	215 nm	20 µL
CEN	10 g sample homogenized in 50 mL water (50°–60°C)	50 mL acetonitrile. Filter through filter paper and membrane filter	IC-PAK A HC (High Capacity) 4.6 × 150 mm (Waters)	Buffer (boric acid, gluconic acid, lithium hydroxide, glycerol) in 12.5% acetonitrile, pH 6.5	1 mL/min	205 nm	100 µL

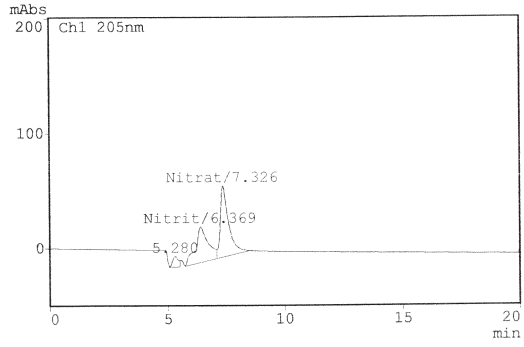

Figure 1. Chromatogram obtained with the weak anionic exchanger column for an aqueous standard solution containing nitrite and nitrate at 5 mg/L.

resolution of the peaks obtained with the NMKL and CEN mobile phases.

The CEN mobile phase (buffer in 12.5% acetonitrile, pH 6.5) shows a better separation of the peaks. In addition, this mobile phase has a practical advantage, i.e., it may be left on the chromatographic column as a storing solvent.

Sample Preparation (Extraction and Clarification)

Meat products, purchased from a local supermarket, were used for the comparison. Both spiked and unspiked meat products were analyzed. One set of samples was extracted and clarified exactly as described in the CEN method. The other set of samples was treated as described in the NMKL method with a modification: the sample was homogenized with water at 60°C (not at 80°C), and it was not placed in a boiling water bath for 15 min.

Table 2 shows the results of the evaluation. Sample preparation affected the recoveries of nitrite and nitrate. The samples pretreated according to the CEN method gave consistently higher recoveries than did samples pretreated according to the modified NMKL method. Application of the paired *t*-test showed a significant difference in the results. The theoretical value of *t* was lower (2.73) than the observed *t* values for nitrite and nitrate (4.73 and 4.10, respectively; $p = 0.05$; 18 degrees of freedom).

Later assays showed that the deproteinizing/clearing Carrez reagents interfere in the analyses (*see* below).

Sample Preparation and Matrix pH

One important difference in the sample preparation procedures of the NMKL and CEN methods is that the former extracts the nitrite/nitrate by using a borax buffer, which is intended to counteract the effect of low pH on the stability of nitrite/nitrate. In order to evaluate if the addition of borax buffer actually enhanced the recovery of the analytes, some assays were performed in which the pH of the matrix was changed before the samples were spiked with nitrite and nitrate ions.

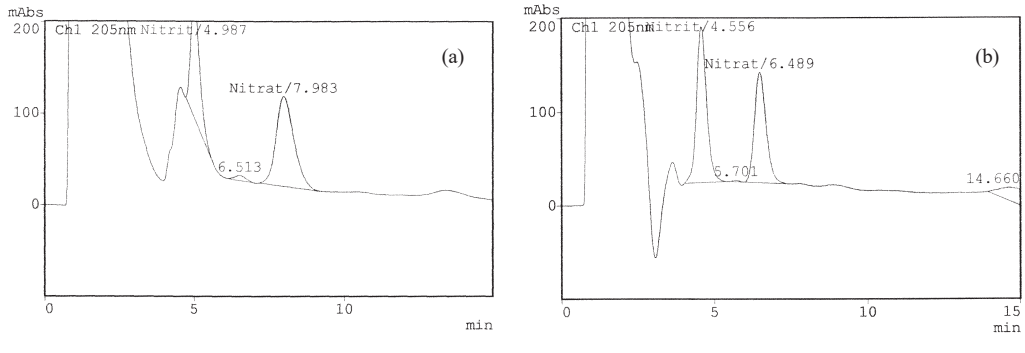

Figure 2. Separation of nitrite and nitrate in sausage extract on a strong anionic exchanger column: (a) with the NMKL mobile phase and (b) with the CEN mobile phase.

Table 2. Recovery of nitrite and nitrate from meat products pretreated according to the CEN and modified NMKL methods

	Nitrite				Nitrate			
	CEN		NMKL		CEN		NMKL	
Sample[a]	mg/kg	%	mg/kg	%	mg/kg	%	mg/kg	%
Pork sausage [b]	NF[c]	—	NF	—	27	—	22	—
Pork sausage + std [b]	112	98	109	95	150	107	133	98
Beef [b]	NF	—	NF	—	NF	—	NF	—
Beef + std	104	90	105	91	127	110	123	107
Ham	NF	—	NF	—	NF	—	NF	—
Ham + std	97	97	93	93	99	99	96	97
Pate [b]	NA[d]	—	NF	—	NA	—	16	—
Pate + std [b]	NA	—	78	78	NA	—	119	105
Chorizo	14	—	12	—	8	—	10	—
Chorizo + std	37	23	26	14	102	94	107	98
Bacon	NF	—	NF	—	NF	—	NF	—
Bacon + std	60	60	47	47	99	99	96	96
Hot dog [b]	80	90	73	—	70	94	61	—
Salami	NF	—	NF	—	NF	—	NF	—
Mortadella + std	NA	—	14	11	NA	—	131	104

[a] Std = standard.
[b] Mean of 2 results.
[c] NF = not found.
[d] NA = not analyzed.

A salami sample with pH < 5 was spiked directly and after pH adjustment with borax, borax plus 1M NaOH, and 5M NaOH. The samples were extracted according to the NMKL and CEN methods. The results are shown in Table 3.

From Table 3 it can be concluded that the higher the pH of the matrix, the higher the recovery obtained. From an analytical point of view, the results suggest that the difference in the pH of the extraction procedures of the methods has a minor influence on recovery, compared with the initial pH of the matrix.

European Prestandard as Horizontal Method; Analysis of Vegetables and Dairy Products

Vegetables (onion, carrot, potato, cucumber, spinach, and lettuce) were analyzed in parallel by using the weak anionic exchanger column (5) and the strong anionic exchanger column (6).

The chromatograms (Figure 3) show that for certain vegetables (i.e., carrot, onion, and potato), which normally have a very low concentration of nitrate, the strong anionic exchanger column is the only one capable of separating this ion. The separation obtained by using the weak anionic exchanger column (Figure 3a) did not allow calculation of the nitrate concentration. However, in the cases for which both methods achieved good chromatographic separation (i.e., cucumber, lettuce, and spinach), the concentrations of nitrate were the same (data not shown).

A cheese sample was also analyzed. Good separation and recovery were obtained.

Method Validation: A Simple and Practical Experimental Design Based on Analysis of Variance (ANOVA)

Many guidelines have been written, most of them describing interesting aspects of the elements of the validation procedure, but the experimental designs to estimate these parameters are omitted or pertain to several replicate analyses of different samples. In general, a good experimental design would enable the analyst to estimate most of the parameters with a minimum of analyses. This task is facilitated by the interrelation between the elements of the validation. However, the diverse approaches in the literature give the impression that to consistently follow the validation procedure proposed, an analyst must undertake enormous and tedious work (7), but this is not the case.

Wilson and Chesseman (8) proposed an experimental design using ANOVA to give an estimate of precision, bias, and limit of detection. The design specifies that analyses ought to be performed in several batches over a period of 1–2 weeks. Each batch consists of replicate analyses of blanks (limit of detection), standard solutions (sensitivity, linearity, and range), and both spiked and unspiked samples (recovery). To estimate precision over a concentration range, at least

Table 3. Influence of matrix pH on recovery of nitrite spike

		Nitrite			
		CEN		NMKL	
Sample	pH	mg/kg	%	mg/kg	%
Salami (sal.)	4.57	NF[a]	—	NF	—
Sal. + standard (std)	4.66	43	52	29	34
Sal. + borax (bx) + std	5.60	79	95	78	94
Sal. + bx + 1M NaOH + std	6.45	81	98	80	95
Sal. + 5M NaOH + std	10.4	NA[b]	—	85	102

[a] NF = not found.
[b] NA = not analyzed.

2 standard solutions are recommended for the upper and lower concentrations of interest. The advantage of this approach is that it estimates the within-batch standard deviation as pooled values from all the batches. This approach is of practical importance because it provides a reasonable indication of what is achievable.

In addition, this basic experimental design includes the internal reproducibility (between-batches standard deviation, S_b), which has an important consequence in relation to the distinction between the validation requirement of a single laboratory and of a regulatory body. Actually, a prerequisite for the acceptance of a method as official by committees or regulatory agencies is estimation of its performance through a collaborative study. Because a collaborative study is an expensive and work-intensive task, it is not a suitable option for all laboratories. However, by estimating the internal reproducibility, the analyst obtains preliminary information about the reproducibility of the method, i.e., the robustness under the "best" conditions.

One general point is worth noting here. The validation procedure should consist of the analysis of n portions in m batches. It is recommended that $n = 2$ and $m = 5$–10. The product $m \times n$ should never be < 10; ≥ 20 is better. Such a design encourages the use of a statistic criterion to estimate the number of analyses, to help to prevent an arbitrary and inappropriate decision.

A detailed discussion of the ANOVA as applied to method validation is beyond the scope of the present article, but the practical utility of ANOVA should not be overlooked. To put it more simply, its role in the experimental design of the validation method should be similar to the importance it has when interlaboratory studies are conducted (9).

Validation of the European Prestandard prENV 12014-4

The application range, linearity, precision, detection limit, and percent recovery were determined for the method.

The procedure was as follows: Foods (sausage, corned beef, ham, chorizo, cheese, potato, and baby food) purchased from a local supermarket were homogenized and spiked (except potato and chorizo) with different concentrations of ni-

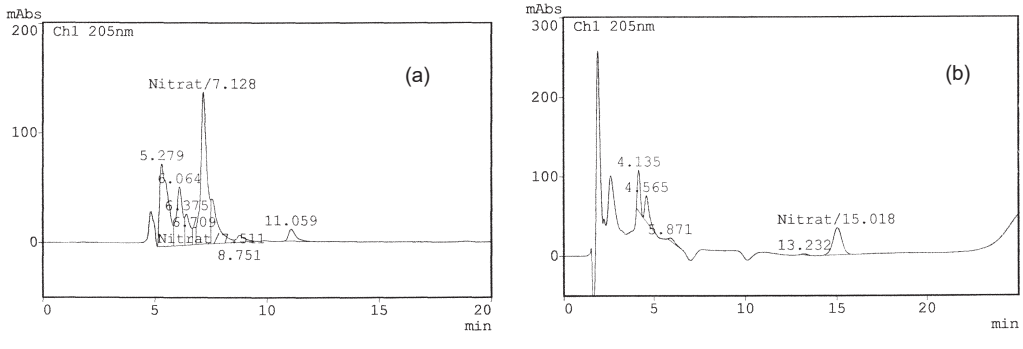

Figure 3. Chromatograms obtained from analyses of carrot samples using (a) a weak anionic exchanger column (CEN, Part 2, 1998) and (b) a strong anionic exchanger column (CEN, Part 4, 1998).

Table 4. Nitrite and nitrate found in sausage (mg/kg)

	NO_2^-		NO_3^-	
Batch	Test 1	Test 2	Test 1	Test 2
1	35.4	34.9	12.2	11
2	30.1	29.9	14.7	12
3	26.2	25.8	16.3	16.6
4	20.3	20.0	12.6	12.5
5	0.0	0.0	29.4	26.1

trite and nitrate ions (data not shown). Samples were stored at 4°C during the study.

Six standard solutions of nitrite and nitrate were used to calculate the calibration curve. In addition, 2 replicate standard solutions of lower (0.5 mg/L) and upper (20 mg/L) concentrations were analyzed as samples. The test consisted of the analyses of replicate samples ($n = 2$) in each batch ($m = 5$). The total of 10 analyses ($m \times n = 10$) gives a reasonable minimum number of degrees of freedom for the calculation of the within-batch standard deviation (S_w) and between-batches standard deviation (S_b) for each sample.

Results and Discussion

Linearity and Range

Linearity was addressed by preparing standard solutions of nitrite and nitrate at 6 levels (0.5, 1.0, 3.0, 5.0, 10.0, and 20.0 mg/L). Standards were injected in a random order into the chromatograph. A linear regression analysis of analyte concentration vs peak response was performed. The coefficients of determination (r^2) for nitrite and nitrate were 0.99999 and 0.99970, respectively. The upper concentration of the range is equivalent to 400 mg nitrite and/or nitrate/kg sample.

Precision and Internal Reproducibility

As an example of the repeatability obtained on precision, only the results for the sausage sample are shown in Tables 4 and 5. The results of the ANOVA are given in Table 5. The critical value of F ($P = 0.01$, $n_1 = 4$, $n_2 = 5$) is 11.4, which is less than 6851.9 and 44.6, the F values for nitrite and nitrate, respectively.

The F-test showed that there is a significant difference between the within-batch and between-batches standard deviations; this difference is explained by the instability of the analytes (Figure 4).

A prerequisite for the experimental design based on ANOVA is the stability of the sample. Unfortunately, we were unable to achieve an acceptable level of control over the instability of the analytes through the use of borax, time, and low temperature. Thus, the only way to estimate the reproducibility of the method was through an interlaboratory study (*see* below, Collaborative Study).

Limit of Detection and Limit of Determination

The NMKL Procedure No. 4 (10) establishes that the detection and determination limits are calculated by multiplying the standard deviation of the blank by 3 and 10, respectively. Because the studied method failed to detect an analytical response for the blank sample, the standard deviation of the standard solution of lowest concentration (0.5 mg/L) was used to calculate these limits (11).

Thus, the detection limits of nitrite and nitrate are 1 and 10 mg/kg, respectively.

Bias and Recovery Test

The experimental recovery is obtained from the difference between 2 measurements (sample and spiked samples), according to the following relationship:

$$\text{Recovery, \%} = (\text{total analyte found} - \text{analyte originally present}) \times 100/\text{analyte spike}$$

The recovery ranges for nitrite and nitrate estimated immediately after sample preparation (date not reported) were 96–108% and 96–107%, respectively. The poor recovery of the later batches was attributed to the instability of the analytes.

Identification of Systematic Errors: Constant-Type and Relative-Type Errors

According to Linnig et al. (12), relative-type and constant-type systematic errors are associated with the slope and intercept of a plot of found vs added concentration of the analyte, respectively. They also state that the effect of relative-type error is generally more important in the determination of major components, whereas constant-type errors are more serious in the determination of trace components. The

Table 5. Summary of sums of squares and degrees of freedom for sausage samples

Sample	Source of variability	Sums of squares	Degrees of freedom	Mean squares (MS)	F[a]
Sausage NO_2^-	Between-batches (B)	1480	4	370.0 (MSB)	6852
	Within-batch (W)	0.27	5	0.054 (MSW)	
Sausage NO_3^-	Between-batches	351.9	4	88	44.6
	Within-batch	9.86	5	1.97	

[a] F = (MSB)/(MSW).

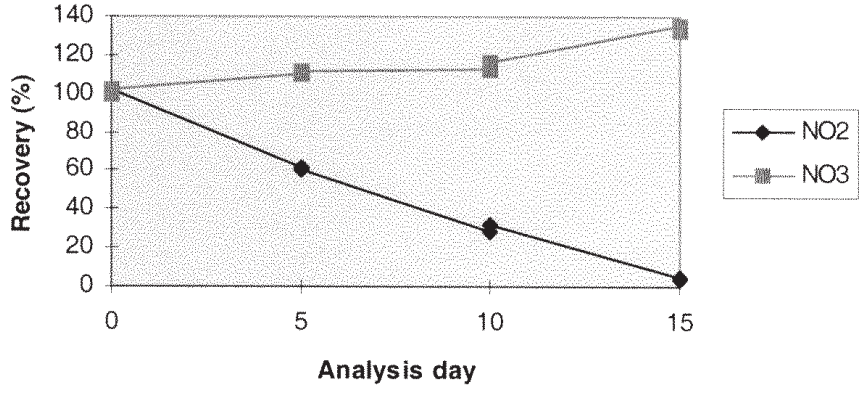

Figure 4. Rate of depletion of nitrite and nitrate spikes in ham.

ability to use the method to analyze other foods, which usually have low concentrations of nitrate and nitrite (baby food, cheese), made it necessary to identify these systematic errors.

An experiment was performed. Minced meat samples were spiked with different concentrations of nitrite and nitrate ions. The samples were analyzed immediately after preparation. Examination of the ratio values (columns 3 and 5, Table 6) shows that the nitrite ratios are distributed around a value close to unity, whereas the nitrate ratios decrease when the amount of analyte increases. This difference suggests the presence of a constant-type systematic error in the results of the nitrate analyses. A correction factor should be used, if meaningful results are to be obtained. The intercept of the regression line for nitrate is 13.4 (Figure 5). This value could be an adequate correction factor for the constant-type error in the analyses for residual nitrate in minced meat.

On the other hand, there is no definitive criterion for judging whether the intercept is an adequate correction factor for the analyses for nitrite, because the observed intercept falls into the confidence interval of the calculated intercept (data not shown).

The existence of a relative-type systematic error (error proportional to the amount of sample analyzed) is related to a slope different from unity. Nitrite and nitrate straight lines show slopes of 1.10 and 1.09, respectively, which indicate a relative error of about 10%.

Comparison of Ion Chromatographic (CEN-Part 4) and Spectrophotometric (CEN-Part 3) Methods for Determination of Nitrite in Minced Meat

Spiked minced meat samples were analyzed for nitrite by both methods. The spectrophotometric method is based on

Table 6. Results[a] of analyses of spiked minced meat samples for nitrite and nitrate

	Nitrite		Nitrate	
NO_2^- + NO_3^- added, mg/kg	Found, mg/kg	Ratio, found/added	Found, mg/kg	Ratio, found/added
5	5.2	1.027	17.1	3.419
10	10.0	1.002	20.4	2.636
30	31.2	1.040	42.9	1.431
50	51.6	1.033	74.2	1.485
100	103.4	1.034	119.2	1.192
200	218.1	1.090	232.6	1.163
400	431.7	1.074	445.1	1.113
600	661.6	1.103	669.6	1.116

[a] Each value is the mean of 2 results.

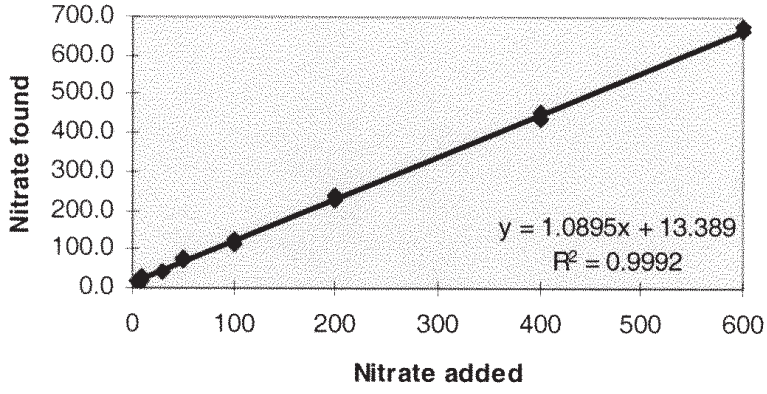

Figure 5. Effect of experimental errors in nitrate determination.

diazotization of sulfanilamide by nitrite, followed by coupling with *N*-(1-naphthyl)ethylenediamine dichloride to form an azo dye, which is measured spectrophotometrically at 540 nm (13).

The paired *t*-test did not give a significant difference (columns 2 and 3, Table 7).

Furthermore, another portion was analyzed simultaneously by the same ion chromatographic method using acetonitrile and the Carrez solution, which are the clarification solutions of the CEN and NMKL methods, respectively (columns 3 and 4, Table 7). The results show the disturbing effect of Carrez solution when the ion chromatographic method is used. These results agree very well with those of previous analyses performed during the evaluation of the methods that led to the choice of the European Prestandard (Table 2). Therefore, the Carrez solution is not recommended as a clarifying solution for the ion chromatographic method.

Collaborative Study

Participating Laboratories, Test Materials, and Statistical Procedure

A total of 17 laboratories participated in the collaborative studies I and II. The participating laboratories were in Denmark, Estonia, Finland, France, Germany, Italy, Norway, Spain, and Sweden.

The procedure established by AOAC INTERNATIONAL to conduct a collaborative study was followed (14).

If acidic, the test materials were neutralized with a solution of 5M NaOH and spiked with a saturated solution of borax to increase the pH. The test materials were sent by courier in a freeze-box. Recommendations were given to the participants to store the test materials in a refrigerator (4ºC) and start the analysis in the morning on the same day. Each laboratory was provided well in advance with the written version of the method, the schedule, instructions, etc.

Table 7. Comparison of residual nitrite levels in minced meat as determined by 2 different methods

	Found, mg/kg[a]		
	Spectrophotometry	Liquid chromatography	
NO_2^- + NO_3^- added, mg/kg	Carrez solution	Acetonitrile	Carrez solution
5	4.8	5.2	ND[b]
10	9.3	10.0	2.3
30	33.7	31.2	21.5
50	50.5	51.6	38.1
100	108.5	103.4	88.4

[a] Each value is the mean of 2 results.
[b] ND = not detected.

Statistical Analysis

The basis of the outliers test is to determine if the candidate outlier values are consistent with the Gaussian distribution assumed a priori. The outliers exclusion was done with the Cochran test and the single and double Grubbs tests at the 2.5% rejection level. The Cochran test is for the removal of laboratories showing significantly greater variability among replicates (blind duplicates). The Grubbs test is for the removal of laboratories with extreme values. The Horrat values were used to judge the reliability of the method. The Horrat value for reproducibility is the observed (RSD_R) value divided by the RSD_R value calculated from the Horwitz equation at the concentration of interest ($RSD_R = 2^{(1-0.5\log C)}$). If the Horrat value is ≤ 2, then the method may be assumed to have a satisfactory reproducibility (15). This is the acceptability criterion.

Collaborative Study I

Results were received from 13 laboratories (Table 8). Data for the test materials were rounded without decimals. The test materials were the following: A = sausage sample spiked with a low concentration of nitrite and nitrate; B, D = sausage sample spiked with a high concentration of nitrite and nitrate; C = baby food sample spiked with nitrite and nitrate; E = cabbage sample; and Std = 10 mg NO_2^-/L and 10 mg NO_3^-/L.

The results from laboratories 9 and 11 were considered as invalid data.When the outliers tests were applied to all laboratories, laboratories 9 and 11 had the most outliers. Furthermore, the results of the check standard solution were significantly different from those of other laboratories. Because the effect of retaining a real outlier is usually more serious than the effect of rejecting a valid point, the decision to eliminate all results of these laboratories as invalid data is correct. Valid data are "the values that the collaborator has no reason to suspect as being wrong" (14).

The outliers tests were applied to the results of the remaining laboratories. Laboratory 2 had Grubbs outliers in 4 of 5 analyses for nitrate. Laboratory 4 had a Grubbs outlier for the sausage sample (test material A).

In all analyses of the preliminary collaborative study, the precision (repeatability and reproducibility) was satisfactory.

Table 8. Reported values of nitrite and nitrate from collaborative study I

		NO_2^-, mg/kg						NO_3^-, mg/kg				
Laboratory code	Std, mg/L[a]	A	B	C	D	E	Std, mg/L	A	B	C	D	E
1	10.1	46	154	58	154	—	10.2	42	152	78	154	324
2	10.1	49	158	58	157	—	10.2	101[b]	210[b]	173[b]	210[b]	316
3	10.4	47	169	59	159	—	10.5	37	148	65	150	305
4	10.3	57	174	62	173	—	10.4	68[b]	175	75	170	322
5	9.8	43	162	53	180	—	8	31	126	54	136	269
6	10.3	48	150	59	160	—	10.2	40	150	76	150	300
7	9.9	45	156	57	149	—	10	36	152	67	144	287
9[c]	6.5	6	104	26	107	—	9.3	98	206	176	216	319
10	10.2	50	168	57	165	—	10.1	25	132	69	135	311
11[c]	10.9	56	186	82	187	7	11.7	138	286	133	231	384
12	10.5	47	158	62	162	—	10.5	39	150	67	151	292
14	9.5	41	144[b]	46	136	—	9.5	30	138	54	128	262
15	—	48	171	65	175	—	—	36	161	71	161	322
Accepted results	10	11	11	11	11	—	10	9	10	10	10	11
No. of outliers	—	0	1	0	0	—	—	2	1	1	1	0
Mean	10.1	47	160	58	161	—	10	35	148	68	148	301
SD_r[d]	—	—	6.1	—	6.1	—	—	—	4.2	—	4.2	—
SD_R[e]	—	4.1	9.3	5.0	12.5	—	—	5.6	14.1	8.2	12.6	21.3
RSD_R, %[f]	—	8.7	5.8	8.6	7.8	—	—	15.9	9.5	12.2	8.5	7.1
Horrat value	—	1	0.8	1.0	1.0	—	—	1.7	1.3	1.4	1.1	1.0

[a] Std = standard.
[b] Grubbs outlier.
[c] Invalid data.
[d] SD_r = repeatability standard deviation (calculated for the blind duplicate test materials).
[e] SD_R = reproducibility standard deviation.
[f] RSD_R, % = reproducibility relative standard deviation.

Collaborative Study II

Results were received from 15 laboratories (Table 9). The test materials were the following: F, K = salami sample spiked with a low concentration of nitrite and nitrate; G = lettuce sample; H = paté sample spiked with nitrite and nitrate; I = salami sample spiked with nitrite and nitrate; J = cheese sample; and Std = 5 mg NO_2^-/L and 5 mg NO_3^-/L. Laboratories 9 and 11 had Cochran outliers.

Laboratories 3, 4, 8, 9, 11, 13, and 14 had Grubbs outliers.

Test materials G and J contained only nitrate. However, some laboratories reported a very low concentration of nitrite. When the retention times were checked, most of them showed a significant difference from retention times reported by the other laboratories. These results (which could indicate the presence of interfering substances) were considered as invalid data.

On the basis of the Horrat values, 3 out of 10 did not fulfill the acceptance criterion (*see* Table 9, Horrat values; values are not given for test materials G and J, which had a nitrite concentration below the limit of detection). Two comments should be made. First, test materials F and K were the same (blind duplicates). In one case the acceptance criterion was fulfilled, and in the other case it was not. Second, the concentration of the nitrite spike in the test materials was very close to the limit of determination (5 and 25 mg/kg for nitrite and nitrate ions, respectively, values obtained in the validation performed by our laboratory), and obviously the uncertainty is higher at this level.

Table 9. Reported values of nitrite and nitrate from collaborative study II

		NO_2^-, mg/kg							NO_3^-, mg/kg					
Laboratory code	Std, mg/L[a]	F	G	H	I	J	K	Std, mg/L	F	G	H	I	J	K
1	4.9	7	—	52	51	—	9	5.0	67	2207	103	108	120	76
2[b]	4.9	—	—	68	50	7	11	5.0	56	2222	104	99	119	54
3	5.0	4	—	56	30[c]	—	7	5.0	56	2132	99	92	116	55
4	5.0	6	—	52	51	—	9	5.0	60	2137	94	96	60[c]	58
6	5.0	10	—	80	59	22	10	5.1	70	2505	150	117	141	70
7	5.0	6	—	70	49	4	8	5.0	78	2264	153	129	165	95
8	5.0	18[c]	—	64	61	—	22[c]	5.0	71	2320	102	116	142	82
9[b]	1.0	36[c]	23	118[c]	58	16	15[c]	3.3	75	2220	123	116	129	127[c]
10	4.8	7	—	66	54	—	9	4.9	76	—[d]	128	134	150	81
11[b]	4.8	43[c]	—	82	72[c]	—	—	4.8	58	2000	100	110	100	61
12	5.0	7	—	68	45	—	9	5.0	63	2200	107	105	108	61
13	4.9	10	4	45	52	—	8	5.0	68	1415[c]	110	101	157	2[c]
14	4.9	15[c]	—	76	52	350	14[c]	4.9	53	460[c]	93	85	273[c]	59
17	5.0	6	—	56	48	—	9	5.0	48	2346	105	99	131	55
18	4.9	10	—	70	49	—	6	4.9	68	2245	152	105	145	92
Accepted results	14	11	—	14	13	—	11	14	15	12	15	15	13	13
No. of outliers	—	4	—	1	2	—	3	—	0	2	0	0	2	2
Mean	4.9	7	<LOD[e]	65	52	<LOD	9	5.0	64	2233	115	107	133	69
SD_r[f]	—	2.2	—	—	—	—	2.2	—	8.3	—	—	—	—	8.3
SD_R[g]	—	2.0	—	11.0	4.7	—	1.5	—	9.0	124.8	21.1	13.3	19.5	14.6
RSD_R, %[h]	—	27.7	—	17.1	8.9	—	17.0	—	13.9	5.6	18.4	12.4	14.7	21.1
Horrat value	—	2.3	—	2.0	1.0	—	1.5	—	1.6	1.1	2.4	1.6	1.9	2.5

[a] Std = standard.

[b] Test materials F and K gave Cochran outliers (nitrite).

[c] Grubbs outlier.

[d] Excess data reported.

[e] LOD = limit of detection.

[f] SD_r = repeatability standard deviation.

[g] SD_R = reproducibility standard deviation.

[h] RSD_R,% = reproducibility relative standard deviation.

Conclusions

Nitrite determination with the weak anionic exchanger column, CEN-Part 2 (5), is not suitable. This method is not reliable for the analysis of certain vegetables with low concentrations of nitrate.

The pH of the matrix at the time of the addition of nitrite/nitrate has the greatest influence on the stability of these ions; the higher the pH, the better the recovery. Sample extraction with a buffer has a minor influence, compared with the pH of the matrix.

Recoveries of nitrite from meat products, vegetables, cheese, and baby food ranged from 96 to 108% with CEN-Part 4 (6). The corresponding range for nitrate recovery was 96–107%.

The detection limits for nitrite and nitrate ions were 1 and 10 mg/kg, respectively. The limits of determination were 5 and 25 mg/kg for nitrite and nitrate ions, respectively.

Constant-type and relative-type systematic errors were found in the analysis for nitrite and nitrate in minced meat. To obtain accurate analytical results, a correction of this systematic error is necessary. Relative-type errors were approximately 10% for both analytes.

In general, the performance of the method differs when nitrite and nitrate are determined. Better performance is obtained for nitrite determinations.

The study shows that the method can be applied to the analysis of foods other than meat and meat products, e.g., baby food, vegetables, and cheese.

In analyses for nitrite, no statistically significant difference was found between the methods studied, CEN-Part 4 (6) and the spectrophotometric method, CEN-Part 3 (13).

The precision of the method fulfills the criteria recommended by AOAC INTERNATIONAL (14) and the European Committee for Standardization (15); therefore, CEN-Part 4 (6) is suitable for the determination of nitrate and/or nitrate in various kinds of matrixes.

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European Standard EN 12014-4. Nordic Committee on F ood Analysis No.165, 2000

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NORDISK METODIKKOMITÉ FOR NÆRINGSMIDLER

NORDIC COMMITTEE ON FOOD ANALYSIS

No 165
2000

Nitrit och nitrat. Jonkromatografisk bestämning av nitrit och/eller nitrat i livsmedel

Denna metod är kollaborativt avprövad.

1. TILLÄMPNING OCH ANVÄNDNINGSOMRÅDE.

Denna metod beskriver en jonkromatografisk metod för bestämning av kvarvarande mängd nitrit och nitrat i olika livsmedel. Metoden kan användas för analys av kött, köttprodukter, barnmat, grönsaker och ost. Detektionsgränsen för nitrit och nitrat i jonform är 1 respektive 10 mg/kg. Utbytet av nitrit/nitrat varierade mellan 96-108%.

2. PRINCIP

Nitrit och nitrat extraheras från provet med hett vatten. Vattenlösningen behandlas med acetonitril för att avlägsna störande substanser. Nitrit- och nitratinnehållet i lösningen bestäms sedan med jonkromatografi (IC) och ultraviolett detektion (UV) vid våglängden 205 nm.

3. REAGENS

Alla reagens skall vara av pro analysi kvalitet och vatten skall vara av minst första gradens kvalitet enligt standard ISO 3696:1987. Destillerat vatten rekommenderas.

3.1 Acetonitril

3.2 Glycerol

3.3 Litiumhydroxid vattenfri, eller litiumhydroxid monohydrat

3.4 Borsyra, med massfraktion 99%

3.5 Saltsyra, HCl (= 1,18 g/ml, 36%)

Nitrite and nitrate. Nitrite and/or nitrate in foodstuffs by ion chromatography

This method is validated collaboratively.

1. SCOPE AND FIELD OF APPLICATION

This method describes an ion chromatographic method for the determination of the residual nitrite and nitrate contents in different foodstuffs. The method can be applied for analysing meat, meat product, baby food, vegetables and cheese. The limits of detection for nitrite and nitrate ions are 1 and 10 mg/kg respectively. The range of percent recovery of residual nitrite/nitrate was 96-108%.

2. PRINCIPLE

Nitrite and nitrate are extracted from the test sample with hot water. The aqueous solution is treated with acetonitrile to remove any interfering substances. The nitrite and nitrate contents of the solution are then determined by ion chromatography (IC) and ultraviolet (UV) detection at a wavelength of 205 nm.

3. REAGENTS

All reagents must be of analytical grade and the water used of at least grade 1 as defined in the standard ISO 3696:1987. Distilled water is recommended.

3.1 Acetonitrile

3.2 Glycerol

3.3 Lithium hydroxide anhydrous, or lithium hydroxide monohydrate

3.4 Boric acid, having a mass fraction of 99%

3.5 Hydrochloric acid, HCl (= 1.18 g/ml, 36%)

3.5.1 Saltsyra 1,8 mol/l. Pipettera 15 ml saltsyra (3.5) i en 100 ml mätkolv, späd med vatten till märket och blanda.

3.5.2 Saltsyra 0,1 mol/l. Späd 5 ml saltsyra (3.5) med vatten till märket i en 100 ml mätkolv och blanda.

3.6 Natriumnitrit Hydroskopiskt ämne. Torkas 1 h vid 110 °C.

3.7 Kaliumnitrat Hydroskopiskt ämne. Torkas 1 h vid 110 °C.

3.8 Stamlösning av nitrit och nitrat. Lös 1,500 g natriumnitrit (3.6) och 1,629 g kalium nitrat (3.7) i vatten i en 1000 ml mätkolv, späd till märket och blanda väl. Stamlösningen är hållbar i minst 2 veckor om den förvaras i kylskåp vid 4 °C.

3.9 Standardlösningar av nitrit och nitrat.
Pipettera 0 ml, 0,1 ml, 0,3 ml, 0,5 ml, 1,0 ml, respektive 2,0 ml av stam lösningen i sex 100 ml mätkolvar, späd till märket med vatten och blanda. 1 liter av dessa lösningar innehåller 0 mg, 1,0 mg, 3,0 mg, 5,0 mg, 10 mg respektive 20 mg av nitrit- och nitratjoner. Det rekommenderas att man bereder standardlösningarna den dag de skall användas.

3.10 Glukonsyralösning, med masskoncentration 50 g/100 ml, optiskt rengjord på fastfas extraktions-kolonn (4.6).
Observera: Glukonsyra har ibland en mörkbrun färg. Om så är fallet rekommenderas det att rena den tills en svagt gul färg erhålles.

3.11 Litiumborat glukonat buffertlösning: Till en 1000 ml mätkolv innehållande 500 ml vatten sätts 34,00 g borsyra (3.4) och 19,6 ml glukonsyra-lösning (3.8). I denna lösning löses helt 11,00 g vattenfri litiumhydroxid eller 19,26 g litiumhydroxid monohydrat (3.3). Tillsätt 125 ml glycerol (3.2), späd till märket med vatten och blanda väl. Lösningen är hållbar i minst 6 månader om den förvaras i kylskåp vid 4 °C.

3.12 Mobil fas: Till en 1000 ml mätkolv innehållande 500 ml vatten sätts 17 ml buffertlösning (3.9) och 125 ml acetonitril (3.1). Späd till märket med vatten. Blanda väl. Justera pH till 6,5 ± 0,1 med saltsyra (3.5.1 eller 3.5.2). Filtrera genom ett membranfilter med porstorlek 0,22 µm (4.3). Lösningen är hållbar i längst en vecka och pH bör konrolleras varje dag före användning. Om pH ligger

3.5.1 Hydrochloric acid 1.8 mol/l: Dilute 15 ml of hydrochloric acid (3.5) in a 100 ml volumetric flask to the mark with water and mix.

3.5.2 Hydrochloric acid 0.1 mol/l: Dilute 5 ml of hydrochloric acid (3.5.1) in a 100 ml volumetric flask to the mark with water and mix.

3.6 Sodium nitrite Hygroscopic substance. Dry 1 hr at 110 °C.

3.7 Potassium nitrate Hygroscopic substance. Dry 1 hr at 110 °C.

3.8 Stock solution of nitrite and nitrate. Dissolve 1.500 g of sodium nitrite (3.6) and 1.629 g of potassium nitrate (3.7) in a 1000 ml volumetric flask in water, dilute to the mark with water and mix well. The stock solution may be used at least for 2 weeks if stored in a refrigerator at 4 °C.

3.9 Standard solutions of nitrite and nitrate.
Pipette 0 ml, 0.1 ml, 0.3 ml, 0.5 ml, 1.0 ml and 2.0 ml, respectively of the stock solutions (3.6) into six 100 ml volumetric flasks, dilute to the mark with water and mix. 1 litre of these solutions contains 0 mg, 1.0 mg, 3.0 mg, 5.0 mg, 10 mg and 20 mg of nitrite/nitrate ions, respectively. It is recommended to prepare the standard solutions on the day of use.

3.10 Gluconic acid solution, having a mass concentration of 50 g/100 ml, optionally clarified on solid phase extraction column (4.6).
Note: Gluconic acid sometimes has a dark brown colour. In this case it is recommended to clarify it until a slight yellow colour is obtained.

3.11 Lithium borate gluconate buffer solution: To 500 ml of water in a 1000 ml volumetric flask 34.00 g of boric acid (3.4) and 19.6 ml of gluconic acid solution (3.8). Dissolve completely in the solution 11.00 g of anhydrous lithium hydroxide or 19.26 g of lithium hydroxide monohydrate (3.3). Add 125 ml of glycerol (3.2), dilute to the mark with water and mix well. This solution is stable for at least 6 months if stored in a refrigerator at 4 °C.

3.12 Mobile phase: To 500 ml of water in a 1000 ml volumetric flask, add 17 ml of buffer solution (3.11) and 125 ml of acetonitrile (3.1). Dilute to the mark with water. Mix well. Adjust the pH to 6.5 ± 0.1 by adding hydrochloric acid (3.5.1 then 3.5.2). Filter through a membrane filter of pore size of 0.22 µm (4.3). The solution can be stored for maximum one week. The pH value should be checked every day before use. If the pH value falls outside the stated range, prepare a new solution.

utanför accepterat intervall görs en ny lösning.

4. APPARATUR

Förutom allmän laboratorieutrustning behövs följande:

4.1 Homogeniseringsutrustning, mekanisk eller elektrisk, lämplig för att homogenisera provet. Denna inkluderar en höghastighetsrotationskniv, eller en kvarn försedd med platta med hål inte större än 4,5 mm i diameter, och en homogenisator.

4.2 Veckfilterpapper, nitratfritt.

4.3 Membranfilter, för vatten- och acetonitril-lösningar, med porstorlek ca. 0,22 µm.

4.4 Filterhållare, för membranfilter.

4.5 Membranfilter, engångs membranfilter (spruta och membranfilter 0,45 µm).

4.6 pH-meter

4.7 Fastfas extraktionskolonn RP C_{18} (100 mg), med passande spruta.

4.8 Jonkromatograf, bestående av följande:

4.8.1 Vätskekromatograf, isokratiskt system utrustat med UV-detektor, pump, injektor och skrivare, integrator eller arbetsstation.

4.8.2 Analytisk separationskolonn, Anjonbytare, 4,6 mm x 150 mm, packmaterial (ex. Waters IC Pak HC); polymetakrylat med kvartenär ammonium funktionell grupp, partikelstorlek 10 µm, kapacitet (30 ± 3)µ eq/ml, med förkolonn, ex. 20 mm, med samma packmaterial för att skydda den analytiska kolonnen.

Anmärkning: Waters IC pak HC är ett exempel på en kommersiellt tillgänglig lämplig kolonn. Denna information ges som en praktisk upplysning och utgör inget godkännande av NMKL av denna produkt.

5. UTFÖRANDE

5.1 Provberedning

Homogenisera provet med lämplig utrustning (4.1). Var försiktig så att temperaturen i provet inte

4. APPARATUS

In addition to normal laboratory equipment the following are required:

4.1 Homogenising equipment, mechanical or electrical, capable of homogenising the test sample, this includes a high-speed rotational cutter, or a mincer fitted with a plate with holes not exceeding 4.5 mm in diameter, and a homogenizer.

4.2 Fluted filter paper, nitrate-free.

4.3 Membrane filter, for aqueous and acetonitrile solutions, with a pore size of approximately 0.22 µm.

4.4 Filter holder for membrane filter.

4.5 Membrane filter, a disposable filter membrane device (syringe and membrane filter 0.45 µm).

4.6 pH meter

4.7 Solid phase extraction column RP C_{18} (100 mg), with suitable syringe.

4.8 IC apparatus, comprising the following:

4.8.1 Liquid chromatograph, isocratic system equipped with UV detector, pump, injector and printer, integrator or work station.

4.8.2 Analytical separating column, Anion exchanger, 4.6 mm x 150 mm, packing material (e.g. Waters IC Pak HC); polymethacrylate resin with a quarternary ammonium functional group, particle size of 10 µm, capacity (30 ± 3) µ eq/ml, with a precolumn, e.g. 20 mm, having the same packing to protect the analytical column.

Note: Waters IC Pak HC is an example of a suitable commercially available column. This information is given for the benefit of the user of this method and does not imply exclusive approval by NMKL.

5. PROCEDURE

5.1 Sample preparation

Homogenise the laboratory sample with the appropriate equipment (4.1). Make sure that the

överstiger 25 °C. Om en kvarn används så mal provet minst två gånger. Väg, på 10 m g när, in 10 g av det homogeniserade provet i en 150 ml vidhalsad E-kolv.

5.2 Extraktion och rening

Analysen måste genomföras på en arbetsdag.

Sätt 50 m l varmt vatten (50-60 °C) till provet i E-kolven. Blanda om sorgsfullt med homogenisator (4.1). Skölj hom ogenisatorn med vatten och tillför sköljvattnet till kolven. Överför kvantitativt provblandningen till en 200 m l mätkolv genom att skölja den vidhalsade kolven m ed vatten. Tillsätt 50 ml acetonitril (3.1). Blanda försiktigt. Låt svalna till rumstemperatur. Späd till märket med vatten.
Filtrera först genom veckfilterpapper (4.2) och sedan genom engångs m embranfiltert (4.5) för att göra en sista rening av provet. Gör en blanklösning där provet ersätts med 10 ml vatten.

5.3 Konstruktion av kalibreringskurva
För att rita kalibreringskurvan injiceras först standardlösningarna (3.7) och sedan blanklösningen.

5.4 Bestämning

5.4.1 Kromatografiska betingelser: Om kolonnen som angivits i (4.8.2) används, har goda resultat erhållits med följande parametrar:
Mobil fas: se (3.10)
Detektion (UV): 205 nm
Injektionsvolym: 100 µl
Flöde: 1 ml/min
Retentionstid (t_r): nitrit ≈ 16 min., nitrat ≈ 26 min.
Om en annan kolonn än den beskriven i (4.8.2) används kan de kromatografiska betingelserna behöva justeras.

5.4.2 Injicering: Injicera först standardlösningar (3.7) och sedan blank och provlösningar med betingelser beskrivna under 5.4.1. En av standardlösningarna skall injiceras efter vart fem te prov i en provserie. Mät topparean och beräkna koncentrationen av analyterna enligt 6.
Kontrollera med blankvärdet att ingen kontamination av nitrat och/eller nitrit finns.

6. BERÄKNING AV RESULTAT

Beräkna massfraktionen av nitrit/nitrat,
W (NO_2^-/NO_3^-), uttryckt som milligram jon per kg:

temperature of the sample material does not rise above 25 ºC. If a m incer is used, grind the sample at least twice. Weigh, to the nearest 10 m g, 10 g of the homogenised sample into an e.g. a 150 m l wide neck conical flask (test portion).

5.2 Extraction and clarification

The analysis should be performed in a single working day.
Add 50 ml of hot water (50 ºC to 60 ºC) to the test portion in the conical flask. Mix thoroughly with the homogenizer (4.1). Rinse the homogenizer with water and add the washings to the flask. Quantitatively transfer this slurry into a 200 m l volumetric flask by rinsing the wide neck flask with water. Add 50 m l of acetonitrile (3.1). Mix gently . Allow cooling to room temperature. Dilute to the mark with water.
Filter first through the fluted filter paper (4.2) and then use the disposable filter m embrane device (4.5) to carry out the final clarification of the sample.
Prepare a blank replacing the test portion by 10 ml of water.

5.3 Preparation of the calibration graph
To plot a calibration graph, inject the standard solutions (3.7) first and then the blank solution.

5.4 Determination

5.4.1 Chromatographic conditions: Using the column specified in (4.8.2) good re sults has been achieved with the following operating conditions:
Mobile phase: see under (3.10)
Detection (UV): 205 nm
Injection volume: 100 µl
Flow rate: 1 ml/min
Retention time (t_r): Nitrite ≈ 16 min; nitrate ≈ 26 min.
If other columns than the one described in (4.8.2) are used, adjust chromatographic conditions.

5.4.2 Injection: Inject the standard solutions (3.7) first and then the blank and the sam ples under the conditions described in 5.4.1. One of the standard solutions should be injected every five samples when performing a series of analyses. Measure the peak area and calculate the concentration of the analy tes as described in 6.
Check the blank value to ensure that there was no nitrate and/or nitrite contamination.

6. CALCULATION OF RESULTS

Calculate the mass fraction of nitrite/nitrate,
W (NO_2^-/NO_3^-), expressed in m illigrams of ion per

$$W_{(NO_2^-/NO_3^-)} = \frac{200 \cdot A_{(NO_2^-/NO_3^-)}}{m} \cdot F$$

där:
$A_{(NO_2^-/NO_3^-)}$ = värdet för nitrit och/eller nitrat, taget ur kalibreringskurvan, i milligram per liter
200 = utspädningsvolymen i milliliter
m = den invägda provmängden, i gram
F = spädningsfaktorn

Avrunda resultatet till heltal utan decimaler.

7. METODENS PÅLITLIGHET

Metodens pålitlighet undersöktes i en intern analytisk kvalitetskontroll och i två metodavprövningsstudier utförda enligt det harmoniserade IUPAC protokollet. Totalt 17 laboratorier från europeiska länder deltog. Data från metodavprövningarna summeras i ett annex.

8. METODENS REFERENTER

Metoden testades (internt och kollaborativt) av Leonardo Merino och Ulla Edberg på Livmedelsverket, Uppsala, Sverige.

Observera
Denna metod liknar i princip metoden beskriven i ”utkast Europeisk Prestandard prEN 12014-Part 4, Bryssel, 1998”.

kilogram from:

$$W_{(NO_2^-/NO_3^-)} = \frac{200 \cdot A_{(NO_2^-/NO_3^-)}}{m} \cdot F$$

where:
$A_{(NO_2^-/NO_3^-)}$ = is the value for nitrite and/or nitrate, read off the calibration graph, in milligrams per litre
200 = is the volume of dilution in millilitres
m = is the initial mass of the test portion, in grams
F = is the dilution factor

Give the results without any decimals.

7. RELIABILITY OF THE METHOD

The reliability of the method was investigated in an internal analytical quality control and two method-performance studies conducted according to the Harmonized IUPAC protocol. A total of 17 laboratories from European countries participated. Data of the method-performance studies are summarised in annex.

8. REFEREES OF THE METHOD

The method was validated (internal and collaboratively) by Leonardo Merino and Ulla Edberg at the Swedish National Food Administration in Uppsala, Sweden

Note
This method is similar in principle to the method described in the “draft European Prestandard prEN 12014-Part 4, Brussels, 1998”

Annex. Data från kollaborativa avprövningar

Metoden har testats i två kollaborativa avprövningar utförda av Livsmedelsverket, Sverige, under 1998 (tabell 1 och 2). Dessutom har samma metod 1994 testats på andra matriser av Centre Technique de la Salaison (CTSCCV), i Frankrike.

Kollaborativ studie I

Tabell 1. Studien utfördes av 13 laboratorier i europeiska unionen

	NO_2^- (mg/kg)			NO_3^- (mg/kg)			
	Korv	Korv	Barnmat	Korv	Korv	Barnmat	Kål
Accepterade resultat	11	11	11	9	10	10	11
Antal avvikande resultat	0	1	0	2	1	1	0
Medelvärde	47	161	58	35	148	68	301
Repeterbarhet standardavvikelse, SD_r	-	6,1	-	-	4,2	-	-
Repeterbarhet relativ SD, RSD_r (%)	-	3,8	-	-	2,8	-	-
Repeterbarhet, r (2,8 x SD_r)	-	17,2	-	-	11,6	-	-
Reproducerbarhet SD, SD_R	4,1	11,2	5,0	5,6	13,4	8,2	21,3
Reproducerbarhet relativ SD, RSD_R (%)	8,7	6,9	8,6	15,9	9,0	12,2	7,1
Reproducerbarhet, R (2,8 x RSD_R)	11,5	31,2	14,0	15,7	37,5	23,0	59,6

SD = standardavvikelse

Kollaborativ studie II

Tabell 2. Resultat inkom från 15 deltagande laboratorier i europeiska unionen

	NO_2^- (mg/kg)			NO_3^- (mg/kg)				
	Salami*	Salami	Paté	Salami*	Salami	Paté*	Ost	Sallad
Accepterade resultat	11	13	14	13	15	15	13	12
Antal avvikande resultat	4	2	1	2	0	0	2	2
Medelvärde	9	52	65	66	107	115	133	2233
Repeterbarhet SD, SD_r	2,2	-	-	8,3	-	-	-	-
Repeterbarhet relativ SD, RSD_r (%)	23,3	-	-	12,4	-	-	-	-
Repeterbarhet, r (2,8 x SD_r)	6,1	-	-	23,1	-	-	-	-
Reproducerbarhet SD, SD_R	4,2	4,7	11,0	12,9	13,3	21,1	19,5	124,8
Reproducerbarhet relativ SD, RSD_R (%)	45,1	8,9	17,1	19,4	12,4	18,4	14,7	5,6
Reproducerbarhet, R (2,8 x RSD_R)	11,9	24,9	47,9	36,0	34,7	51,5	41,2	15,7

* På basis av Horrat värden uppfyller inte data för precisionen acceptanskriterierna för reproducerbarhet och ges endast som information.

Annex. Data from method performance studies

The method has been studied in two interlaboratory tests carried out by the Swedish National Food Administration in 1998 (Table 1 and 2). Furthermore the same method was tested in 1994 with other matrixes by the Centre Technique de la Salaison (CTSCCV), France.

Collaborative study I

Table 1. The study was conducted by 13 laboratories in the European community

	NO_2^- (mg/kg)			NO_3^- (mg/kg)			
	Sausage	Sausage	Baby food	Sausage	Sausage	Baby food	Cabbage
Accepted results	11	11	11	9	10	10	11
Number of outliers	0	1	0	2	1	1	0
Mean	47	161	58	35	148	68	301
Repeatability standard deviation, SD_r	-	6.1	-	-	4.2	-	-
Repeatability relative SD, RSD_r (%)	-	3.8	-	-	2.8	-	-
Repeatability value, r (2.8 x SD_r)	-	17.2	-	-	11.6	-	-
Reproducibility SD, SD_R	4.1	11.2	5.0	5.6	13.4	8.2	21.3
Reproducibility relative SD, RSD_R (%)	8.7	6.9	8.6	15.9	9.0	12.2	7.1
Reproducibility value, R (2.8 x RSD_R)	11.5	31.2	14.0	15.7	37.5	23.0	59.6

SD = Standard deviation

Collaborative study II

Table 2. Results were received from 15 collaborating laboratories in the European community

	NO_2^- (mg/kg)			NO_3^- (mg/kg)				
	Salami*	Salami	Paté	Salami*	Salami	Paté*	Cheese	Lettuce
Accepted results	11	13	14	13	15	15	13	12
Number of outliers	4	2	1	2	0	0	2	2
Mean	9	52	65	66	107	115	133	2233
Repeatability SD, SD_r	2.2	-	-	8.3	-	-	-	-
Repeatability relative SD, RSD_r (%)	23.3	-	-	12.4	-	-	-	-
Repeatability value, r (2.8 x SD_r)	6.1	-	-	23.1	-	-	-	-
Reproducibility SD, SD_R	4.2	4.7	11.0	12.9	13.3	21.1	19.5	124.8
Reproducibility relative SD, RSD_R (%)	45.1	8.9	17.1	19.4	12.4	18.4	14.7	5.6
Reproducibility value, R (2.8 x RSD_R)	11.9	24.9	47.9	36.0	34.7	51.5	41.2	15.7

* Based on the Horrat values, the precision data do not fulfil the acceptance criteria of reproducibility, and are only given for the purpose of information.

3.1.3 Paper III - Levels of nitrate in Swedish lettuce and spinach over the past 10 years

Food Additives and Contaminants, 2006, 23(12): 1283-1289

Food Additives and Contaminants, December 2006; 23(12): 1283–1289

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Levels of nitrate in Swedish lettuce and spinach over the past 10 years

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Abstract
Monitoring of nitrate in Swedish-produced lettuce and spinach over the past 10 years (1996–2005) showed that more than 95% of the samples were below the maximum levels established by the European Commission in 1997. The good agricultural practices used by Swedish farmers may partly explain these results. Analytical results of organic farming production of lettuce from 2000 showed low nitrate levels compared with conventional production. The 10 years of Swedish experience has shown good compliance with the European Union maximum levels, but even lower nitrate levels may be achieved by organic farming methods, at least regarding fresh lettuce grown under cover.

Keywords: *Nitrate, vegetables, eutrophication, good agricultural practice, organic farming*

Introduction

Our intake of nitrate is from three main sources: vegetables, drinking water and food additives. Among them, vegetables are generally the major source (75–91%), yet in some areas drinking water can account for the major contribution (European Commission Scientific Committee for Food 1997). Nitrate is a natural component of vegetables originating from the uptake of nitrate ions in excess of its reduction and subsequent assimilation. The concentration of nitrate in vegetables depends on genetic factors, environmental variables and agricultural practice (Maynard et al. 1976).

When vegetables are classified according to their capacity of nitrate accumulation, genetic factors are most pronounced and commonly mask the effects of the other factors. In 1995, the National Food Administration (NFA), based on a survey of nitrate levels in vegetables from the Swedish retails market, made the following categorization: high levels ($>1000\,mg\,kg^{-1}$) — fresh lettuce, spinach; intermediate levels ($350–1000\,mg\,kg^{-1}$) — Chinese cabbage, iceberg lettuce, leek, beetroot, white cabbage; and low levels ($<350\,mg\,kg^{-1}$) — broccoli, cucumbers, carrot, cauliflower, potato, tomato (Merino et al. 1997). Thus, lettuce and spinach are those with relatively high nitrate accumulation capacity.

The environmental factors affecting nitrate levels in vegetables are light, temperature, humidity, carbon dioxide atmosphere, water supply, etc. It is well documented that a low light intensity is often associated with increased nitrate concentration in plants (Cantliffe 1972). Hence, nitrate levels tend to be higher in samples from Northern Europe than those from Mediterranean countries. The nitrate content in vegetables grown under cover is usually considerably higher than in those grown in the open air in the same seasons (European Commission Scientific Committee for Food 1997). In general, environmental variables may exert a marked effect on nitrate accumulation, but they are difficult to handle (Maynard et al. 1976).

With the use of Good Agricultural Practices (GAP), it may be easier to control the levels of nitrate in vegetables and much work is focused on this topic. Light, plant density, watering, fertilizing, sprinkling, etc., are some of the variables considered in GAP (European Commission 1997b–e).

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The scientific discussion about the potential risk with the usage of nitrate in the agricultural and food sector is focused on two major topics, namely environmental and health aspects. In the 1970s, there was an intense nitrate debate due to the potential relationship between the use of nitrates and nitrites as additives and the formation of carcinogenic nitrosamines. Nitrate — and its derivate nitrite — is also associated to a disease affecting the oxygen transport in blood (methaemoglobinaemia), of which babies are most susceptible (Walker 1990). Hence, the Scientific Committee for Food (SCF), based on toxicological studies of the increase in circulating methaemoglobin (using a safety factor of 500), established an acceptable daily intake (ADI) for nitrate of $3.7\,mg\,kg^{-1}$ body weight (European Commission Scientific Committee for Food 1997).

The intake of nitrate and nitrite from food are generally well within the ADIs. Thus, studies performed in the UK showed that taking into account nitrate intake from the rest of the diet, even the highest nitrate levels found in the UK Monitoring Programme are not likely to cause even high-level consumers of lettuce or of spinach to exceed the ADI (Department of Health 2004). An estimation of Swedish intake showed a mean intake of 18–21 $mg\,day^{-1}$ and a 95% percentile intake of 63–72 $mg\,day^{-1}$ (Fernlöf and Darnerud 1996), to be compared with the ADI = $222\,mg\,day^{-1}$. However, there may be a considerable range in nitrate intake levels depending on other sources than vegetables, and the presence of nitrate-contaminated drinking water could drastically increase the total intake. Small children are especially sensitive to the effects of methaemoglobinaemia, and that infant formula made with water with high nitrate levels could be a serious health risk. To eliminate potential risks, the Swedish NFA also recommends that small children should not be given juices made of nitrate-rich vegetables, such as beetroot.

To conclude, consumers seem in general to be sufficiently protected, as regards health effects, by the present regulation. High nitrate intake levels, which could be harmful especially in small children, may primarily be the result of factors other than nitrate in vegetables, e.g. contaminated drinking water. From a risk–benefit viewpoint, the beneficial health effects of vegetable consumption must also be taken into account. Moreover, a recently published study stresses evidence of the beneficial effects of some derivatives of nitrate, e.g. nitric oxides to help regulate blood pressure and protect the stomach against harmful substances (gastric juice, strong spices, alcohol, bacteria, etc.) (Lundberg et al. 2004).

As indicated above, the concern for the environmental and health aspects of nitrate as a water pollutant is still of great interest. The European Union monitoring networks indicate that over 20% of groundwater and between 30 and 40% of lakes and rivers show excessive nitrate concentration and agricultural sources account for between 50 and 80% of the nitrate entering Europe's water (European Commission 2005). Nutrient emissions from agriculture are the main reason for eutrophication. Rivers are now discharging several hundred thousand tonnes of nitrogen a year into the Baltic Sea. Swedish watercourses, however, account for only a minor proportion of this input. Instead, the greater part comes from Eastern Europe, where population and agricultural acreage are considerably greater. In Sweden, about 100 000 people today are dependent on drinking water that contains nitrate concentrations in excess of the limit ($10\,mg\,NO_3$-$N\,L^{-1}$) (Swedish EPA 2005). Hence, it is important to control the pollution caused by nitrates from agricultural sources to which codes of GAP play a key role (European Commission 1991).

The evaluation of the results of a Monitoring Programme for nitrate in lettuce and spinach, together with the adopted GAP codes and an updated scientific risk assessment, will be used by the European Commission to establish a longer-term strategy for managing the risk from nitrate in vegetables. This includes the review of the maximum levels lay down in 1997 (European Commission 2001).

In 1995, the Swedish NFA evaluated the content of nitrates in vegetables (Merino et al. 1997), and in 1996, it started a monitoring programme for nitrate in lettuce and spinach cultivated in Sweden. The result of this study is presented in this paper.

Materials and methods

Chemicals

All reagents must be of analytical grade and the water used of at least grade 1 as defined in the ISO Standard 3696:1987.

Equipment

The liquid chromatograph was an isocratic system equipped with a ultraviolet light detector. The analytical separating column was an anion exchanger, 4.6×150 mm, with packing material (e.g. Waters IC Pak HC); polymethacrylate resin with a quaternary ammonium functional group, with a particle size of 10 μm, was used.

Samples

The samples studied were fresh lettuce, iceberg lettuce, fresh and frozen spinach.

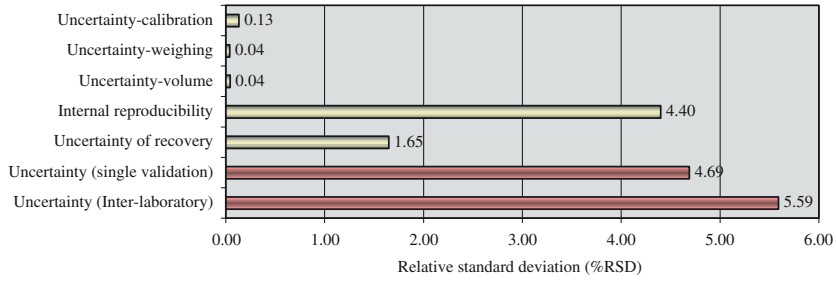

Figure 1. Estimation of uncertainty using the step-by-step method and its comparison with the uncertainty calculated from data of an interlaboratory study. The single validation combined uncertainty ($\mu_c = 4.69$) includes the overall precision ($\mu_{iR} = 4.40$) and the bias uncertainty ($\mu_{Rec} = 1.65$). The uncertainties of calibration, weighing and volume are included in the overall precision. The interlaboratory uncertainty ($\mu = 5.59$) was used to interpret the results of the Monitoring Programme.

Sample collection

The samples collected during the Monitoring Programme followed European Commission guidelines (European Commission 1997a), i.e. one sample per 2 metric tons of production with a minimum of 12 samples per year for product. Fresh lettuces, iceberg lettuce together with fresh and frozen spinach representative of the seasonal growing and the geographical areas of the country were collected annually by the Swedish NFA. Each collected laboratory sample consisted of at least ten individual vegetables. The samples were homogenized as a whole and frozen to −18°C until analysis.

In the summer of 2000, a project was started by the NFA to compare organic, conventional and integration farming in relation to the quality of the food produced. The project considered the analysis of content of bacteria, vitamins, metals, pesticides, nitrate, etc. (Staffas and Grönhholm 2002). From 100 samples, 24% of the fresh and iceberg lettuces came from organic farming. These results are included for comparison and are not a part of the Monitoring Programme.

Sample preparation

Nitrate was extracted from the sample with hot water (50–60°C) and the test solution treated with acetonitrile to remove suspended material (Nordic Committee on Food Analysis 2000).

Analytical method

The ion was determined by ion chromatography with ultraviolet detection at 205 nm (Nordic Committee on Food Analysis 2000).

Quality control

The method accomplishes the criteria for analytical performance established by the European Commission (1996). The authors' laboratory has previously evaluated this method. Thus, extraction, clean-up and chromatographic conditions were some of the parameters studied. Furthermore, an internal validation and two collaborative studies with the participation of 15 European laboratories were performed (Merino et al. 2000). The recovery rate calculated in the single validation was 103%. The recovery factor was not statistically significant; therefore, it was not used to correct the results. The uncertainty calculated in a single validation study was lower than the uncertainty obtained from an interlaboratory study (Figure 1). Because the confidence of the statistical parameters from interlaboratory studies is higher than the single validation (Codex Alimentarius Commission 2005), the former was used to interpret the results.

Throughout the Monitoring Programme, the Swedish NFA laboratory followed the recommended internal quality control procedure and participated annually in proficiency testing schemes with satisfactory performances.

Results and discussion

The yearly nitrate levels in lettuce and spinach are shown in Figures 2 and 3, respectively. The summary of the nitrate content, expressed as median, is presented in Table I. No significant differences were observed between the median and mean values in any of the studied vegetable groups, indicating a fairly normal distribution of the sample values. Some differences in levels were observed during the 10-year observation time, but no clear-cut time trend was obvious.

Table I. Incidence of nitrate above the maximum level (ML) detected through the Monitoring Programme, 1996–2005.

Type	Number of samples	Median ($mg\,NO_3\,kg^{-1}$)	Range ($mg\,NO_3\,kg^{-1}$)	Number above ML
Fresh lettuce	159	2684	58–5406	4
Iceberg lettuce	71	931	94–2298	1
Fresh spinach	63	1747	47–5975	12
Frozen spinach	70	551	213–1862	0
Fresh lettuce (organic farming)*	14	826	442–2038	0

*Samples not included in the Monitoring Programme.

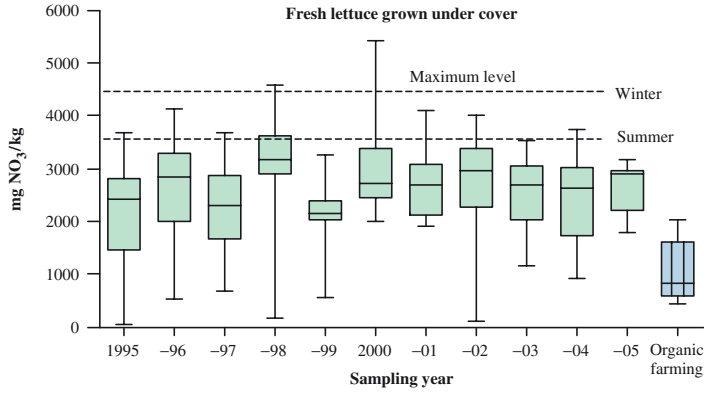

Figure 2. Nitrate content of fresh lettuce during 1995–2005. Four samples exceeded the maximum levels (ML), one in summer 1996, one in summer 1998, and two in winter 2000 (ML summer = 3500 mg kg^{-1}, ML winter = 4500 mg kg^{-1}).

A total of 159 samples of fresh lettuce were analysed. These samples were cultivated under cover during winter and summer. The median value found was of 2684 mg $NO_3\,kg^{-1}$, which is below the maximum limits established by the European Commission. However, four samples exceeded the regulatory limits: two were summer samples from 1996 and 1998 and two winter samples from 2000 (Table I and Figure 2). The producers were informed for corrective actions.

The median of the iceberg lettuce 931 mg $NO_3\,kg^{-1}$ was well below the regulatory limit (Table I and Figure 3). However, in 1996, one high value was observed. All the Swedish production of iceberg lettuce is produced on uncovered fields during the summer season.

The results from organic farming showed that the median nitrate levels in fresh lettuce was 826 mg $NO_3\,kg^{-1}$ and that in iceberg lettuce 672 mg $NO_3\,kg^{-1}$. Although only comparing nitrate data from one year (2000), the results indicate that organic farming, at least when compared with conventional farming of vegetables with fairly high nitrate levels (i.e. fresh lettuce), could lead to a substantial decrease in these levels. However, this is not the case with iceberg lettuce where it seems that the genetic factor is more important and masks the influence of the environmental factors and agricultural practices. In the study from 2000, fresh lettuce from organic production (826 mg $NO_3\,kg^{-1}$; $n = 14$) could be compared both with those from integrated production (1708 mg $NO_3\,kg^{-1}$; $n = 28$) and from conventional production (2484 mg $NO_3\,kg^{-1}$; $n = 5$), which may give further evidence for the importance of the production manner for the nitrate levels. There are other studies showing that organic farming in average has the potential of reducing nitrate levels in vegetables products (Brand and Molgaard 2001). However, the literature also reports studies showing inconsistent or not significant difference in nitrate content in conventional and organically grown crops (Lyons et al. 1994; Woese et al. 1997).

A total of 63 samples of fresh spinach were analysed with a median concentration of 1747 mg $NO_3\,kg^{-1}$ (Table I and Figure 4).

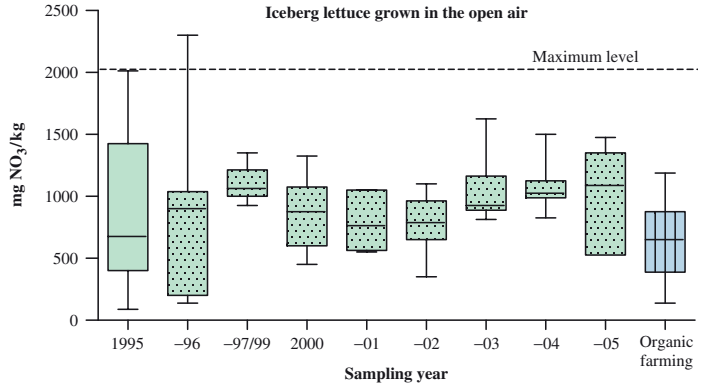

Figure 3. Nitrate content in iceberg lettuce during 1995–2005. One value in 1996 exceeded the maximum level. Due to few samples taken, the results of the 1997–99 are merged.

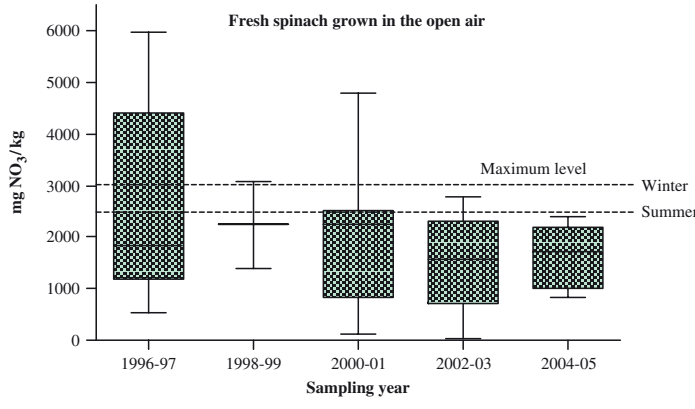

Figure 4. Nitrate content in open air fresh spinach during 1996–2005. Twelve samples exceeded the ML in 1996–97 and one sample in 2000–01. During 1998–99 only two samples of fresh spinach were analysed (ML summer = 2500 mg kg^{-1}).

Twelve samples from 1996 exceeded the maximum level specified in the regulation. All those samples came from the same producer who was using the vegetables for further processing as frozen spinach and not for direct consumption in the market.

The results of 70 frozen spinach samples with a median concentration of 551 mg NO_3 kg^{-1} (Table I and Figure 5) confirm that, as is well known, the nitrate content in processed vegetables is usually much less than in fresh vegetables due to the loss of nitrate during the blanching process (Maynard et al. 1976).

In theory, the adverse climatic conditions in Sweden, with low light intensity, may promote high levels of nitrates in plants. However, concentrations below the maximum levels of nitrate were observed in 96% of the samples analysed during the 10 years of the Monitoring Programme. The good agriculture practices, which in Sweden could be performed as Integrated Production (IP) (Green Production 2005) or organic farming, controlled by KRAV (http://www.krav.se) or Demeter (http://www.krav.se), allows Swedish farmers to produce lettuce and spinach fulfilling European regulations. The Swedish IP criteria are revised every 2 years and contain instructions that specifically address how to reduce nitrate levels in lettuce. Today, about half (lettuce under cover) or 75% (iceberg lettuce) of the production area for commercial lettuce production belongs to growers connected to the Swedish organization for IP production.

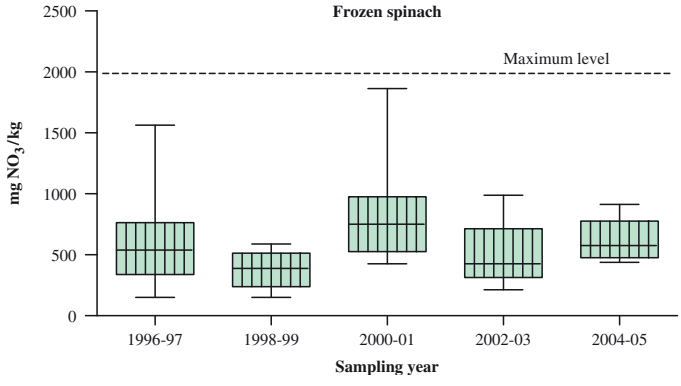

Figure 5. Nitrate content in frozen spinach during 1996–2005. All samples were below the maximum level (ML = 2000 mg kg^{-1}).

The adopted quality criteria for the analytical methods and sampling to be used by monitoring laboratories should minimize the adverse influence of these factors in the variability of the data. Consequently, the comparison of the levels of nitrate between Member States will be easier. In addition, a better insight of the role of agricultural practice on the nitrate levels in vegetables is gained.

There is a potential public health risk with elevated nitrate levels in food because of possible nitrosamine formation, as well as methaemoglobin. On the other hand, in the light of increasing evidence that the reduction of nitrate to nitrite and the subsequent formation of biologically active nitrogen oxides could be beneficial, the previous view emphasizing only the harmful effects of the intake of high nitrate may be reconsidered (Lundberg et al. 2004). However, although these new studies are discovering interesting aspects of the biological chemistry of the nitrogen cycle, they do not yet deny that the pollution of the environment with nitrate is a major public health problem (European Commission Scientific Committee for Food 1997).

Conclusions

Former surveys made by the NFA in Sweden in 1985 and 1995 (Lönberg and Everitt 1985; Merino et al. 1997) and the results of the Monitoring Programme reported in this paper show a sustained low concentration of nitrate in lettuce and spinach cultivated in Sweden. During the last 5 years, no samples with nitrate levels over the maximum limits established by the European Commission have been found. The specific instruction regarding nitrate reduction in lettuce production introduced by Swedish producer organizations may be an important factor in explaining these results.

Lower nitrate levels in organic farming were found in fresh lettuce but not in iceberg lettuce. This could indicate that the suitability of the agricultural practice as a regulator of the utilization of nitrogen could be limited by genetic factors. The Monitoring Programme is an effective tool to map out the nitrate levels in lettuce and spinach and its continuation would allow final conclusions about the role played by the GAP in the decrease of the nitrate content. The 10 years of Swedish experience strengthen the opinion that there is no technical reason to increase the European maximum level established in 1997.

Acknowledgements

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3.2 Work related to development of an analytical method: Control and exposure estimate of nitrite in meat products (Papers IV-VI)

3.2.1 Paper IV - Development and Validation of a Method for Determination of Residual Nitrite/Nitrate in Foodstuffs and Water After Zinc Reduction

Food Analytical Methods, 2009, Vol. 2:212-220

Food Anal. Methods (2009) 2:212–220
DOI 10.1007/s12161-008-9052-1

Development and Validation of a Method for Determination of Residual Nitrite/Nitrate in Foodstuffs and Water After Zinc Reduction

Leonardo Merino

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Abstract An environmentally friendly and cost-effective spectrophotometric method to analyze nitrate and/or nitrite was developed. The method is based on reduction of nitrate with zinc powder (instead of the cadmium or enzymes used in the standard methods approved by ISO/CEN). The initial nitrite concentration and total nitrite after reduction are determined by the very sensitive and widely used diazotization-coupling Griess reaction. A single-laboratory validation was applied in five different matrices (vegetable, meat product, baby food, dairy product, and surface water). The results show that the new method fulfills the international criteria for precision and recovery. The limit of detection for several matrices, calculated using fortified samples, ranged from 3 to 5 mg/kg for both nitrite and nitrate. Furthermore, the results obtained were in good agreement with those obtained using the CEN method (HPLC) and the ISO method (Cd reduction).

Keywords Nitrate and Nitrite Analysis · Environmentally Friendly · Spectrophotometry · Validation · Analysis of Variance

Introduction

The importance of monitoring nitrate/nitrite has long been recognized, but the reasons for this have changed in recent years. There was initial concern about the harmful effects of nitrate/nitrite on health, especially regarding the risk of infantile methemoglobinemia and formation of carcinogenic N-nitroso compounds. Now, some scientists are emphasizing the beneficial effects of dietary intake of nitrate after the discovery that nitric oxide (derived from nitrate and nitrite) plays an essential role in the immune system (Benjamin 2000; Dock 2005). Whatever the underlying scientific aim and the future implications in the legislation laid down by official bodies, there is still a need for suitable analytical methods so that these ions can be reliably monitored.

In 1993, the European Committee for Standardisation [CEN (European Organisation for Standardisation) 1993] started the work of adopting European Standard methods to analyze nitrate and nitrite in foodstuffs. From the beginning, the member experts expressed a desire to have HPLC methods as the European Standard. They also expressed a request for alternative methods to the international standard [ISO (International Organisation for Standardisation) 1985], which uses the hazardous cadmium (Cd). It had been argued that the Cd reduction method was the only method suitable for analyzing nitrate in dairy products [ISO (International Organisation for Standardisation) 2003]. However, an enzymatic method has been successfully validated for dairy products and was approved as international standard in 2006 [ISO (International Organisation for Standardisation) 2006].

The spectrophotometric (enzymatic) method and the HPLC standard methods employ relatively expensive reagents and instruments that are not easily available to laboratories with meager resources. Therefore, from a global perspective, an environmentally friendly and economical method for analyzing nitrate/nitrite in foodstuffs is required.

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The aim of this study was to develop a new sensitive method for use in routine determination of nitrite and nitrate in different kinds of samples without the use of cadmium. The analytical applicability was examined through a single-laboratory validation study.

Analytical Methods

A variety of methods proposed to analyze nitrate and nitrite involve spectrophotometric procedures (visible wavelength) and, more recently, HPLC methodologies (UV wavelength). The spectrophotometric methodology is generally based on the reduction of nitrate to nitrite. Earlier works report a large number of strategies to ensure a quantitative reduction of nitrate, but attention should be paid to some questionable statements found in the literature on this issue. For example, it is suggested (Chow and Johnstone 1962) that the further reduction of nitrite can be avoided if the nascent nitrite, after the reduction of nitrate, is diazoted with sulphanilamide at pH 2, but this recommendation is not suitable at the pH 11 required for the quantitative reduction of nitrate by zinc. Due to the fact that a number of variables are involved in the reduction, for example pH, oxidation–reduction potential, surface area of the reductant, time, temperature, etc. (Nydahl 1976), most of these being interdependent, it was difficult to distinguish the reasons why several procedures that were recommended did not function in our laboratory. Nevertheless, they were studied in order to measure the influence on performance of the proposed method and their feasibility.

Also, it is claimed in the literature that the determination of nitrate in the presence of nitrite is unreliable, because the metal reduction is not specific for nitrate. Several techniques have been proposed to overcome the over-reduction of nitrite, such as removing the nitrite for resin exchange (Lambert 1960), diazotization and boiling (Tsaihwa and Johnstone 1962), urea treatment and boiling (Mir 2007), etc. Here, it was demonstrated that the over-reduction of nitrite is not of practical importance if the reduction of nitrate to nitrite is optimized, and independent calibration curves are employed to calculate the concentration of nitrate and nitrite in the samples. The minor concentration of nitrite relative to nitrate usually found in foodstuffs and water strengthens this conclusion.

Likewise, the effect of manganese as reported by many studies (Bray 1945; Middleton 1957; Heanes 1975) was evaluated. Experiments conducted in our laboratory showed that use of manganese, either as catalyst or complexing agent, did not have significant effect on the reduction of nitrate, but it showed a definite disruptive effect on nitrite determination. Therefore, this reagent was not included in the improved procedure.

Materials and Methods

Scope and Field of Application

A spectrophotometric method was developed for the determination of nitrate/nitrite content in foodstuffs and water after zinc reduction and Griess reaction.

Principle

Nitrate (NO_3^-) is reduced quantitatively to nitrite (NO_2^-) in the presence of zinc powder (Zn). The nitrite (that originally present plus reduced nitrate) is determined by diazotizing with sulphanilamide and coupling with *N*-(1-naphthyl)-ethylenediamine dihydrochloride to form a highly colored azo dye that is measured at 540 nm. The nitrite present in the sample is determined by analyzing without the reduction step. The nitrate is calculated as the difference between the total nitrite content after reduction and the initial nitrite concentration.

Reagents

All reagents must be of analytical grade, and the water must be of deionized quality.

1. Sodium nitrite, $NaNO_2$ (Merck, Germany). Dried in a desiccator for 24 h.
2. Potassium nitrate, KNO_3 (Merck, Germany). Dried in an oven at 105 °C for 24 h.
3. Nitrite stock solution, 2,000 mg NO_2^-/L. Dissolve 0.6003 g of sodium nitrite (reagent #1) in water and dilute to 200 mL in a volumetric flask. At a temperature of 4 °C, this solution is stable for at least 3 months.
4. Nitrate stock solution, 2,000 mg NO_3^-/L. Dissolve 0.6521 g of potassium nitrate (reagent #2) in water and dilute to 200 mL in a volumetric flask. At a temperature of 4 °C, this solution is stable for at least 6 months.
5. Nitrite and nitrate working solutions, 100 mg/L. Dilute 5 mL of the stock solutions of nitrite (reagent #3) and nitrate (reagent #4) to 100 mL in separate volumetric flasks. Prepare daily.
6. Hydrochloric acid, HCl (=1.19 g/mL, 37%; Merck, Germany).
7. Hydrochloric acid, 1.0 mol/L. Dilute 83 mL HCl (reagent #6) to 1,000 mL.
8. Ammonia, NH_3 (=0.91 g/mL, 25%; Merck, Germany).
9. Ammonia buffer solution, pH 11.0. Add 75 mL ammonia (reagent #8) to 825 mL of water. Adjust pH to 11.0 with hydrochloric acid (reagent #7). Transfer the solution to a volumetric flask and dilute to 1,000 mL.

10. Carrez solution I. Dissolve 150 g of potassium hexacyanoferrate (II) trihydrate, $K_4[Fe(CN)_6]\cdot 3\ H_2O$ (Merck, Germany) in water and dilute to 1,000 mL. Store the solution in a brown bottle.
11. Carrez solution II. Dissolve 230 g of zinc acetate dihydrate, $Zn(CH_3COO)_2\cdot 2\ H_2O$ (Merck, Germany) in water and dilute to 1,000 mL.
12. Color reagent I. Dissolve 2.0 g sulphanilamide (Merck, Germany) in water and add 105 mL HCl (reagent #6). Dilute to 200 mL with water.
13. Color reagent II. Dissolve 0.2 g *N*-(1-naphthyl)-ethylenediamine dihydrochloride (Merck, Germany; Hopwin & Willian, England) in water and dilute to 200 mL water. Store in a dark bottle. Replace monthly or as soon as a brown color develops.
14. Zinc powder (Merck, Germany; Mallinckrodt, USA).

Apparatus

In addition to normal laboratory equipment, the following are required:

1. Milling device (Ultra-Turrax, Germany), appropriate for milling the sample.
2. Filter paper (Whathman, Germany), both nitrite and nitrate free.
3. Funnel.
4. pH meter (Orion, Switzerland).
5. Erlenmeyer flask, 100 mL.
6. Volumetric flask, 100 mL.
7. Centrifuge cups.
8. Centrifuge (Heraeus, Germany).
9. Spectrophotometer (Unicam, UK) for use at 540 nm.
10. Shaker (Edmund Buhler, Germany; optional).
11. Glass bottle, 100 mL.

Procedure

Preparation of the Test Sample

Homogenization and Extraction Weigh into an Erlenmeyer flask (apparatus #5), to the nearest 1 mg, 5–10 g of the homogenized laboratory sample. Add 60 mL hot water (50–60 °C). Homogenize with a grinding device (apparatus #1). Avoid producing undue heat.

Clarification Add in the following order, with swirling after addition of each reagent: 4 mL Carrez solution I (reagent #10) and 4 mL Carrez solution II (reagent #11). Transfer to centrifuge cups (apparatus #7) and centrifuge for 10 min at approximately 4,000 rpm (apparatus #8). Filter the clear supernatant quantitatively through the filter paper (apparatus #2). Dilute to 100 mL volumetric flask (apparatus #6) with water.

Determination of Nitrite

Transfer 20 mL of the test sample to a 100-mL volumetric flask (apparatus #6). Add 10 mL ammonia buffer and mix (reagent #9).

Color Development and Measurement Add 2 mL of color reagent I (reagent #12) and mix. Let stand for 5 min at room temperature. Add 2 mL of color reagent II (reagent #13), mix, and dilute to volume. Between 10 min and 2 h later, measure absorbance at 540 nm (apparatus #9). Adjust the instrument against water.

Determination of Nitrate

Reduction of Nitrate to Nitrite Transfer 20 mL of the test sample to a glass bottle (apparatus #11). Add 10 mL ammonia buffer (reagent #9) and 0.1 g of zinc powder (reagent #14). Shake vigorously for 5 min, manually or with the use of a shaker (apparatus #10). Filter the clear supernatant quantitatively through the filter paper (apparatus #2). Collect the filtrate in a 100-mL volumetric flask (apparatus #6).

Color Development and Measurement Add to the filtrate (Reduction of Nitrate to Nitrite found in "Determination of Nitrate") 2 mL of color reagent I (reagent #12) and mix. Let stand for 5 min at room temperature. Add 2 mL of color reagent II (reagent #13), mix, and dilute to volume. Between 10 min and 2 h later, measure absorbance at 540 nm (apparatus #9). Adjust the instrument against water.

Preparation of Calibration Graph

Calibration Graph for Nitrite (0–1.2 mg NO_2^-/L) Pipette 0, 1, 2, 3, 4, and 6 mL of the nitrite working solution (reagent #5) into separate Erlenmeyer flasks (apparatus #5). Add 60 mL hot water (50–60 °C). Carry out the procedure exactly as described in Clarification found in "Preparation of the Test Sample" and "Determination of Nitrite". Plot absorbance of the test portions against their nitrite concentration is in milligram NO_2^- per liter. Calculate the equation of the calibration graph $y_1=b_1x_1+c_1$.

Calibration Graph for Nitrate (0–1.2 mg NO_2^-/L) Pipette 0, 1, 3, 4, 6, and 8 mL of the nitrate working solution (reagent #5) into separate Erlenmeyer flasks (apparatus #5).

Add 60 mL hot water (50–60 °C) and carry out the procedure exactly as described in Clarification found in "Preparation of the Test Sample" and "Determination of Nitrate". Plot absorbance of the test portions against their nitrite concentration after reduction is in milligram NO_2^- per liter. Calculate the equation of the calibration graph $y_2 = b_2x_2 + c_2$.

Calculation of Results

Nitrite Concentration

Nitrite Concentration in the Test Sample Read from calibration graph [Calibration Graph for Nitrite (0-1.2 mg NO_2^-/L) found in "Preparation Of Calibration Graph"] the concentration of nitrite in the test sample, $x_{NO_2^-}$, in milligrams per liter:

$$x_{NO_2^-} = \frac{Abs_{(s1)} - Abs_{(bl1)}}{b_1} \times F$$

where:

$Abs_{(s1)}$	absorbance of the test portion
$Abs_{(bl1)}$	absorbance of the blank
b_1	gradient of the calibration graph
F	dilution factor, 5 in the method (plus any dilution of the test sample)

Nitrite Concentration in the Laboratory Sample Calculate the nitrite concentration in the laboratory sample, $w_{NO_2^-}$, in milligrams per kilogram:

$$w_{NO_2^-} = \frac{x_{NO_2^-} \times V}{m}$$

where:

$x_{NO_2^-}$	nitrite concentration in the test sample, in milligrams per liter
V	volume of the test sample, in milliliters (100 in the method)
m	mass of laboratory sample, in grams

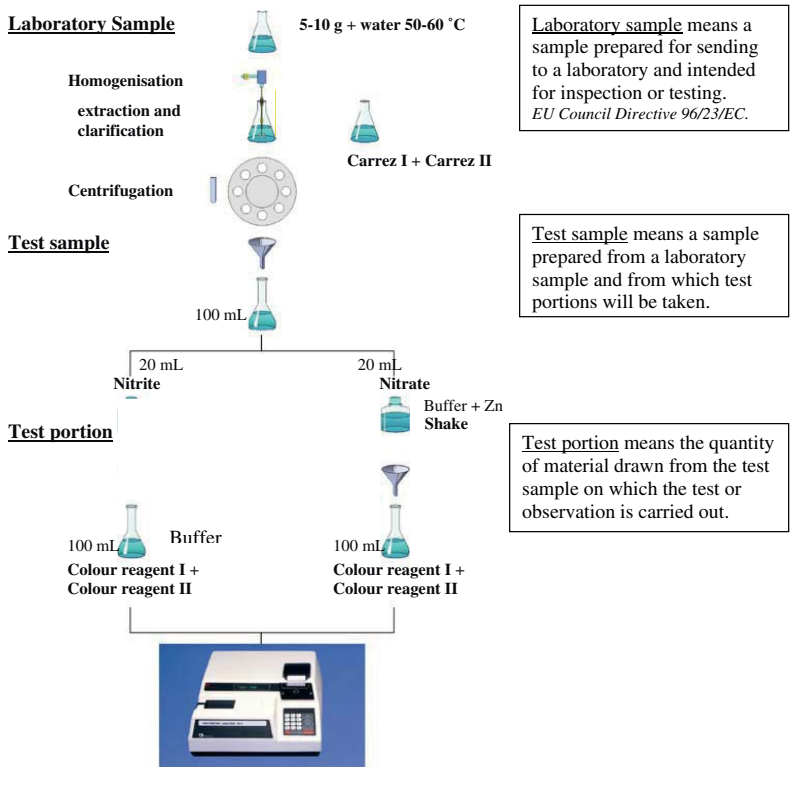

Fig. 1 Flow chart of the analytical procedure

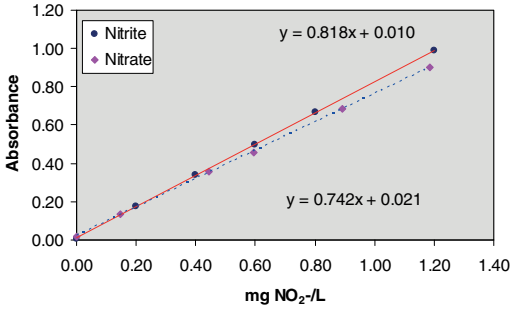

Fig. 2 Comparison of standard curves for nitrate and nitrite

Nitrate Concentration

The nitrate concentration is calculated as the difference between the total nitrite content after reduction ("Determination of Nitrate") and the nitrite concentration ("Determination of Nitrite").

Total Nitrite Concentration in the Test Sample Read from the calibration graph [Calibration graph for nitrate (0-1.2 mg NO_2^-/L) found in "Preparation of Calibration Graph"] the concentration of total nitrite in the test sample, $x_{\text{total NO}_2^-}$, in milligrams per liter:

$$x_{\text{total NO}_2^-} = \frac{\text{Abs}_{(s2)} - \text{Abs}_{(bl2)}}{b_2} \times F$$

where:

$\text{Abs}_{(s2)}$	absorbance of the test portion
$\text{Abs}_{(bl2)}$	absorbance of the blank
b_2	gradient of the calibration graph
F	dilution factor, 5 in the method (plus any dilution of the test sample)

Nitrate Concentration in the Laboratory Sample Calculate the nitrate concentration in the laboratory sample, $w_{NO_3^-}$, in milligrams per kilogram (Fig. 1):

$$w_{NO_3^-} = 1.35 \times \frac{\left(x_{\text{total NO}_2^-} - x_{NO_2^-}\right) \times V}{m}$$

where:

$x_{\text{total NO}_2^-}$	total nitrite concentration in the test sample, in milligrams per liter
$x_{NO_2^-}$	nitrite concentration in the test sample, in milligrams per liter
V	volume of the test sample, in milliliters (100 in the method)
m	mass of laboratory sample, in grams
1.35	factor of conversion of NO_3^- to NO_2^-

Method Development

The investigation in the laboratory sought to establish (a) the optimal conditions for the reduction of nitrate to nitrite so as to avoid incomplete reduction or over-reduction of nitrate and (b) the optimal pH for the diazotization-coupling of nitrite. To that end, the effect of buffer solution, pH range, amount and supplier of zinc powder, shaking time, speed, and time of centrifugation on the efficiency of reduction was examined.

Method Validation

A single-laboratory validation using five different matrices (vegetable, meat products, baby food, dairy product, and surface water) was performed. An experimental design based on analysis of variance was used (Wilson and Hunt 1986). Key performance parameters such as intermediate precision (repeatability and reproducibility), recovery, uncertainties, and limit of detections were calculated. The analyses were carried out in 11 batches over a time period

Table 1 Output from a regression analysis for nitrite and nitrate (milligrams per liter)

	Coefficients	Standard error	*p* value	Lower 95%	Upper 95%
Nitrite					
Intercept	0.010	0.008	2.34E−01	−0.007	0.027
Slope	0.818	0.013	6.55E−58	0.792	0.843
Nitrate					
Intercept	0.022	0.004	1.05E−05	0.013	0.031
Slope	0.742	0.007	2.87E−69	0.729	0.755

Table 2 Comparison of the calibration curves for standard solutions and a fortified sample

Analyte	Slope	Confidence interval (95%)
Nitrite (standard solution)	0.818	0.792–0.843
Nitrite (fortified sample)	0.764	0.734–0.793
Nitrate (standard solution)	0.742	0.729–0.755
Nitrate (fortified sample)	0.653	0.614–0.691

of 6 months. Each batch consisted of replicate analysis of samples, fortified samples, blanks, and standard solutions.

Preparation of the Samples

Samples of minced meat, baby food, milk, lettuce, and surface water were adjusted to pH 10 by adding successively 5 M NaOH to the whole batch of each matrix. Afterward, the samples were spiked with standard solutions of nitrite and nitrate to give levels of 57, 48, 33, 29, and 30 mg/kg, respectively. All samples were thoroughly mixed to homogeneity and stored in portions of 50 g at −18 °C. It is interesting to note that the instability of nitrite complicates validation according to recommended approaches, but this problem was overcome by increasing the pH and storing samples at low temperature (Merino et al. 2000).

Results and Discussion

Calibration Using Standard Solutions

In each batch of analyses, a calibration function was calculated from the regression analysis of five standard solutions of nitrate and nitrite and a blank. The concentration range for nitrite and nitrate was up to 1.2 mg/L, expressed as NO_2^- (Fig. 2).

The calibration curves prepared in the 11 batches show a low random scatter of slopes for both nitrite and nitrate, and therefore, the method would allow the use of a fixed calibration curve (Table 1). If this approach is used, the uncertainty effect resulting from calibration should be included in the uncertainty budget.

Calibration Using Fortified Samples (Matrix Effect)

To evaluate the matrix effect, calibration curves were prepared using fortified blank materials. The bias arising from the effect of the matrix was measured throughout the comparison of the slope of nitrite and nitrate for the calibration curves of standard solutions and a fortified meat product. The observed slope of the calibration curve for nitrite falls into the confidence interval of the calculated slope for the fortified sample (Table 2). Therefore, there is no definitive criterion for judging the presence of a bias caused by the matrix in the analysis of nitrite. On the other hand, the bias for the analysis of nitrate calculated by the difference between the slopes is approximately 12%. Both conclusions agree (taking into account the uncertainty) with the results of recovery test for the meat product (see Tables 6 and 7, columns 3, meat product).

Limit of Detection

The limit of detection for several matrices ranged from 3 to 5 mg/kg of nitrite and nitrate, respectively. They were calculated from the intercept of the calibration curves using fortified samples (Miller and Miller 2000; EU Decision 2002/657/EWC 2002).

Robustness Test

A robustness test based on the Plackett–Burman design was carried out to identify the potential source of variability of

Table 3 Results of the Plackett–Burman design to identify statistically significant effects

Parameter	Normal	Alternative	Absorbance nominal	Absorbance alternative	Diff	$2\times s_R$ Significant?
pH buffer	11.0	11.1	0.535	0.532	0.002	No
Zn quantity	0.10 g	0.13 g	0.531	0.536	0.005	No
Shaking	Apparatus	Manual (1 min)	0.673	0.394	0.278	Yes
Centrifugation	4,000 rpm	3,000 rpm	0.557	0.510	0.046	No
Zn quality	Merck	Mallinckrodt	0.596	0.471	0.125	Yes
Elapsed time	0 min	5 min	0.528	0.539	0.012	No
Wavelength	540 nm	538 nm	0.529	0.538	0.009	No

Eight combinations of seven parameters
Normal values described in the operating procedure, *Alternative* extreme variation that could occur when transferring the method

Table 4 Comparison of nitrite/nitrate levels as determined by three different methods

Sample	NO_2^- (mg/kg)			NO_3^- (mg/kg)		
	Zn	Cd	HPLC	Zn	Cd	HPLC
Vegetable (lettuce)	20.9	15.9	11.0	1,579	1,449	1,498
Minced meat	58.1	43.3	53.0	47.5	49.8	31.8
Reference material[a]	203	143	152	322	347	314
Baby food	51.4	38.1	47.3	37.1	56.0	57.8
Dairy product (milk)	28.8	22.3	25.8	26.7	28.8	44.8
Surface water	34.3	27.2	33.5	34.8	32.4	35.5

The data are mean values
[a] Test material for a proficiency test—minced meat (Proficiency Test 2006)
Nitrite: Assigned value=202 mg/kg, robust standard deviation=30.1 mg/kg. Nitrate: Assigned value=440 mg/kg, robust standard deviation=66.5 mg/kg

seven parameters (Table 3). Since a robustness test intentionally exaggerates potential sources of variability, the error estimated under reproducibility conditions of the validation study should be used to identify the physically relevant variations (Dejaegher and Vander Hayden 2006).

The difference between the average normal and alternative absorbance values for shaking type and Zn quality (supplier) exceeded twice the standard deviation of the internal reproducibility (Table 3). Accordingly, these parameters have a significant effect, and they should be carefully monitored when the method is transferred (King 2003).

Comparison with Standard Methods

The results obtained were in good agreement with those obtained using a Cd reduction method [ISO (International Organisation for Standardisation) 2003] and HPLC method [CEN (European Committee for Standardisation) 2004b]. The paired *t* test showed no statistical differences between the Zn method vs ISO method (Cd) and the Zn method vs CEN method (HPLC) for the analysis of nitrite and nitrate (Table 4).

The sensitivity of the new method compared favorably with that of state-of-the-art HPLC methods. As Table 5 shows, for analysis of samples of meat products with low concentration of residual nitrate and nitrite, the Zn-spectrophotometric method is the effective option.

Precision

The repeatability and within-laboratory reproducibility were estimated from the duplicate analyses of 11 batches. All samples gave Horrat values less than 2, which provides reasonable evidence that the factors affecting the analytical system were sufficiently well controlled.

Recovery

The differences between the pairs of results obtained from the spiked sample and the unspiked sample were used to calculate the recovery. The overall mean recovery was the average of the 11 mean recoveries for each batch.

The range of recovery of residual nitrite for the six samples was 70–110% (Table 6). The lettuce sample gave the lowest recovery and highest Horrat value for nitrite, probably because the concentration of added nitrite was small compared with the nitrate already in the sample and the relatively low weight of sample used in the validation

Table 5 Comparison of the new Zn-spectrophotometric method and the CEN method (HPLC)

Sample	Zn mg NO_2^-/kg	method mg NO_3^-/kg	CEN mg NO_2^-/kg	method mg NO_3^-/kg
Sausage	10±1	14±3	ND	ND
Sausage (chorizo)	15±1	4±1	ND	ND
Ham	0.4±0.04	6±2	ND	ND
Liver pâté	8±1	24±7	10±2	10±3
Baby food (meat)	1.5±1	2.2±1	1.5±0.3	0.8±0.2

The recommendation given by the Analytical Methods Committee to reporting results even though fall close or below the detection limited was followed (Anal Methods Comm Analyst 2001)
ND not detected (LOD≈5 mg NO_2^-/kg, 10 mg NO_3^-/kg; Merino et al. 2000)

Table 6 Reported values of nitrite from the single-laboratory validation study

	NO_2^- (mg/kg)					
	Vegetable (lettuce)	Meat product	Reference material	Baby food	Dairy prod. (milk)	Surface water
Accepted results	18	20	18	20	20	22
Number of outliers (Cochran and Grubbs test)	4	2	4	2	2	0
Mean	20.9	58.1	203	51.4	28.8	34.3
Repeatability standard deviation, s_r	2.99	0.72	8.64	0.91	0.45	0.38
Repeatability rel. standard deviation RSD_r (%)	14.3	1.24	4.26	1.77	1.56	1.10
Reproducibility standard deviation, s_R	4.22	1.25	5.18	0.80	1.11	1.00
Reproducibility rel. standard deviation RSD_{iR} (%)	20.2	2.15	2.55	1.56	3.65	2.92
Recovery (%)	70	102	101	109	99	104
Combined relative uncertainty, u_c (%)	25.0	2.59	4.46	2.50	4.10	3.23
Horrat value	2.0	0.2	0.4	0.2	0.4	0.3

The combined relative uncertainty (u_c) was calculated by taking the square root of the sum of the relative standard uncertainty of the intermediate precision (RSD_{iR}) and the relative standard uncertainty of the recovery ($u_{\%R}$) calculated in the validation study

study (0.4 g). Independent analyses for only nitrite carried out on approximately 10 g of the same lettuce sample gave recovery of 96%.

The range of recovery for nitrate was 73–105% (Table 7), which fulfills the recommended criteria established by the EU Commission for the official control of nitrate in foodstuffs, i.e., for a concentration range <500 mg/kg, the recommended recovery value is 60–120%, while for ≥500 mg/kg, the recommended recovery value is 90–110% CEN [EU Commission Regulation (EC) 1882/2006 2006].

Uncertainty

The estimation of uncertainty in measurements was based on the random effect (measured in terms of within-laboratory reproducibility) and systematic effect (measured as the uncertainty associated with the recovery test carried out along the validation study; King 2003; Wilson and Hunt 1986). The uncertainty due to sample non-homogeneity is already accounted for in the overall uncertainty.

Conclusions and Comments

- The results of the single-laboratory validation (in-house) study show that the method fulfills the internationally accepted fitness for purpose criteria for precision and recovery.
- The comparison of the proposed method and other standard methods shows no significant statistical difference for the analysis of nitrate.
- The sensitivity of the Zn-spectrophotometric method (visible wavelength) was compared favorably with the HPLC methods (UV wavelength). The Zn method can be a good alternative for analyses of samples with a low

Table 7 Reported values of nitrate from the single-laboratory validation study

	NO_3^- (mg/kg)					
	Vegetable (lettuce)	Meat product	Reference material	Baby food	Dairy prod. (milk)	Surface water
Accepted results	22	20	22	20	22	22
Number of outliers (Cochran and Grubbs test)	0	2	0	2	0	0
Mean	1,579	47.5	322	37.1	26.8	34.8
Repeatability standard deviation, s_r	52.4	2.41	21.2	5.21	1.89	3.47
Repeatability rel. standard deviation RSD_r (%)	3.32	5.07	6.58	14.0	7.05	9.97
Reproducibility standard deviation, s_R	109	5.63	30.7	6.17	3.13	3.69
Reproducibility rel. standard deviation RSD_{iR} (%)	6.90	11.9	9.53	16.6	11.7	10.6
Recovery (%)	94	83	73	–	92	105
Combined relative uncertainty, u_c (%)	7.80	13.1	13.9	22.1	13.9	14.9
Horrat value	1.3	1.3	1.4	1.8	1.2	1.1

concentration of residual nitrate/nitrite, e.g., meat products.

- The Zn method can be suitable for laboratories that need/want to substitute the method based on cadmium reduction [ISO (International Organisation for Standardisation) 2003].
- The Zn method can be suitable for laboratories that need/want to substitute methods based on the enzymatic principle [CEN (European Committee for Standardisation) 2004a; ISO (International Organisation for Standardisation) 2006; CEN (European Committee for Standardisation) 2004c].
- The new method proposed uses inexpensive reagents and instruments and can be helpful to laboratories in developing countries that want to adapt to international environmental requirements.

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3.2.2 Method resulting from Paper IV. Test method - Nitrite and nitrate. Determination of nitrate and/or nitrite in foodstuffs and water by spectrophotometry after zinc reduction and Griess reaction
Nordic Committee on Food Analysis, 2013, No.194

Nr. 194
2013

NORDISK METODIKKOMITÉ FOR NÆRINGSMIDLER

NORDIC COMMITTEE ON FOOD ANALYSIS

No. 194
2013

Nitrat och nitrit. Spektrofotometrisk bestämning av nitrat och/eller nitrit i livsmedel och vatten efter reduktion med zink och Griess reaktion.

Nitrate and nitrite. Determination of nitrate and/or nitrite in foodstuffs and water by spectrophotometry after zinc reduction and Griess reaction.

1. TILLÄMPNING OCH ANVÄNDNINGS-OMRÅDE

Detta dokument beskriver en spektrofotometrisk metod som kan användas för att bestäm ma nitrat- och nitritinnehållet i livsmedel samt vatten efter reduktion med zink och Griess reaktion. Metoden har validerats för sallat, kött produkter, barnm at, mejeri produkter samt vatten. Valideringen visar att metoden ullfyller internationellt accepterad metodkriterier för precision och bias.

1. SCOPE AND FIELD OF APPLICATION

This document specifies a spectrophotom etric method for the determ ination of nitrate/nitrite content in foodstuffs and water after zinc reduction and Griess reaction. The m ethod has been validated in vegetables (lettuce), meat products, baby food, dairy product (milk) and surface water. The validation studie shows that the m ethod fulfills the internationally accepted fitness for purpose criteria for precision and bias.

2. PRINCIP

Zinkpulver (Zn) används för att kvantitativt reducera nitrat (NO_3^-) till nitrit (NO_2^-). Nitriten (ursprunglig och reducerad nitrat) diazoteras med sulfanilamid och kopplas m ed N-(1-naftyl)-etyldiamindihydro-klorid för att bilda ett starkt färgat azofärgämne. Absorbansen för färgäm net mäts vid 540 nm. Mängden nitrit i provet bestäms genom att analysera provet utan reduktionssteget. Koncentrationen av nitrat beräknas genom att ta skillnaden mellan den totala nitritkoncentrationen efter reduktion och den ursprungliga nitritkoncentrationen.

2. PRINCIPLE

Nitrate (NO_3^-) is reduced quantitatively to nitrite (NO_2^-) in the presence of zinc powder (Zn). The nitrite (originally present plus reduced nitrate) is determined by diazotizing with sulfanilamide and coupling with N-(1-naphtyl)-ethylenediamine dihydrochloride to form a highly colored azo dye that is measured at 540 nm. The nitrite present in the sample is determined by analyzing without the reduction step. The nitrate is calculated as the difference between the total nitrite content after reduction and the initial nitrite concentration.

3. REAGENS

Alla reagens skall vara av pro analys i kvalitet och vattnet av avjoniserat kvalitet.

3.1 Natriumnitrit, $NaNO_2$. Torkad i en exsickator i 24 timmar.

3.2 Kaliumnitrat, KNO_3. Torkad i ugn vid 105 °C i 24 timmar.

3. REAGENTS

All reagents must be of analytical grade and the water shall be of deionised quality.

3.1 Sodium nitrite, $NaNO_2$. Dried in a desiccator for 24 hr.

3.2 Potassium nitrate, KNO_3. Dried in an oven at 105 °C for 24 hr.

3.3 Stamlösning av nitrit, 2000 mg NO_2^-/l. Lös upp 0,6003 g natriumnitrit (3.1) i vatten i en mätkolv och späd till 200 m l. Denna lösning är stabil i åtm instone 3 m ånader om den förvaras vid en temperatur av 4 °C.

3.4 Stamlösning av nitrat, 2000 mg NO_3^-/l. Lös upp 0,6521 g kalium nitrat (3.2) i vatten i en mätkolv och späd till 200 m l. Denna lösning är stabil i åtm instone 6 m ånader om den förvaras vid en temperatur av 4 °C.

3.5 Arbetslösningar av nitrit och nitrat, 100 m g/l. Späd 5 ml av nitritstam lösningen (3.3) och 5 ml av nitratstam lösningen (3.4) till 100 ml i separata mätkolvar. Nya arbetslösningar ska beredas dagligen.

3.6 Saltsyra, HCl (= 1,19 g/ml, 37 %).

3.7 Saltsyra, 1,0 mol/l. Späd 83 m l saltsyra (3.6) till 1000 ml.

3.8 Ammoniak, NH_3 (= 0,91 g/ml, 25 %).

3.9 Ammoniakbuffert, pH 11,0. Tillsätt 75 m l ammoniak (3.8) till 825 ml vatten. Justera pH till 11,0 med saltsyra (3.7). Överför lösningen till en mätkolv och späd till 1000 ml.

3.10 Carrezlösning I. Lös upp 150 g kaliumhexacyanoferrat(II)trihydrat, $K_4[Fe(CN)_6]\cdot 3\ H_2O$, i vatten och späd till 1000 ml. Förvara lösningen i en brun flaska.

3.11 Carrezlösning II. Lös upp 230 g zinkacetatdihydrat, $Zn(CH_3COO)_2\cdot 2\ H_2O$, i vatten och späd till 1000 ml.

3.12 Färgreagens I. Lös upp 2,0 g sulfanilam id i vatten och tillsätt 105 m l saltsyra (3.6). Späd till 200 ml med vatten.

3.13 Färgreagens II. Lös upp 0,2 g N-(1-nafty l)-etyldiamindihydroklorid i vatten och späd till 200 ml. Förvara lösningen i en brun flaska. Bered en ny lösning varje månad eller när lösningen brunfärgas.

3.14 Zinkpulver < 150 µm.

4. APPARATUR

Förutom allmän laboratorieutrustning behövs följande:

3.3 Nitrite stock solution, 2000 m g NO_2^-/l. Dissolve 0.6003 g of sodium nitrite (3.1) in water and dilute to 200 m l in a volum etric flask. At a temperature of 4 °C this solution is stable for at least 3 months.

3.4 Nitrate stock solution, 2000 m g NO_3^-/l. Dissolve 0.6521 g of potassium nitrate (3.2) in water and dilute to 200 m l in a volum etric flask. At a temperature of 4 °C this solution is stable for at least 6 months.

3.5 Nitrite and nitrate working solutions, 100 mg/l. Dilute 5 ml of the stock solutions of nitrite (3.3) and nitrate (3.4) to 100 m l in separate volumetric flasks. Prepare daily.

3.6 Hydrochloric acid, HCl (= 1.19 g/ml, 37 %).

3.7 Hydrochloric acid, 1.0 m ol/l. Dilute 83 m l HCl (3.6) to 1000 ml.

3.8 Ammonia, NH_3 (= 0.91 g/ml, 25 %).

3.9 Ammonia buffer solution, pH 11.0. Add 75 m l ammonia (3.8) to 825 ml of water. Adjust pH to 11.0 with hy drochloric acid (3.7). Transfer the solution to a volumetric flask and dilute to 1000 ml.

3.10 Carrez solution I. Dissolve 75 g of potassium hexacyanoferrate (II) trihy drate, $K_4[Fe(CN)_6]\cdot 3\ H_2O$ in water and dilute to 500 m l. Store the solution in a brown bottle.

3.11 Carrez solution II. Dissolve 115 g of zinc acetate dihydrate, $Zn(CH_3COO)_2\cdot 2\ H_2O$, in water and dilute to 500 ml.

3.12 Color reagent I. Dissolve 2.0 g sulfanilam ide in water and add 105 ml HCl (3.6). Dilute to 200 ml with water.

3.13 Color reagent II. Dissolve 0.2 g N-(1-naphty l)-ethylenediamine dihydrochloride in water and dilute to 200 m l water. Store in a dark bottle. Replace monthly or as soon as a brown color develops.

3.14 Zinc powder < 150 µm.

4. APPARATUS

In addition to normal laboratory equipment the following are required:

4.1 Homgeniseringsutrustning, lämplig för att homogenisera provet.

4.2 Filterpapper, nitrit- och nitratfritt.

4.3 Tratt.

4.4 pH-meter.

4.5 Erlenmeyerkolv, 100 ml.

4.6 Mätkolv, 100 ml.

4.7 Centrifugrör.

4.8 Centrifug.

4.9 Spektrofotometer, lämplig för att mäta absorbansen vid 540 nm.

4.10 Skakapparat

4.11 Glasflaska med skruvlock, 100 ml.

4.1 Grinding device, appropriate to grind the sample.

4.2 Paper filter, nitrite and nitrate free.

4.3 Funnel.

4.4 pH-mete.r

4.5 Erlenmeyer flask, 100 ml.

4.6 Volumetric flask, 100 ml.

4.7 Centrifuge cups.

4.8 Centrifuge.

4.9 Spectrophotometer, for use at 540 nm.

4.10 Shaker

4.11 Glass bottle, 100 ml.

5. UTFÖRANDE

5.1 Beredning av testprov

5.1.1 Homogenisering och extraktion
Väg, med en noggrannhet på 1 mg, in 5 – 10 g av det homogeniserade laboratorieprovet (se Annex 2) i en E-kolv (4.5). Tillsätt 60 ml varmt vatten (50-60 °C). Homogenisera provet varsamt med homogeniseringsutrustningen (4.1).

5.1.2 Rening
Tillsätt i följande ordning: 4 ml Carrezlösning I (3.10) och 4 ml Carrezlösning II (3.11). Skaka om efter varje tillsatts. Överför till centrifugrör (4.7) och centrifugera i 10 minuter vid ungefär 4000 rpm (4.8). Filtrera den klara supernatanten kvantitativt genom ett filterpapper (4.2) och samla upp filtratet i en 100 ml mätkolv (4.6). Späd lösningen till 100 ml med vatten.

5.2 Bestämning av nitrit

Överför 20 ml av testprovet till en 100 ml mätkolv (4.6). Tillsätt 10 ml ammoniakbuffert (3.9) och blanda.

5.2.1 Färgframkallning och mätning
Tillsätt 2 ml färgreagens I (3.12) till filtratet och blanda. Låt lösningen stå 5 minuter i rumstemperatur. Tillsätt 2 ml färgreagens II (3.13), blanda och späd till 100 ml (4.6). Efter 10

5. PROCEDURE

5.1 Preparation of the test sample

5.1.1 Homogenization and extraction
Weigh in an Erlenmeyer flask (4.5), to the nearest 1 mg, 5 – 10 g of the homogenized laboratory sample (see Annex 2). Add 60 ml hot water (50-60 °C). Homogenize gently with a grinding device (4.1).

5.1.2 Clarification
Add in the following order, with swirling after addition of each reagent: 4 ml Carrez solution I (3.10) and 4 ml Carrez solution II (3.11). Transfer to centrifuge cups (4.7) and centrifuge for 10 min at approximately 4000 rpm (4.8). Filter quantitatively the clear supernatant through the filter paper (4.2). Dilute to 100 ml volumetric flask (4.6) with water.

5.2 Determination of nitrite

Transfer 20 ml of the test sample to a 100 ml volumetric flask (4.6). Add 10 ml ammonia buffer (3.9) and mix.

5.2.1 Color development and measurement
Add to the filtrate 2 ml of color reagent I (3.12) and mix. Let it stand for 5 min at room temperature. Add 2 ml of color reagent II (3.13) mix and dilute to volume. Between 10 min and 2 hr afterward, measure absorbance at 540

minuter upp till 2 tim mar mät absorbansen vid 540 nm (4.9). Nollställ instrumentet med vatten.

5.3 Bestämning av nitrat

5.3.1 Reducering av nitrat till nitrit
Överför 20 m l av testprovet till en flaska (4.11). Tillsätt 10 ml ammoniakbuffert (3.9) och 0,1 g zinkpulver (3.14). Skaka kraftigt i 5 minuter, manuellt eller med hjälp av en skakmaskin (4.10). Filtrera den klara supernatanten kvantitativt genom ett filterpapper (4.2). Sam la upp filtratet i en 100 ml mätkolv (4.6).

5.3.2 Färgframkallning och mätning
Tillsätt 2 m l färgreagens I (3.12) till filtratet (5.3.1) och blanda. Låt lösningen stå 5 m inuter i rumstemperatur. Tillsätt 2 ml färgreagens II (3.13) och blanda. Späd till 100 ml. Mät absorbansen vid 540 nm (4.9) efter 10 m inuter upp till 2 timmar. Nollställ instrumentet med vatten.

5.4 Beredning av kalibreringskurva

5.4.1 Kalibreringskurva för nitrit (0-1.2 mg/L)
Pipettera 0, 1, 2, 3, 4 och 6 m l av nitritarbetslösningen (3.5) till separata E-kolvar (4.5). Följ därefter utförandet som beskrivs i 5.1 och 5.2. Avsätt testportionernas absorbans som en funktion av deras nitritkoncentration i m g NO_2^-/l. Beräkna ekvationen fö r kalibreringskurvan $y_1 = b_1x_1 + c_1$.

5.4.2 Kalibreringskurva för nitrat (0-1.2 mg/L)
Pipettera 0, 1, 3, 4, 6 och 8 m l av nitratarbetslösningen (3.5) till separata E-kolvar (4.5). Följ därefter utförandet som beskrivs i 5.1 och 5.3. Avsätt testportionernas absorbans som en funktion av deras nitritkoncentration efter reduktion i mg NO_2^-/l. Beräkna ekvationen för kalibreringskurvan $y_2 = b_2x_2 + c_2$.

6. BERÄKNING AV RESULTAT

6.1 Koncentration av nitrit

6.1.1 Koncentration av nitrit i testprovet
Använd kalibreringskurvan (5.4.1) för att bestämma koncentrationen av nitrit i testprovet, $x_{NO_2^-}$, i mg/l:

$$x_{NO_2^-} = \frac{\mathrm{Abs}_{(s1)} - \mathrm{Abs}_{(bl1)}}{b_1} \cdot F$$

där:
$\mathrm{Abs}_{(s1)}$ = testportionens absorbans
$\mathrm{Abs}_{(bl1)}$ = blankens absorbans

nm (4.9). Adjust the instrument with water.

5.3 Determination of nitrate

5.3.1 Reduction of nitrate to nitrite
Transfer 20 ml of the test sam ple to a glass bottle (4.11). Add 10 m l ammonia buffer (3.9) and 0.1 g of zinc powder (3.14). Shake vigorously for 5 min, manually or with the use of a shaker (4.10). Filter quantitatively the clear supernatant through the filter paper (4.2). Collect the filtrate in a 100 m l volumetric flask (4.6).

5.3.2 Color development and measurement
Add to the filtrate (5.3.1) 2 m l of color reagent I (3.12) and mix. Let it stand for 5 min at room temperature. Add 2 ml of color reagent II (3.13) m ix and dilute to volume. Between 10 min and 2 hr afterward, measure absorbance at 540 nm (4.9). Adjust the instrument against water.

5.4 Preparation of calibration graph

5.4.1 Calibration graph for nitrite (0-1.2 mg/L)
Pipette 0, 1, 2, 3, 4 and 6 m l of the nitrite working solution (3.5) into Erlenm eyer flasks (4.5). Carry out the procedure exactly as described in 5.1 and 5.2. Plot absorbance of the test portions against their nitrite concentration in m g NO_2^-/l. Calculate the equation of the calibration graph $y_1 = b_1x_1 + c_1$.

5.4.2 Calibration graph for nitrate (0-1.2 mg/L)
Pipette 0, 1, 3, 4, 6 and 8 ml of the nitrate working solution (3.5) into separate Erlenmeyer flasks (4.5). Carry out the procedure exactly as described in 5.1 and 5.3. Plot absorbance of the test portions against their nitrite concentration after reduction in m g NO_2^-/l. Calculate the equation of the calibration graph $y_2 = b_2x_2 + c_2$.

6. CALCULATION OF RESULTS

6.1 Nitrite concentration

6.1.1 Nitrite concentration in the test sample
Read from calibration graph (5.4.1) the concentration of nitrite in the test sample, $x_{NO_2^-}$, in mg/l:

$$x_{NO_2^-} = \frac{\mathrm{Abs}_{(s1)} - \mathrm{Abs}_{(bl1)}}{b_1} \cdot F$$

where:
$\mathrm{Abs}_{(s1)}$ = absorbance of the test portion
$\mathrm{Abs}_{(bl1)}$ = absorbance of the blank

b_1 = kalibreringskurvans lutning
F = utspädningsfaktor, 5 i metoden, (samt eventuell utspädning av testprovet)

6.1.2 Koncentration av nitrit i laboratorieprovet

Beräkna koncentrationen av nitrit i laboratorieprovet, $w_{NO_2^-}$, i mg/kg:

$$w_{NO_2^-} = \frac{x_{NO_2^-} \cdot V}{m}$$

där:
$x_{NO_2^-}$ = koncentration av nitrit i testprovet, i mg/l
V = testprovets volym, i milliliter (100 i metoden)
m = laboratorieprovets vikt, i gram

6.2 Koncentration av nitrat

Koncentrationen av nitrat beräknas genom att ta skillnaden mellan den totala nitritkoncentrationen efter reduktion (5.3) och nitritkoncentrationen (5.2).

6.2.1 Total koncentration av nitrit i testprovet

Använd kalibreringskurvan (5.4.2) för att bestämma den totala koncentrationen av nitrit i testprovet, $x_{totalNO_2^-}$, i mg/l.

$$x_{totalNO_2^-} = \frac{\mathrm{Abs}_{(s2)} - \mathrm{Abs}_{(bl2)}}{b_2} \cdot F$$

där:
$\mathrm{Abs}_{(s2)}$ = testportionens absorbans
$\mathrm{Abs}_{(bl2)}$ = blankens absorbans
b_2 = kalibreringskurvans lutning
F = utspädningsfaktor, 5 i metoden, (samt eventuell utspädning av testprovet)

6.2.2 Koncentration av nitrat i laboratorieprovet

Beräkna koncentrationen av nitrat i laboratorieprovet, $w_{NO_3^-}$, i mg/kg:

$$w_{NO_3^-} = 1{,}35 \cdot \frac{\left(x_{totalNO_2^-} - x_{NO_2^-}\right) \cdot V}{m}$$

där:
$x_{totalNO_2^-}$ = total koncentration av nitrit i testprovet, i mg/l

b_1 = gradient of the calibration graph
F = dilution factor, 5 in the method, (plus any dilution of the test sample)

6.1.2 Nitrite concentration in the laboratory sample

Calculate the nitrite concentration in the laboratory sample, $w_{NO_2^-}$, in mg/kg:

$$w_{NO_2^-} = \frac{x_{NO_2^-} \cdot V}{m}$$

where:
$x_{NO_2^-}$ = nitrite concentration in the test sample, in mg/l
V = volume of the test sample, in milliliters (100 in the method)
m = mass of laboratory sample, in grams

6.2 Nitrate concentration

The nitrate concentration is calculated as the difference between the total nitrite content after reduction (5.3) and the nitrite concentration (5.2).

6.2.1 Total nitrite concentration in the test sample

Read from the calibration graph (5.4.2) the concentration of total nitrite in the test sample, $x_{totalNO_2^-}$, in mg/l:

$$x_{totalNO_2^-} = \frac{\mathrm{Abs}_{(s2)} - \mathrm{Abs}_{(bl2)}}{b_2} \cdot F$$

where:
$\mathrm{Abs}_{(s2)}$ = absorbance of the test portion
$\mathrm{Abs}_{(bl2)}$ = absorbance of the blank
b_2 = gradient of the calibration graph
F = dilution factor, 5 in the method, (plus any dilution of the test sample)

6.2.2 Nitrate concentration in the laboratory sample

Calculate the nitrate concentration in the laboratory sample, $w_{NO_3^-}$, in mg/kg:

$$w_{NO_3^-} = 1.35 \cdot \frac{\left(x_{totalNO_2^-} - x_{NO_2^-}\right) \cdot V}{m}$$

where:
$x_{totalNO_2^-}$ = total nitrite concentration in the test sample, in mg/l

$x_{NO_2^-}$ = koncentration av nitrit i testprovet, i mg/l
V = testprovet volym, i milliliter (100 i metoden)
m = laboratorieprovets vikt, i gram

OBS: För att säkerställa jämförbarheten mellan resultaten för analys av köttprodukter rekommenderas korrigering för matriseffekter. En empirisk bestämning av denna effekt beräknas i valideringen till 12% (proportionell bias) [1].

7. METODENS PÅLITLIGHET

Metodens pålitlighet undersöktes genom en intern validering.

Data från intern validering summeras i annex 1.

8. METODENS REFERENTER

Denna metod har utarbetats av Leonardo Merino i samarbete med Mailani Åström vid Livsmedelsverket, Sweden.

$x_{NO_2^-}$ = nitrite concentration in the test sample, in mg/l
V = volume of the test sample, in milliliters (100 in the method)
m = mass of laboratory sample, in grams

Note: To ensure the comparability of the results for the analysis of meat products, a correction for matrix effects is recommended. An empirical determination of this effect calculated in the validation study was 12% (proportional bias) [1].

7. RELIABILITY OF THE METHOD

The reliability of the method was evaluated in a single validation study.

Data of the single validation study are summarized in Annex 1.

8. REFEREES OF THE METHOD

This method was developed by Leonardo Merino in co-operation with Mailani Åström at the National Food Agency, Sweden.

[1] Merino, L. (2009). *Development and Validation of a Method for Determination of Residual Nitrite/Nitrate in Foodstuffs and Water after Zinc Reduction*. Food Anal. Methods, 2:212-220

Annex 1. Data from the single-laboratory study

Table 1. Reported values of nitrite from the single-laboratory validation study carried out in 11 batches over a time period of 6 months

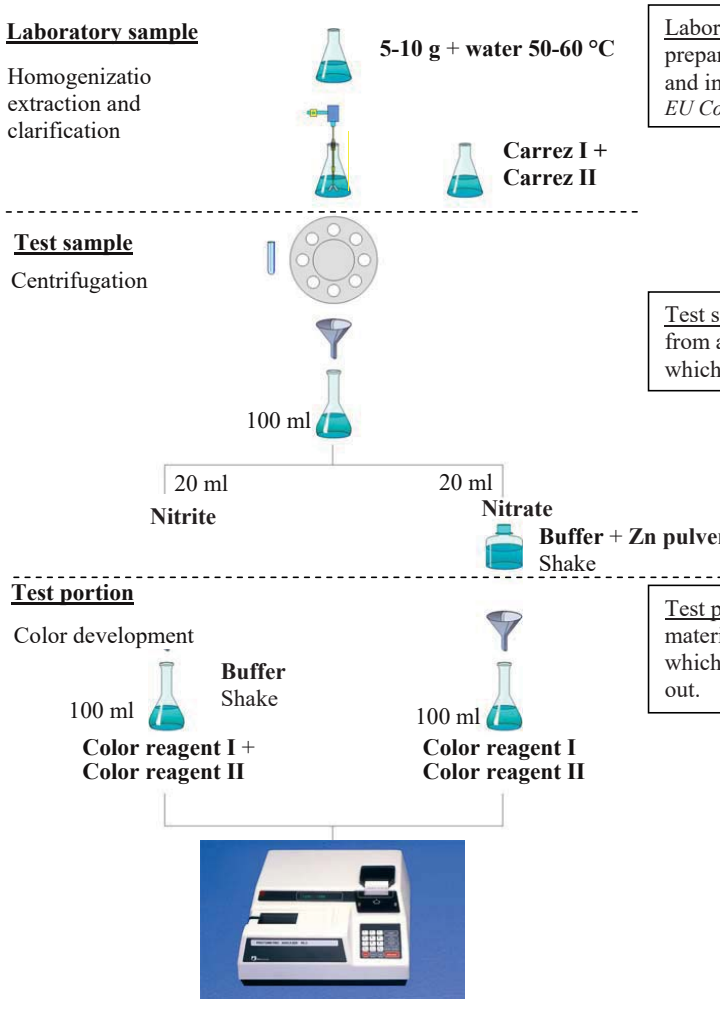
	NO_2^- (mg/kg)					
	Vegetable (lettuce)	Meat product	Meat Ref. Material	Baby food	Dairy prod. (milk)	Surface water
Accepted results	18	20	18	20	20	22
Number of outliers (Cochran and Grubbs test)	4	2	4	2	2	0
Mean	20.9	58.1	203	51.4	28.8	34.3
Repeatability standard deviation, s_r	2.99	0.72	8.64	0.91	0.45	0.38
Repeatability rel. standard deviation RSD_r (%)	14.3	1.24	4.26	1.77	1.56	1.10
Reproducibility standard deviation, s_R	4.22	1.25	5.18	0.80	1.11	1.00
Reproducibility rel. standard deviation RSD_{iR} (%)	20.2	2.15	2.55	1.56	3.65	2.92
Recovery (%)	70	102	101	109	99	104
Combined relative uncertainty, u_c (%)	25.0	2.59	4.46	2.50	4.10	3.23
Horrat value	2.0	0.2	0.4	0.2	0.4	0.3

Table 2. Reported values of nitrate from the single-laboratory validation study carried out in 11 batches over a time period of 6 months

	NO_3^- (mg/kg)					
	Vegetable (lettuce)	Meat product	Meat Ref. Material	Baby food	Dairy prod. (milk)	Surface water
Accepted results	22	20	22	20	22	22
Number of outliers (Cochran and Grubbs test)	0	2	0	2	0	0
Mean	1579	47.5	322	37.1	26.8	34.8
Repeatability standard deviation, s_r	52.4	2.41	21.2	5.21	1.89	3.47
Repeatability rel. standard deviation RSD_r (%)	3.32	5.07	6.58	14.0	7.05	9.97
Reproducibility standard deviation, s_R	109	5.63	30.7	6.17	3.13	3.69
Reproducibility rel. standard deviation RSD_{iR} (%)	6.90	11.9	9.53	16.6	11.7	10.6
Recovery (%)	94	83	73	-	92	105
Combined relative uncertainty, u_c (%)	7.80	13.1	13.9	22.1	13.9	14.9
Horrat value	1.3	1.3	1.4	1.8	1.2	1.1

The combined relative uncertainty (u_c) was calculated by taking the square root of the sum of the relative standard uncertainty of the intermediate precision (RSD_{iR}) and the relative standard uncertainty of the recovery ($u_{\%R}$) calculated in the validation study.

Annex 2. Flow chart of the analytical procedure



Laboratory sample means a sample prepared for sending to a laboratory and intended for inspection or testing *EU Council Directive 96/23/EC.*

Test sample means a sample prepared from a laboratory sample and from which test portions will be taken.

Test portion means the quantity of material drawn from the test sample on which the test or observation is carried out.

3.2.3 Paper V - Estimate dietary intake of nitrite and nitrate in Swedish children

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Estimated dietary intake of nitrite and nitrate in Swedish children

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This study examines the intake of nitrate and nitrite in Swedish children. Daily intake estimates were based on a nationwide food consumption survey (4-day food diary) and nitrite/nitrate content in various foodstuffs. The mean intake of nitrite from cured meat among 2259 children studied was 0.013, 0.010 and 0.007 mg kg^{-1} body weight day^{-1} in age groups 4, 8–9 and 11–12 years, respectively. Among these age groups, three individuals (0.1% of the studied children) exceeded the acceptable daily intake (ADI) of 0.07 mg nitrite kg^{-1} body weight day^{-1}. The mean intake of nitrate from vegetables, fruit, cured meat and water was 0.84, 0.68 and 0.45 mg kg^{-1} body weight day^{-1} for children aged 4, 8–9 and 11–12 years, respectively. No individual exceeded the ADI of 3.7 mg nitrate kg^{-1} body weight day^{-1}. However, when the total nitrite intake was estimated, including an estimated 5% endogenous conversion of nitrate to nitrite, approximately 12% of the 4-year-old children exceeded the nitrite ADI. Thus, the intake of nitrite in Swedish children may be a concern for young age groups when endogenous nitrite conversion is included in the intake estimates.

Keywords: exposure assessment; nitrate; nitrite; meat; drinking water; vegetables

Introduction

Nitrite (NO_2^-) and nitrate (NO_3^-) are natural constituents of food, but they are also used as additives to improve food quality and protect against microbial contamination. However, there are health concerns due to their ability to form carcinogenic N-nitroso compounds (NNOC) and cause methaemoglobinaemia. Methaemoglobinaemia, manifested as cyanosis, has been associated with nitrate or nitrite intake since the 1940s (Comly 1945). Infants are known to be more susceptible to this adverse effect than older children and adults (Filer et al. 1970; World Health Organization (WHO) 2007). Nitrate can be partly converted to nitrite in the body and the toxicity of the two compounds is primarily ascribed to nitrite.

Nitrite in particular and sometimes nitrate are used as food additives in cured meat products due to their preservative, antibacterial, flavouring and colour fixative properties. The levels of residual nitrite and nitrate in these products are variable because they depend on the time and temperature used during processing and storing, the initial addition of nitrite and nitrate, the composition of the meat, pH, addition of antioxidant components such as ascorbate and the presence of micro-organisms (Gibson et al. 1984; Honikel 2008). Accordingly, there may be considerable depletion of nitrite in nitrite-cured meat products over time during storage (Merino, unpublished data; Pérez-Rodríguez et al. 1996). In addition to cured meat, nitrite can be detected in vegetables, especially after storage in conditions that favour bacterial growth. Some studies have also reported the presence of nitrite in other foodstuffs, e.g. cereals, cheese and bread, but the content of nitrite in these food groups is often lower than the limit of detection (LOD) (Knight et al. 1987; Dich et al. 1996; Petersen and Stoltze 1999; Jakszyn et al. 2006; Thomson et al. 2007; Menard et al. 2008; Griesenbeck et al. 2009).

Nitrate, which is ingested in much higher amounts than nitrite, is mainly found in vegetables, especially in green leafy vegetables such as spinach and lettuce (Petersen and Stoltze 1999; Tamme et al. 2006). Furthermore, nitrate is found in cured meat either through being used as a food additive or formed from conversion of initially added nitrite. Nitrate is also present in limited amounts in other foodstuffs such as bread, cereals and dairy products (Dich et al. 1996; Ysart et al. 1999; Menard et al. 2008; Griesenbeck et al. 2009). In addition, nitrate is normally found in low concentrations in tap water, while private wells may contain considerable amounts of nitrate. An acceptable daily intake (ADI) for nitrate of 3.7 mg kg^{-1} body weight has been established by the World Health Organization (WHO) and the European Union

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Scientific Committee for Food (SCF). The ADI for nitrite is 0.07 mg kg^{-1} body weight according to the Food and Agriculture Organization/World Health Organization (FAO/WHO) (2003a) and 0.06 mg kg^{-1} body weight according to the SCF (1995).

The conversion of nitrate to nitrite in the body mainly takes place in the oral cavity, and it has been estimated that 5–7% of nitrate ingested is converted to nitrite by bacteria normally occurring in the mouth (Eisenbrand et al. 1980; FAO/WHO 2003b). However, it has been reported that there are individuals with an even higher conversion rate, up to 20% (European Food Safety Authority (EFSA) 2008). In addition to the dietary intake, nitrate is formed endogenously, with nitric oxide as the precursor, at a daily rate of approximately 60 mg in adults, with increasing amounts during inflammatory processes (WHO 2007).

Most intake assessments made previously have considered adults. Therefore, the present study focused on the intake of nitrite and nitrate in Swedish children. The intake estimates presented cover the consumption of cured meat, vegetables, fruit and drinking water.

Materials and methods

Dietary survey

A nationwide dietary survey, including 56 Swedish counties, was performed in 2003 by the Swedish National Food Administration (NFA). The participants were 590 children aged 4 years, 713 school children aged 8–9 years, and 956 school children aged 11–12 years, resulting in a total of 2259 children. The children aged 4 years were randomly selected from a register of families with children of this age, while the school children were randomly selected as whole school classes. To account for possible seasonal differences, the survey was conducted in two steps, one in spring and one in autumn. All food and drink ingested were recorded by the children or their parents in a food diary for 4 consecutive days. A picture book was used to help subjects estimate portion sizes. Prior to the study, all participants attended a meeting where they were informed about the procedures (Enghardt Barbieri et al. 2006).

Nitrite and nitrate levels in food and drinking water

Cured meat products

In 2008, the Swedish NFA analysed the contents of nitrite and nitrate in a total of 40 samples of cured meat products, which were randomly collected from different supermarkets in Uppsala, Sweden. Each analysed sample was pooled from two separate packages of the same product. The meat products were selected from a list of products preferentially consumed by children in Sweden.

All samples were analysed for nitrite and nitrate using a spectrophotometric method based on reduction of nitrate with zinc powder developed at the Swedish NFA (Merino 2009). The initial nitrite concentration and total nitrite after reduction are determined by the very sensitive and widely used diazotization-coupling Griess reaction. The results of a single-laboratory validation applied to five different matrices, including vegetables, meat products and water, have shown that the method meets the international criteria for precision and recovery (Merino 2009).

In addition, all samples were analysed in parallel by an HPLC ion chromatography method (European Committee for Standardisation (CEN) 2005). For the analysis of meat products with low concentrations of nitrite and nitrate, a comparison between the two methods showed that the HPLC ion chromatography method had lower sensitivity and gave more biased results than the spectrophotometric method. Hence, the nitrite and nitrate concentrations analysed by the spectrophotometric method were used to estimate intake (Merino 2009).

The content of cured meat in a variety of common dishes was calculated and these data were included in the calculations (Table 1). When the meat product was unspecified, e.g. 'sausage', the mean value of all sausages sampled was used. For products that are normally boiled, fried or heated prior to consumption, possible losses of nitrite and nitrate due to cooking were not taken into consideration.

Fruit and vegetables

Data on the content of nitrate in common vegetables on the Swedish market were obtained from HPLC analyses carried out at the Swedish NFA in 1995 (Merino et al. 1997, 2000; CEN 1998). Since these analyses did not cover all vegetables, the database was complemented with a list provided by EFSA containing data on the nitrate content in various vegetables in 20 European Union member states and Norway, analysed between 2000 and 2007 (EFSA 2008).

The definition of vegetables used for the intake calculations included raw and processed vegetables (e.g. mashed and cooked) but not vegetable dishes (e.g. gratins and stews). The definition of potatoes included processed potatoes, as well as dishes with potatoes as a main ingredient. The changes in levels of nitrate due to cooking were not estimated.

There was no information on the content of nitrate in fruit in either the Swedish NFA or EFSA compilation, so it was estimated at 10 mg kg^{-1}, based on analytical data presented by Sušin et al. (2006). Fruit was defined as a group that included fresh, dried, frozen and preserved fruit. Juices and dishes that contain fruit were not included.

Table 1. Nitrite and nitrate concentrations ($mg\,kg^{-1}$) in a selection of Swedish vegetables and cured meat products.

Sample	Number of samples	NO_2^-, mean	NO_2^-, range	NO_3^-, mean	NO_3^-, range
Meat products					
Bacon	4	2.2	0.4–6.0	8.7	7.4–10.7
Sausage	10	7.7	0.5–16.5	15.3	6.1–44.9
Salami and sandwich sausage	6	0.7	0.3–1.2	6.2	0.0–17.7
Chicken sausage	1	34.1	34.1	12.6	12.6
Turkey (smoked)	1	23.5	23.5	6.9	6.9
Ham (cooked)	1	0.6	0.6	4.0	4.0
Ham (smoked)	2	1.1	0.8–1.3	3.5	3.2–3.8
Black pudding	1	0.6	0.6	6.9	6.9
Liver pâté	1	20.5	20.5	18.4	18.4
Vegetables					
Butterhead lettuce	9			1724	59–3332
Spinach (frozen, whole leaves, blanched)	3			1010	782–1161
Chinese cabbage	9			899	281–1456
Iceberg lettuce	10			864	98–2102
Leek	10			535	16–1000
Spinach (frozen, chopped, blanched)	6			486	268–812
Beetroot	9			486	102–1418
White cabbage	10			379	43–681
Broccoli	8			301	30–832
Cucumber	10			179	8–350
Carrot	10			165	0–280
Cauliflower	10			139	15–299
Potato	10			47	0–137
Tomato	10			4	0–16

The contents of nitrite in fruit and vegetables reported in the literature show great variability and are often reported to be lower than the LOD. Due to these inconclusive data, the nitrite contribution from fruit and vegetables was not considered in the present intake estimation.

Drinking water

Data on nitrate content in drinking water were obtained from the Swedish Water and Wastewater Association and contained analyses from 238 local water plants. No sample exceeded the highest permitted value of $50\,mg\,l^{-1}$ (Svensson et al. 2009). Among the samples analysed, 46% had concentrations lower than the LOD (varying from 0.44 to 4.4 mg nitrate ion l^{-1}) (Svenska Vatten och Avloppsföreningen (VAV) 1996). Samples with nitrate contents below the LOD were allocated a value of half the LOD. In the calculations, a mean nitrate concentration of $3.2\,mg\,l^{-1}$ was used and the daily intake of drinking water was estimated to be 1 L in 4-year-old children and 1.5 L in the older age groups.

The concentration of nitrite in drinking water is concluded to be very low, and the nitrite exposure from water consumption was therefore excluded from the estimated dietary intake of nitrite.

Dietary intake assessment

The intake calculations were based on the consumption data from the food diaries and analytical data on nitrite and nitrate contents in different foodstuffs as presented above. The intake was calculated as the average daily intake for each respondent and expressed in $mg\,day^{-1}$ and $mg\,kg^{-1}$ body weight day^{-1}. The latter was based on individual body weight, which meant that children who had not reported their body weight were excluded from these calculations, leaving 527, 644 and 912 children in age groups 4, 8–9 and 11–12 years, respectively. Estimation of the contribution of different food groups to the total nitrate and nitrite intake was based on food weight.

To account for the total intake of nitrite from the diet, the estimated conversion of dietary nitrate to nitrite was added to the direct intake of nitrite from cured meat products. In the calculations, a conversion factor of nitrate to nitrite of 5% was used and adjustment was made for the difference in molecular weight between nitrite and nitrate.

There are two ADIs for nitrite, i.e. 0.06 and $0.07\,mg\,kg^{-1}$ body weight, as established by SCF (1995) and FAO/WHO (2003a), respectively. The toxicological data used to reach the early and lower ADI were later considered irrelevant by the WHO. In the present calculations, the newer ADI set by the

WHO was adopted. All values reported in the following sections are expressed as nitrate and nitrite ions.

Results

Intake of nitrite from cured meat

A summary of the concentrations of nitrite and nitrate in the meat products most frequently consumed by Swedish children is presented in Table 1. Using these data, the mean intake of nitrite from cured meat products was estimated at 0.013, 0.010 and 0.007 mg kg^{-1} body weight day^{-1} in children aged 4, 8–9 and 11–12 years, respectively (Table 2). There was no considerable difference in nitrite intake between boys and girls. One child in each age group exceeded the ADI. In two of these three children, the major source of nitrite intake was chicken sausage, i.e. the product with the highest nitrite content, which was consumed at a rate of 35 and 90 g day^{-1} in the younger and older child, respectively. In the third child who exceeded the ADI, liver pâté was the main contributor to the high nitrite intake. All three children had a lower weight than average for their respective age. The mean body weights were 18.2 (range = 11–29), 30.6 (range = 18–59) and 42.4 (range = 25–77) kg in age groups 4, 8–9 and 11–12, respectively.

It has been suggested that nitrite intake estimations should include a calculation where children who did not consume cured meats are excluded (Pennington 1998). Accordingly, separate calculations were made that included all participants, as well as only children who reported an intake of cured meat. However, the difference in nitrite intake between the calculations used was small. Therefore, only the calculations including all children are given in the present study.

The intake of cured meat products per kg body weight decreased with increasing age. The food group that included various sausages was the most consumed type of cured meat, and contributed approximately 77% of the total intake of nitrite from this food group. Liver pâté accounted for approximately 11% of the total nitrite intake, while ham and poultry products contributed approximately 5% each. Poultry products had the highest nitrite concentration, thus accounting for a relatively significant part of the total nitrite intake even though the consumption of such products was very small. In contrast, the consumption of ham was quite high, but the low concentration of nitrite in ham resulted in a low nitrite intake from this specific foodstuff.

Table 2. Nitrite intake from cured meat products by children in the three age groups studied.

	mg day^{-1}		mg kg^{-1} body weight day^{-1}	
Age (years)	Mean	95th percentile	Mean	95th percentile
4	0.23	0.58	0.013	0.034
8–9	0.29	0.84	0.010	0.028
11–12	0.28	0.75	0.007	0.019

Intake of nitrate from vegetables, fruit, cured meat and drinking water

The contents of nitrate in Swedish vegetables and cured meat are presented in Table 1. Based on these data, and previous presumptions on fruit and water levels, the total estimated intake of nitrate from vegetables, fruit, cured meat and water is presented in Table 3. The daily intake per kg body weight decreased with increasing age. No individual exceeded the ADI of 3.7 mg kg^{-1} body weight day^{-1}. Drinking water contributed 21–26% of the total nitrate intake. Of the nitrate intake from food excluding water, approximately 98% originated from fruit and vegetables, while the remaining 2% came from cured meat products.

Approximately 59% of the total nitrate intake from fruit and vegetables came from vegetables (excluding potatoes), 34% from potatoes and 7% from fruit. The total consumption of this food group was quite consistent among the three age groups, although the intake of different items within the category of fruit and vegetables varied. Younger children consumed more fruit but fewer potatoes than older children,

Table 3. Total intake of nitrate from vegetables, fruit, cured meat and water by children in the three age groups studied.

	mg day^{-1}				mg kg^{-1} body weight day^{-1}			
	Mean		95th percentile		Mean		95th percentile	
Age (years)	Including water	Excluding water	Including water	Excluding water	Including water	Excluding water	Including water	Excluding water
4	14.9	11.7	28.4	25.2	0.84	0.66	1.62	1.42
8–9	20.3	15.5	38.1	33.3	0.68	0.51	1.24	1.06
11–12	18.4	13.6	36.9	32.1	0.45	0.33	0.92	0.78

Table 4. Total exposure to nitrite of children in the three age groups studied, including the intake of nitrite from cured meat products and 5% conversion of nitrate to nitrite from other foodstuffs.

	mg day^{-1}			mg kg^{-1} body weight day^{-1}			
Age (years)	Mean	95th percentile	Maximum	Mean	95th percentile	Maximum	Exceeding the ADI
4	0.78	1.46	2.37	0.044	0.083	0.148	12%
8–9	1.05	1.88	3.78	0.035	0.061	0.145	3%
11–12	0.96	1.79	5.02	0.023	0.045	0.140	1%

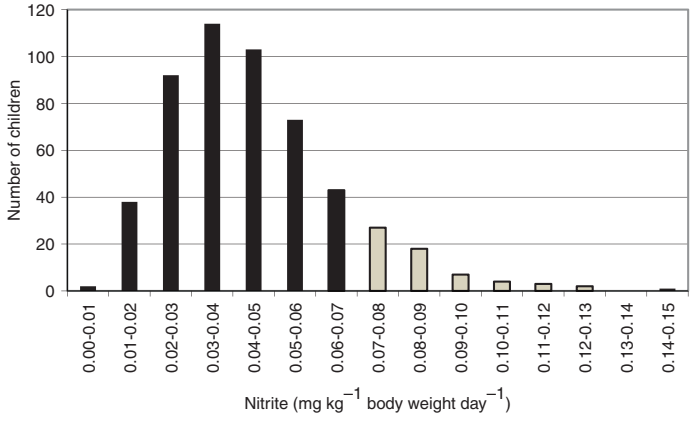

Figure 1. Distribution diagram of total nitrite exposure in children aged 4 years included in the present study ($n = 527$). Grey columns represent children with nitrite intake exceeding the ADI. The upper limits of the units for each bar represent values 'less (<) than', e.g. 0.00– < 0.01.

whereas the intake of vegetables was fairly constant throughout the ages.

Total intake of nitrite

Total nitrite intake, including 5% conversion of dietary nitrate from vegetables, fruit, water and cured meat and direct nitrite intake from cured meat, is presented in Table 4. In addition, a distribution diagram of the total nitrite intake among children aged 4 is presented in Figure 1. The results show that approximately 12%, 3% and 1% of the children in age groups 4, 8–9 and 11–12, respectively, exceeded the ADI. The estimated contribution from the conversion of dietary nitrate was approximately 70% of the total nitrite intake.

Discussion

A summary of previous intake estimations in different countries is presented in Table 5. However, the results of these studies are not easily compared due to differences in study design, food groups studied and selection of participants. For example, in the present study, analyses were preformed on a small number of samples from each category of cured meat. Consequently, a single sample may have influenced the results. In any case, the estimated intake of nitrite from cured meat products was slightly higher in the present study than in a Danish study that included children of the same age groups (Leth et al. 2008). Conversely, the intake was three to five times lower than that reported in Estonian and Finnish children of comparable ages (Laitinen et al. 1993; Reinik et al. 2005).

The total daily intake of nitrate from food and water in the present study was one-half to one-third of the intake reported in Estonian and Finnish children (Laitinen et al. 1993; Tamme et al. 2006). The lower intake in the present study was probably due to the relatively low content of nitrate in Swedish vegetables, especially potatoes and tomatoes, in comparison with the levels reported in other countries. In addition, the reported intake of vegetables (excluding fruit and potato) was low in the present study, in comparison with the mean vegetable consumption in European 11-year-olds (Yngve et al. 2005).

Table 5. Estimated daily intake (mg kg^{-1} body weight day^{-1}) of nitrite and nitrate in children and adults in different countries.

Study	Country	Sources of intake	Age (years)		Nitrite (mg kg^{-1} bw day^{-1})	Nitrate (mg kg^{-1} bw day^{-1})
Leth et al. (2008)	Denmark	Meat products	4–5	Men	0.011	
				Women	0.009	
			6–14	Men	0.006	
				Women	0.005	
			15–75	Men	0.005	
				Women	0.002	
Reinik et al. (2005)	Estonia	Meat products	1–16		0.028[b]	
Jakszyn et al. (2006)	Spain	Various foods	Adults		0.014[a]	
Present study (2010)	Sweden	Meat products (nitrite), various foods and water (nitrate)	4		0.013	0.8
			8–9		0.010	0.7
			11–12		0.007	0.4
Laitinen et al. (1993)	Finland	Various foods and water	9–24		0.028[c]	1.1[c]
Dich et al. (1996)	Finland	Various foods	Adults	Men	0.093[a]	1.1[a]
				Women	0.057[a]	1.1[a]
Thomson et al. (2007)	New Zealand	Meat products and vegetables	Adults		0.009[d]	0.5[d]
Ministry of Agriculture, Fisheries and Food (MAFF) (1998)	UK	Various foods and water	Adults		0.020[a]	1.3[a]
Tamme et al. (2006)	Estonia	Vegetables	Population			0.8[a]
			1–3			1.7[e]
			4–6			1.5[f]
Fernlöf and Darnerud (1996)	Sweden	Vegetables	Adults			0.3[a]
Petersen and Stoltze (1999)	Denmark	Vegetables	Adults			0.6[a]

Notes: [a]Applying an average body weight of 70 kg.
[b]Applying an average body weight of 30 kg.
[c]Applying an average body weight of 51 kg.
[d]Applying an average body weight of 74.8 kg.
[e]Applying an average body weight of 15 kg.
[f]Applying an average body weight of 20 kg.

When evaluating the risk of nitrite exposure, the issue is how to estimate intake correctly. Because all humans are concomitantly exposed to nitrate and nitrite, it seems logical that the ADI for nitrite should include both direct exposure to nitrite and endogenous conversion from dietary nitrate. The mean intake of nitrite from cured meat products alone ranged between 10% and 19% of the ADI for the age groups studied here, and only three of 2259 children exceeded the ADI for nitrite. The average intake of nitrate from food and water was 12–23% of the ADI and no individual exceeded the ADI for nitrate. However, when 5% endogenous conversion of dietary nitrate to nitrite was added to the nitrite intake from cured meat, it was found that 12% of the children in the youngest age group studied exceeded the ADI for nitrite. It has been suggested that some individuals may convert up to 20% of dietary nitrate to nitrite (EFSA 2008), and if such a high conversion factor were used the majority of Swedish children would hypothetically exceed the ADI.

In this study, the nitrite intake from cured meat alone was low and of no health concern in relation to the ADI. In fact, the nitrite intake from cured meat was overshadowed by the endogenous conversion of dietary nitrate to nitrite. However, when considering the nitrite intake from cured meat alone, it was shown that the ADI for nitrite could be exceeded if the average 4-year-old child, on a daily basis, consumed more than 35 g of chicken sausage, i.e. the meat

product with the highest nitrite content. However, it is unlikely that the same individual would continually consume a high amount of the same meat product for a long period of time.

The observation in the present study of a high nitrite content in chicken sausage and smoked turkey prompted further analyses of cured poultry products. Analyses at the Swedish NFA showed a higher average amount of nitrite in sausages containing poultry than in sausages made from red meat (unpublished observation). A higher level of residual nitrite in products containing poultry has also been reported in a previous study (Cassens 1997). This may be an important finding to consider in future studies of nitrite intake because the use of poultry meat may increase in various food categories.

Another way to evaluate better the intake of nitrite and nitrate in future studies would be to include the nitrite and nitrate from other food groups such as dairy products, cereals and bread, as well as nitrite in vegetables and water. Some studies have reported a significant contribution from these food groups to the total nitrate and nitrite intake (Laitinen et al. 1993; Ministry of Agriculture, Fisheries and Food (MAFF) 1998; Jakszyn et al. 2006; Thomson et al. 2007).

Even though nitrite derived from nitrate in various vegetables substantially increased the nitrite intake in the children studied here, it is known that vegetables are beneficial to health for various reasons. In addition, vegetables contain compounds that inhibit formation of NNOC (Dietrich et al. 2005). Therefore, it does not seem reasonable to limit the intake of vegetables in general in order to lower the nitrate exposure. On the other hand, since infants are more susceptible to methaemoglobinaemia, the Swedish NFA recommend avoiding large quantities of spinach, beetroot, nettle, chard and celery in food given to children during their first year. The ADI values on nitrite and nitrate are based on adverse toxic effects in experimental animals and do not take into account possible beneficial health effects of these compounds or transformation, e.g. blood pressure lowering, antibacterial and anti platelet aggregation effects of nitric oxide (Lamas et al. 1998; Lundberg et al. 2008; Webb et al. 2008), However, there is no conclusive evidence to justify intakes exceeding the ADI.

Conclusions

The intake of nitrite by Swedish children from cured meat products was generally low and the ADI was only exceeded in three of the 2259 children studied. This implies that the intake of nitrite from cured meat alone is tolerable. Furthermore, the intake of nitrate from food and drinking water combined was well below the safety margins covered by the ADI for nitrate. However, when an estimated 5% conversion of nitrate to nitrite was included, nitrite intake from cured meat contributed to only 30% of the total intake. It is noteworthy that 12% of children in the youngest age group (4 years) exceeded the ADI when the total exposure to nitrite was considered. Consequently, the intake of nitrite in Swedish children may be of concern for young age groups when endogenous nitrite conversion is included in the intake estimates.

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3.2.4 Paper VI - Time-dependence depletion of nitrite in pork/beef and chicken meat products and its effect on nitrite intake estimation

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Time-dependent depletion of nitrite in pork/beef and chicken meat products and its effect on nitrite intake estimation

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ABSTRACT

The food additive nitrite (E249, E250) is commonly used in meat curing as a food preservation method. Because of potential negative health effects of nitrite, its use is strictly regulated. In an earlier study we have shown that the calculated intake of nitrite in children can exceed the acceptable daily intake (ADI) when conversion from dietary nitrate to nitrite is included. This study examined time-dependent changes in nitrite levels in four Swedish meat products frequently eaten by children: pork/beef sausage, liver paté and two types of chicken sausage, and how the production process, storage and also boiling (e.g., simmering in salted water) and frying affect the initial added nitrite level. The results showed a steep decrease in nitrite level between the point of addition to the product and the first sampling of the product 24 h later. After this time, residual nitrite levels continued to decrease, but much more slowly, until the recommended use-by date. Interestingly, this continuing decrease in nitrite was much smaller in the chicken products than in the pork/beef products. In a pilot study on pork/beef sausage, we found no effects of boiling on residual nitrite levels, but frying decreased nitrite levels by 50%. In scenarios of time-dependent depletion of nitrite using the data obtained for sausages to represent all cured meat products and including conversion from dietary nitrate, calculated nitrite intake in 4-year-old children generally exceeded the ADI. Moreover, the actual intake of nitrite from cured meat is dependent on the type of meat source, with a higher residual nitrite levels in chicken products compared with pork/beef products. This may result in increased nitrite exposure among consumers shifting their consumption pattern of processed meats from red to white meat products.

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Introduction

Nitrite (E249, E250) and nitrate (E251, E252) are approved food additives in the European Union (EU Regulation No. 1129/2011/EC 2011) and are widely used in meat preservation. The amount of nitrite permitted for use as a food additive in cured meat is currently 150 mg kg^{-1} (expressed as $NaNO_2$), except for somewhat higher levels in some traditional cured products. Nitrite has long been a widely used curing agent for meat products owing to its favourable properties, but during the 1970s the debate on the formation of carcinogenic nitrosamines resulted in strong pressure to decrease the use of nitrite for curing in order to reduce the risk of nitrosamine formation and thereby the potential health risks (Sindelar & Milkowski 2012). However, opinion differs within the EU regarding the need to use nitrite in meat processing. Thus, Denmark still maintains national legislation specifying a maximum amount of 60 mg kg^{-1}, instead of 150 mg kg^{-1} according to EU legislation. The Danish authorities state that the necessary preservative effect and microbiological safety can be achieved at the lower maximum levels of nitrite in the Danish regulations, while at the same time reducing the risk of nitrosamine formation (EU Commission Decision 2010/561/EU 2010; Herrmann 2014).

A well-known health effect of nitrite in humans is methaemoglobinaemia, which is the binding of nitrite transformation products to haemoglobin with resulting impairment of oxygen transport capacity. However, the acceptable daily intake (ADI) of nitrite is not based on nitrosamines or methaemoglobinaemia. The ADI for nitrite is 0.07 mg kg^{-1} body weight (b.w.) and is based on adverse effects on the lung and cardiovascular system in rodents (Joint FAO/WHO Expert Committee on Food Authorities 2003). According to the Scientific Committee

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for Food (SCF) (1997), this ADI is applicable to all sources of dietary exposure. There are high levels of nitrate/nitrite also in other foodstuffs than meat products (Larsson et al. 2011; Iammarino et al. 2013). However, based on the possible formation of carcinogenic nitroso compounds, the SCF recommends that the levels of nitrite added to food be lowered to the minimum required to achieve the necessary preservative effect and ensure microbiological safety.

Due to the potential health effects of nitrite, it is of the utmost importance to determine total exposure from all food sources. Even if meat products are the single most important nitrite-containing food for the average consumer, nitrite formation in the body from dietary nitrate (vegetables and water) has also to be considered. It has been estimated that about 25% of ingested nitrate is secreted in human saliva, of which about 20% is reduced to nitrite, i.e., about 5% of the overall dose of nitrate, clearly establishing saliva as a major site of nitrite production in the body (Walker 1990). Using this as the basis for calculation, a Swedish study on nitrate/nitrite intake in children, based on a dietary survey from 2003, showed that about 70% of nitrite exposure originated from estimated *in vivo* transformation of dietary nitrate to nitrite (Larsson et al. 2011). Another potential nitrite source is endogenous production in the body, but this source was not included in our calculation of nitrite exposure. Thus, lower nitrite exposure may be beneficial for human health, but lower nitrite content in cured meat could also increase the health risks arising from microbial contamination.

Different independent meta-analyses of epidemiological studies demonstrate a significantly increased risk of development of colorectal cancer associated with higher consumption of red meat, especially processed red meat (World Cancer Research Fund [WCRF] 2007). There is evidence from the literature that haem–Fe is involved in this epidemiological association and that it may play a central role in colon carcinogenesis associated with red meat intake (Bastide et al. 2015; Hammerling et al. Forthcoming). Poultry, which contains lower amounts of haem–Fe than pork and beef, has not been associated with an increase in colorectal cancer. Nitrite per se is not carcinogenic, but under conditions that result in endogenous nitrosation, the possibility that nitrite is involved in the carcinogenic process cannot be excluded (Habermeyer et al. 2015). Inorganic nitrite, through intestinal conversion of precursor compounds to N-nitrosation compounds, has been proposed as one of several potential causative agents in food-borne risks of contracting colorectal cancer.

Several previous studies have reported that the level of added nitrite in processed meat products decreases over time from the moment of addition to the point of consumption (Hill et al. 1973; Pérez-Rodríguez et al. 1996). This will influence dietary exposure assessments and highlights the importance of reliable analytical measurements of these products at the consumer level. Therefore, the aim of this study was to monitor the nitrite levels in some processed meat products frequently consumed by Swedish children, namely red (pork/beef) and white (chicken) meat-based sausages and liver paté. The specific objective was to determine how nitrite levels change time-dependently from the time point of addition at production stage to the use-by date. In addition, scenario calculations were made on nitrite intake among children using the residual nitrite levels determined in processed meat and estimated conversion of nitrate to nitrite from other foodstuffs. The estimated intakes obtained were compared with the ADI values established for nitrite.

Materials and methods

Sampling and sample preparation

Four Swedish cured meat products were used in this study. The formulations fell within the following specifications: pork/beef sausage (pork and beef meat 61%, fat 19%, protein 12%, E250, E300, carbohydrates 11%), chicken lunch sausages (poultry meat 54%, fat 14%, E250), chicken grill sausages (poultry meat 59%, fat 14%, E250) and liver paté (pork liver 31%, pork meat 16%, fat 23%, protein 12%, carbohydrate 9%, E471, salt 2.3%, E250, E330, E300). These products are frequently consumed in Sweden, especially by children (Enghardt Barbieri et al. 2003).

All cured samples were prepared by three Swedish manufacturers following their standard recipe and commercial method of manufacturing (grinding, mixing, curing, stuffing and thermal processing). The amount of sodium nitrite added to pork/beef sausage (grill type), lunch chicken sausages, grill chicken sausages and liver paté (106, 118, 112 and 119 mg kg^{-1}, respectively, expressed as $NaNO_2$) was below the maximum permitted (150 mg kg^{-1}) by EU regulations (EU Directive 2006/52/EC 2006). Samples were dispatched from the producers to the National Food Agency (NFA) laboratory within 24 h of production and stored in a refrigerator at 2–3°C until analysis. Thereafter the products were analysed for various lengths of time (Figure 1) from processing to the use-by date of each product, and even after that date. The initial nitrite levels at the time of food production were determined by using data of the actual quantities added during formulation of the products, whereas nitrite levels were determined by chemical analyses at all other time points. On each analysis occasion, two packs of pork/beef sausages (10 sausages, 500 g), chicken lunch

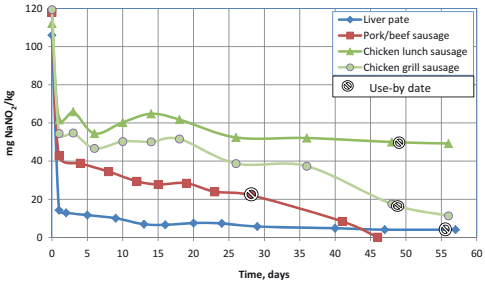

Figure 1. (colour online) Measured changes in nitrite content (mg $NaNO_2$ kg^{-1}) in samples of pork/beef sausage, chicken sausages and liver paté from production date to use-by date. Day 0 is the day on which a calculated initial amount of nitrite was added by the manufacturer. The beginning of the storage period is designated as day 1. The storage temperature was 2–3°C.

sausage (10 sausages, 600 g), chicken grill sausage (10 sausages, 400 g) and liver paté (500 g) were homogenised separately in a blender.

Cooking procedures

Two additional experiments were conducted in a pilot study to determine the effect of boiling (simmering in salted water to serving temperature) and frying on residual nitrite levels in the sausage products. Five pork/beef grill sausages were heated in water for 10 min at 75 ± 1°C. After simmering, all samples were blotted to remove excess water, homogenised and analysed. Six pork/beef grill sausages from another pack were fried in maize oil. After frying, the samples were allowed to cool, drained on absorbent paper, homogenised and analysed for nitrite. As control samples, two packages of pork/beef sausage were homogenised separately and analysed for nitrite. The tap water used in the boiling study was cooled and the nitrite content before and after cooking was also determined.

Chemical analysis

In order to determine the effect of depletion of nitrite on exposure estimates, all samples were analysed for nitrite using a spectrophotometric method based on the sensitive and widely used diazotisation-coupling Griess reaction (NMKL 2013). In this, nitrite is determined by diazotising with sulphanilamide and coupling with N-(1-naphthyl)-ethylenediamine di-hydrochloride to form a highly coloured azo dye that is measured at 540 nm. It has been demonstrated in our laboratory at NFA that the use of the Griess reaction provides more reliable results in determination of low concentrations of nitrate and nitrite ions in meat products than the available HPLC techniques (European Committee for Standardization 2005). Thus, several analyses in parallel of meat products showed that the HPLC-UV method was not able to detect or measure lower concentrations of residual nitrate/nitrite than the spectrophotometric method (Merino 2009).

Duplicate analyses were carried out to estimate the within- and between-sample precision of the results. The combined relative uncertainty for meat products, calculated as the sum of intermediate precision and the uncertainty of recovery within the validation study, was 2.6%. The study followed the recommended internal quality control procedure, including successful participation in proficiency testing.

Intake calculations

The intake scenario in this paper is based on NFA's earlier Swedish national dietary survey on children, performed in 2003 (Enghardt Barbieri et al. 2003; Larsson et al. 2011). As our aim was to study the most sensitive consumer group we used data on 4-year-old children. Data from that survey (n = 590; mean weight = 18.2 kg), and specifically their individual intake of nitrite-containing processed meat, were used. However, we replaced actual consumption of all types of processed meat with either pork/beef sausage consumption, or chicken sausage consumption, in both cases using the levels measured in the present study. Moreover, different factors (0%, 5% and 20%) for endogenous conversion of nitrate to nitrite (from vegetables, fruit and drinking water) were also added to the children's total nitrite intakes, which were given as median and 95th percentile values.

Results

The decrease in nitrite levels as a function of time after addition of $NaNO_2$ to the meat products studied is shown in Figure 1. Already at 24 h after the addition of nitrite to lunch chicken sausage, grill chicken sausage, pork/beef sausage and liver paté, the nitrite levels had decreased to approximately 55%, 45%, 35% and 15% of the initial level, respectively. However, the decline in added nitrite was less pronounced in chicken sausage than in pork/beef sausage and liver paté, so that at the use-by date the nitrite levels were still approximately 40% in chicken lunch sausage and 15% in chicken grill sausage (Figure 1). A noteworthy feature was that the accepted storage period (until use-by date is reached) was almost twice as long for chicken sausage (48 days) as for pork/beef sausage (28 days). In

fact, on applying the accepted period for chicken sausage, there was almost no nitrite left in the pork/beef sausage after 48 days.

The results of the pilot study showed that boiling did not alter the residual nitrite level at all (data not shown). However, frying decreased the mean nitrite level from 11 to 5.6 mg kg^{-1}, i.e., to approximately 50% of the initial level. This decrease did not appear to be related to the weight loss of the fried samples. Hence, it could be attributable to formation of unknown nitrogen-containing compounds, an issue that would require further research. The residual nitrite level in the assays was 11 mg kg^{-1} or less. Consequently, even though frying significantly affected the level of residual nitrite, it had a minor influence on the estimated total dietary intake of nitrite.

Intake scenarios on the calculated nitrite intake in 4-year-old Swedish children were also formulated (Table 2), combining basal consumption data from an earlier intake study (Enghardt Barbieri et al. 2003; Larsson et al. 2011) with the nitrite levels determined in pork/beef and chicken sausages in the present study (Table 1) to represent the levels in all processed meats registered in the survey. The intake calculations used median and 95th percentile values and, depending on the nitrate–nitrite conversion factor applied, this resulted in different exposure levels. Also, these estimated intake data clearly showed the influence of storage time on actual exposure levels. With this method of calculation, it is obvious that not many alternatives resulted in nitrite intake below the ADI of 0.07 mg kg^{-1} b.w. (Table 2). Note that in Figure 1 nitrite levels are given as $NaNO_2$ (mg kg^{-1} product) but in the scenarios for intake calculations (mg kg^{-1} b.w. day^{-1}) the nitrite ion was used.

Table 1. Basal data used in the scenario calculations (see Table 2). The nitrite levels are taken from data points representing samples analysed or extrapolated from adjacent data points.

Type of product	Timeline	As $NaNO_2$ (mg kg^{-1})	As NO_2^- (mg kg^{-1})
Pork/beef sausage	At commercial formulation	120	80
	At half the accepted storage time	30	20
	At use-by date	22	15
Chicken sausage	At commercial formulation	115	77
	At half the accepted storage time	63	42
	At use-by date	52	35

Table 2. Intake scenarios for nitrite ion in 4-year-old Swedish children based on a Swedish consumption survey (Enghardt Barbieri et al. 2003).

Factor (%) converting dietary nitrate to nitrite	At commercial formulation	At half the accepted storage time	At use-by date
Pork/beef sausage (median/95th percentile)			
0	*0.15/0.46*	0.04/*0.12*	0.03/*0.08*
5	*0.19/0.51*	*0.08/0.17*	0.07/*0.14*
20	*0.31/0.69*	*0.19/0.39*	*0.18/0.37*
Chicken sausage (median/95th percentile)			
0	*0.14/0.44*	*0.08/0.24*	0.06/*0.20*
5	*0.18/0.49*	*0.12/0.29*	*0.11/0.25*
20	*0.31/0.67*	*0.24/0.48*	*0.23/0.44*

Note: In the scenarios, reported consumption of all processed meat products was assigned the nitrite levels found in pork/beef or chicken sausages in this study. Italic entries indicate the intake exceeding the ADI for nitrite (0.07 mg kg^{-1} b.w.) (see the Materials and methods for further information).

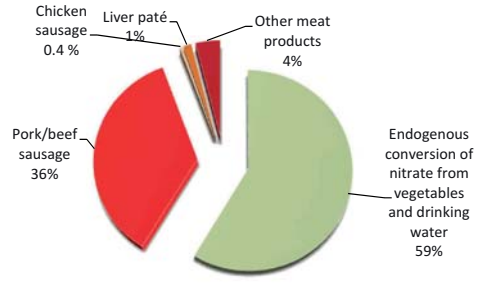

Figure 2. (colour online) Total nitrite exposure in children in the youngest consumer group (4 years), including intake from cured meat products and 5% conversion of nitrate in other foodstuffs to nitrite.

The calculated total dietary intake of nitrite among 4-year-old children (Figure 2) was based on our measured data on nitrite in various cured meat products, estimated conversion (5%) of nitrate in vegetables and fruit to nitrite, and previously published food consumption data (Larsson et al. 2011). This clearly showed that nitrate to nitrite conversion from vegetables and fruit was the major source of nitrite exposure. However, a significant amount also originated from beef/pork sausage (36%). Even if intake from chicken sausage is currently low, our finding of considerably higher residual nitrite levels in chicken sausage compared with beef/pork sausage suggests that a switch in consumption pattern to cured chicken products may result in a significant increase in total nitrite exposure.

Discussion

Our analysis of commonly consumed processed meat products on the Swedish market showed that during the early product life, there was a rapid initial decrease in nitrite from the point of sodium nitrite addition. The nitrite decrease continued in later stages of product life, but at a much slower rate, and at the use-by date the residual level of nitrite in the products was 5–19% of the amount initially added, depending on the food product.

After the manufacturing process, which included a dry heating step, by their use-by date chicken sausages contained consistently higher nitrite levels (approximately 50% higher) than liver paté and pork/beef sausage (4% and 20% of added nitrite, respectively). These levels were based on analysis of processed products that are not cooked before consumption. Cooking may further decrease nitrite levels. For example, our pilot study showed that frying, but not boiling, decreased the nitrite content in sausages. Whether this was associated with a change in nitrosamine formation is not known (Josefsson & Nygren 1981).

In our scenarios, calculated nitrite intake in children based on the nitrite levels measured in sausage was within the ADI when based only on processed meat consumption, but exceeded the ADI when nitrite formed from dietary nitrate was included (Table 2).

Earlier studies have presented results on the complex chemistry of $NO_3/NO_2/NO$ in various meat-based systems. A review by Skibsted (2011) describes in detail nitric oxide chemistry in muscle-based foods, including the chemical reaction behind the colour of cured meat. Cassens et al. (1979) has shown that nitrite added to meat homogenates is partly transformed to other nitrogen-containing compounds and bound to myoglobin and other meat constituents, e.g., lipids and proteins. The formation of nitrate from nitrite may also have to be considered (Pegg & Honikel 2015). In the present study, as well as in several earlier studies, it has been shown that nitrite depletion increases with time in meat-based systems (Pérez-Rodríguez et al. 1996; Armenteros et al. 2012). In addition to heating during the initial processing phase, factors that have been shown to influence the nitrite levels in meat products include pH, temperature and ascorbate addition (Gibson et al. 1984). Honikel (2008) estimated that the decline in nitrite levels due to heating during manufacturing is about 35% of the added level, and thereafter there is a continuing decrease in nitrite levels during storage. Special features of poultry meat regarding nitrite levels during storage have also been described (Kilic et al. 2001, 2002), with a difference between nitrite levels in poultry and pork/beef meat possibly due to pH differences between the products. However, owing to the much higher abundance of haem in red meat compared with chicken, it is also possible that nitrite-induced formation of nitrosylhaem causes greater and faster loss of added nitrite in red meat products (Hammerling et al. Forthcoming).

In our scenarios of time-dependent depletion of nitrite, the calculated nitrite intake in children in most cases exceeded the ADI for nitrite when conversion from dietary nitrate was included and when the nitrite levels recorded in sausages in the present study were used to represent all processed meat. However, actual intake of nitrite via cured meat is highly dependent on time after production start and type of meat. The current trend of increased consumption of meat raises concerns, as well as the potential increase in nitrite exposure among consumers as a consequence of a shift to white meat cured products. The recommendation made by WCRF (2007) in its report to limit intake of red meat and avoid processed meat and the growing Islamic population in Western countries will probably result in increased sales and consumption of different white meat-based cured products, bringing about a change in the consumption pattern of meat mainly from red meat to chicken and turkey. Consequently, it is reasonable to assume that there will be a future increase in nitrite intake among consumers, but it is difficult to estimate the possible health risk from this higher intake. In addition, it is likely that most (59%) of the nitrite originates from conversion of nitrate in other food items, especially vegetables (Figure 2) (European Food Safety Authority 2013). Thus, if conversion is considered, this difference in nitrite content between chicken and pork/beef meat may have a limited impact on total nitrite exposure.

Increased consumption of vegetables is widely recommended because of their generally recognised beneficial health effects. WCRF (2007) rates the evidence as 'convincing to probable' that diets high in vegetables and/or fruits protect against a variety of cancers, but it is not clear whether this effect is related to their high nitrate content. Other beneficial effects of nitrate have been described in experimental and human intervention studies and in epidemiology regarding effects on blood pressure, myocardial infarction and stroke (Habermeyer et al. 2015). Within the body, nitrate and nitrite may function as an alternative source of nitric oxide, an important and multifaceted physiological signalling molecule (Weitzberg & Lundberg 2013). The biological activities of nitric oxide related to secondary products may include pharmacological effects, e.g., on blood vessels and blood pressure and on the induction of oxidative stress/inflammation. However, it is concluded in the literature that available evidence of these effects is inadequate for comprehensive and reliable assessment of positive or negative health effects of nitrate/nitrate, especially long-term effects (Habermeyer et al. 2015).

The conversion of nitrate to nitrite in the body was a crucial factor in estimation of nitrite exposure in this study. However, the usual conversion factor of 5% is an approximate figure and it has been estimated to be as high as 20% for some individuals, so both values were

included here. As also pointed out by several other expert groups (Thomson et al. 2007; Leth et al. 2008; Menard et al. 2008), for consistency the conversion factor should be better defined to achieve more reliable nitrite exposure estimates. At the same time, it is obvious that the scientific basis for establishing the conversion factor must be improved.

To conclude, the present study examined time-dependent depletion of nitrite in various cured meat products and how this affects nitrite intake estimations. We selected food products on the Swedish market that are especially popular among children, i.e., sausages based on pork/beef or chicken and liver paté. We found a strong initial decrease in added nitrite (during processing), followed by a gradual decline during subsequent storage and cooking, a decrease that was higher in pork/beef than in chicken products. These findings, together with a factor for conversion of dietary nitrate, were used in scenarios on estimated nitrite dietary exposure in children. In these scenarios, the intake in 4-year-old children exceeded in most cases ADI for nitrite. The findings could be used in a more general perspective as an argument for improving sources of uncertainty affecting dietary exposure assessments but also for a discussion on the basis and methods for risk assessment of nitrate and nitrite.

Conclusions

- A rapid decrease in nitrite levels occurred initially after the addition of nitrite during production of the actual meat products.
- The depletion of nitrite depended on time and the type of cured meat product, with higher residual nitrite levels in cured chicken products than in cured pork/beef products.
- Nitrite intake from all dietary sources, including nitrate–nitrite conversion, led to the ADI being exceeded in all scenarios calculated. This suggests that an approach to estimating the ADI not accounting for conversion of dietary nitrate causes an underestimation of the true nitrite intake.
- The WCRF recommendation on limiting consumption of red/processed meat could result in increased nitrite exposure among consumers as a consequence of a shift in consumption pattern towards white meat cured food products.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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4. Discussion

4. Discusión general

4.1 Normalización de un método analítico: Monitorización de nitratos en vegetales

4.1.1 *Determinación de nitrito/nitrato resdual en alimentos mediante cromatografía líquida: Estudio colaborativo NMKL.*

El método analítico que todavía es muy empleado internacionalmente utiliza el metal tóxico cadmio como agente reductor. A solicitud del Comité Nórdico de Análisis de Alimentos (NMKL) se desarrolló un proyecto para obtener un método cromatográfico alternativo para analizar nitrito y nitrato residual en productos cárnicos. El estudio se realizó en tres etapas: a) evaluación comparativa del desempeño de tres métodos de cromatografía líquida, b) validación del método de cromatografía iónica seleccionado y c) estudio colaborativo en el que participaron 17 laboratorios de países europeos.

a) Evaluación comparativa y selección del método analítico

Se realizaron diversos ensayos para evaluar comparativamente el tipo de columna (columnas aniónicas débil y fuerte), preparación de muestras (extracción, clarificación) y fases móviles de los métodos seleccionados.

Selectividad.- La resolución de los picos de nitrato y nitrito mostró una pobre selectividad de la columna aniónica débil (Figura 6). Todos los experimentos de ensayo y error con la intención de separar los picos de nitrato y nitrito de las interferencias en soluciones patrones y muestras de zanahoria, cebolla, pepino y patata no tuvieron éxito. Se concluyó que la columna aniónica débil no es apropiada para el análisis de concentraciones bajas de nitrato (<100 mg / kg).

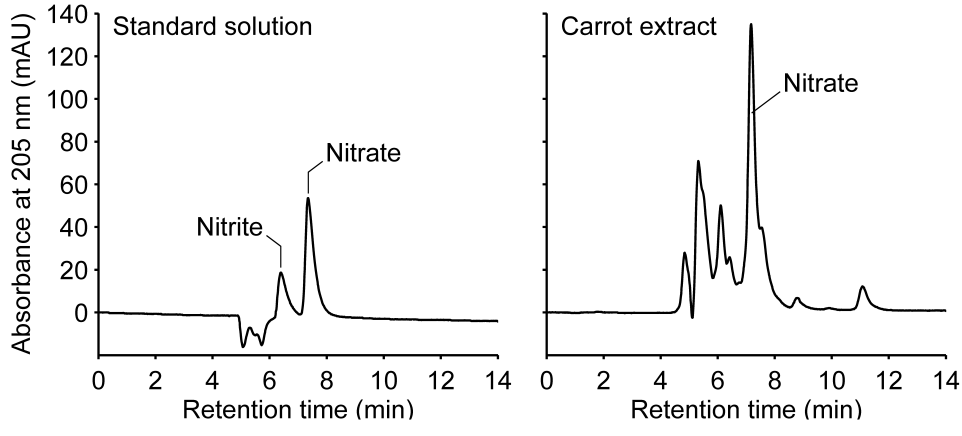

Fig. 6 Separación de nitrato y nitrito en soluciones patrón e ión nitrato en extractos de zanahoria utilizando una columna aniónica débil.

Por otro lado, una buena separación de los picos de nitrato y nitrito fueron obtenidas al usarse una columna aniónica fuerte en soluciones patrón y de extractos de cebolla, ajos, zanahoria, pepino y patatas (Fig. 7).

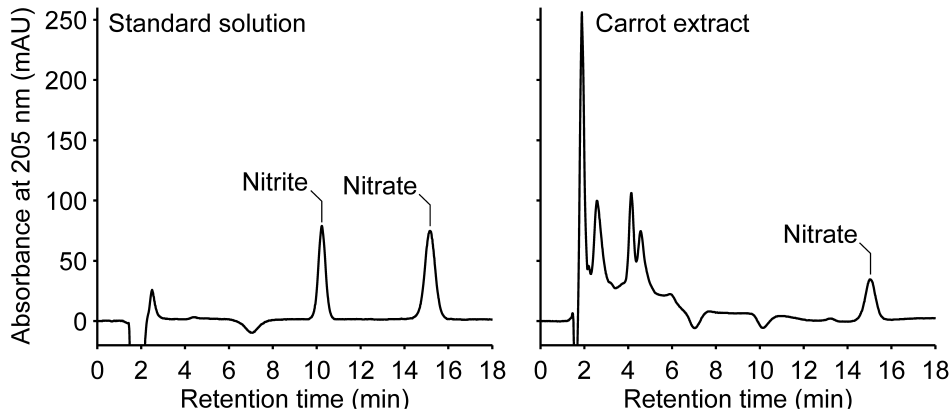

Fig. 7 Separación de nitrato / nitrito en solución patrón y nitrato en extracto de zanahoria en una columna aniónica fuerte.

Clarificación.- Los resultados del ensayo mostraron que la solución de Carrez, que es una solución clarificante adecuada en los métodos de espectrofotometría basados en la reacción de Griess (Paper III), tiene efectos perturbantes con el método de cromatografía iónica.

Efecto del pH. Se investigó el efecto del pH sobre la estabilidad de los analitos y sus correspondientes porcentajes de recuperación. Se pudo determinar que a mayor pH inicial de la matriz, mayor es la recuperación de la muestra fortificada. Estos resultados confirmaron la importancia del pH para garantizar la estabilidad de los iones nitrito/nitrato, lo cual fue de crucial importancia para la correcta evaluación del desempeño del método analítico en la organización del estudio colaborativo.

b) Estudio de validación interno

La aplicabilidad del método en varias matrices fue examinada en un estudio de validación interna usando un diseño experimental basado en el análisis de varianza (ANOVA). Se incluyeron en el estudio productos de carne (salchichas, carne molida, jamón y chorizo), vegetales (patatas), productos lácteos (queso) y alimentos para bebés.

Recuperación.- El rango de recuperación de nitrito en muestras fortificadas de salchicha, carne molida, jamón, alimentos para bebés y queso fue de 96-108%. El intervalo de recuperación para los nitratos fue de 96-107%.

Precisión.- Los análisis duplicados en el día de preparación mostraron buena repetibilidad. Sin embargo, la inestabilidad de las muestras, debido a que no se dio la debida atención al rol de pH en la estabilidad de la muestra, no permitió determinar la precisión intermedia en el estudio de validación interno.

Límite de detección.- El límite de detección (LOD) de nitrito y nitrato fue de 0.06 y 0.36 mg/L en solución, respectivamente. Esta cantidad corresponde a 1 mg de NO_2/ kg y 10 mg de NO_3/kg expresada como fracción masa.

En general, el desempeño del método es diferente al analizarse nitrito y nitrato. Mejores resultados son obtenidos en los análisis de nitrito.

c) Estudio colaborativo

El estudio colaborativo demuestra que el método HPLC-ion cromatográfico puede aplicarse para analizar diversos alimentos incluyendo productos cárnicos (por ejemplo, alimentos para bebés, verduras y queso). La precisión (repetibilidad y reproducibilidad) del método fue satisfactoria.

El procedimiento fue propuesto como método estándar horizontal Europeo (es decir, para analizar diversas matrices). La propuesta contó con el apoyo de Francia (Grupo de Trabajo Francés, 1999), y la oposición de Alemania (Grupo de Trabajo Alemán, 1999) con el argumentó de que el estudio colaborativo no había incluído el suficiente número de matrices y niveles de concentración. Finalmente, el Comité Europeo lo aprobó como método normalizado también aplicable en el análisis de nitrito/nitrato en otras matrices distintas de los productos cárnicos (CEN European Standard N 12014-part 4, 2005).

4.1.2 –Niveles de nitrato de los últimos 10 años en lechugas y espinacas Suecas

Los resultados de la monitorización de vegetales (lechuga y espinaca) producidos en Suecia en los últimos 20 años (1995-2016) confirman que más del 98% de las muestras estuvieron por debajo de los niveles máximos establecidos en 1997 por la Comisión Europea.

Resultados de la monitorización de verduras (lechugas)

La confiabilidad del método analítico utilizado en la monitorización fue demostrada inicialmente por los estudios de validación interno y externo.

Todos los valores medios del contenido de nitratos en lechugas y espinacas estaban por debajo de los niveles máximos establecidos por la Comisión Europea (Fig. 8).

Las lechugas fueron cultivadas bajo cubierta y campos descubiertos durante el invierno y el verano, respectivamente. La variabilidad de los resultados durante los 20 años de la monitorización no muestran ninguna tendencia clara en el tiempo.

Teóricamente, las condiciones climáticas adversas en Suecia (baja radiación solar), deberían favorecer altos contenidos de nitratos en las plantas. Sin embargo, las buenas prácticas agrícolas (GPA), que en Suecia se aplica como Producción

Integrada (PI) (Producción Ecológica, 2005) o a gricultura ecológica (http: /www.krav.se), podría explicar estos resultados satisfactorios.

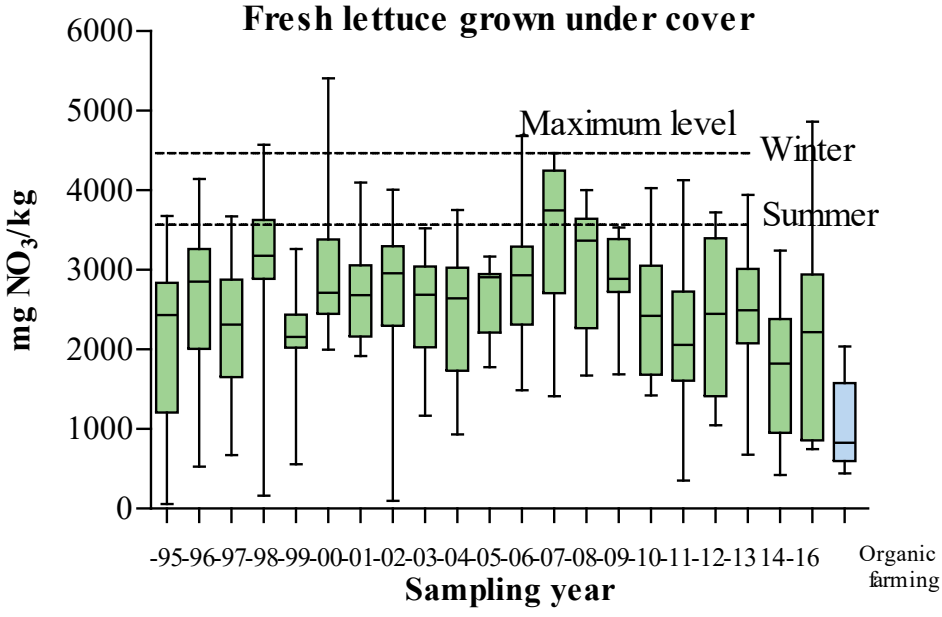

Fig. 8. Contenido de nitrato en lechugas producidas en Suecia entre 1995 de 2016. Cuatro muestras excedieron el límite máximo: una en el verano de 1996, 1998 y 2016, dos en el invierno de 2000. Las lechugas de cultivo ecológico mostraron un contenido menor.

La producción ecológica de lechugas dieron muestras con un c ontenido promedio de nitratos de 826 mg NO_3/kg. La comparación de las lechugas producidas ecológica y convencionalmente muestran que el cultivo ecológico favorecería una disminución sustancial del contenido de nitratos (datos sólo del año 2000).

Protección contra los nitratos

Existen opiniones controvertidas sobre el peligro que representa los nitratos para la salud humana (Sección 1). Por lo tanto, es difícil sacar conclusiones definitivas sobre esta cuestión. Sin embargo, hay menos desacuerdo y controversia sobre la magnitud del daño al medio ambiente que el exceso de nitrato puede causar ante la creciente evidencia de la eutrofización de las aguas.

Es aceptado que todas las formas de actividad agrícola eliminan nitratos, y que el aumento continuo durante décadas de la aplicación de fertilizantes, es u n factor importante en la contaminación con nitratos. Se estima que, en promedio, el 33% de los fertilizantes nitrogenados aplicados a l as tierras arables no aporta ningún beneficio, ya que no es absorbido por los vegetales y permanece en las tierras de

cultivo como residuos orgánicos. Más de la mitad de este excedente es lexiviado en las aguas circundantes (Naturvårdsverket, 2008). Considerando que nuestra dependencia de los fertilizantes para la producción de alimentos es actualmente inevitable, es de fundamental importancia reducir la contaminación de nitratos al medio ambiente a través de la optimización de la relación de alimentos producidos y nitrato liberado. Esto podría ser logrado mediante el uso racional y eficiente de fertilizantes con buenas prácticas agrícolas (GAP), en lugar de sólo buscar el máximo rendimiento de la producción agrícola.

En muchos países de la comunidad europea se están adoptando una serie de medidas para reducir la contaminación por nitratos. Aunque datos recientes muestran que la carga antropogénica total de nutrientes -incluyendo la lixiviación de nutrientes de las tierras agrícolas en Suecia y algunas otras partes de Europa- está disminuyendo (Naturvårdsverket, 2017), siguen siendo necesarias nuevos compromisos y acciones a nivel internacional para continuar con la actual tendencia decreciente.

Sin embargo, hay que reconocer que no existe una solución fácil para el complejo problema de la eutrofización. Los aspectos políticos, económicos y sociales implicados dificultan la resolución de este problema.

4.2 Trabajo Estimación de control y exposición del nitrito en productos cárnicos

4.2.1 *–Desarrollo y validación de un método para la determinación de nitrito/nitrato residual en alimentos y aguas tras la reducción de zinc.*

Se desarrolló un método espectrofotométrico económico y ambientalmente inocuo. El método se basa en la reducción de nitrato con zinc en polvo en lugar del cadmio o enzimas utilizados en los métodos normalizados ISO/CEN donde las concentraciones de nitritos (inicial y total) son determinadas por la reacción de Griess.

Estudio de validación interno

Se realizó una validación interna evaluando cinco matrices (vegetales, productos cárnicos, alimentos para bebés, productos lácteos y aguas superficiales). Los resultados muestran que el método propuesto cumple los criterios internacionales de precisión y recuperación.

Estudio del efecto matriz. Se prepararon curvas de calibración utilizando blancos fortificados. El sesgo del efecto de la matriz se cuantificó de la relación de pendientes de las curvas de calibración con soluciones patrón y con muestras fortificadas. La pendiente de la curva de calibración de nitritos está dentro del intervalo de confianza de la pendiente calculada para la muestra fortificada. Por lo tanto, no hay criterio definitivo para juzgar la presencia de un sesgo causado por la matriz en el análisis de nitritos. Por otro lado, el sesgo para el análisis de nitratos, calculado por la relación de las pendientes, es de aproximadamente el 12%. Se propone utilizar este porcentaje, como una primera aproximación de un factor de recuperación, en el análisis de nitratos en productos cárnicos.

Límite de detección.- El límite de detección de varias matrices varía entre 3 y 5 mg/kg de nitritos y nitratos, respectivamente, calculados de la intersección de las curvas de calibración de muestras fortificadas con el eje de las ordenadas.

Comparación con métodos estándar.- La comparación entre pares de métodos no muestra diferencias estadísticamente significativas entre el Zn-método *vs* Cd-método y entre el Zn-método *vs* el HPLC-método.

Precisión.- Todas las muestras dieron valores de Horrat menores que 2, lo que da evidencia razonable del desempeño satisfactorio del sistema analítico.

Recuperación.- El intervalo de recuperación de nitritos residuales para las seis muestras fue de 70-110%. La recuperación de nitratos fue del 73-105%. Ambos rangos cumplen los criterios recomendados por la Comisión Europea para el control oficial de los nitratos en alimentos, esto es, para un intervalo de concentración <500 mg/kg el valor de recuperación recomendado es 60-120%, y para ≥500 mg/kg el rango de recuperación recomendable debe ser de 90-110%.

Incertidumbre.- La estimación de la incertidumbre en las mediciones se basó en la suma de las fuentes de error aleatorio (precisión intermedia) y error sistemático (recuperación). Las incertidumbres calculadas cumplen con el límite máximo de incertidumbre (target uncertainty) propuesto como criterio de aceptabilidad de un método analítico.

4.2.2 –*Estimación de los nitritos y nitratos presentes en la dieta de los niños suecos.*

La Ingesta Diaria Admisible (IDA) de nitratos y nitritos en niños suecos fue calculada utilizando una encuesta nacional de consumo de alimentos y en el contenido de nitrito/nitrato en diversos alimentos incluídos en la dieta.

Ingesta de nitritos de la carne curada. Se determinó la ingesta media de nitritos de carne curada entre 2259 niños en los grupos de edad 4, 8-9 y 11-12, utilizando el contenido de nitrito/nitrato en los productos cárnicos más consumidos. Usando estos datos, la ingesta media de nitritos de productos cárnicos curados se estimó en 0.013, 0.010 y 0.007 mg/kg de peso corporal día-1 en niños de 4, 8-9 y 11-12 años, respectivamente. No hubo marcadas diferencias en la ingesta de nitritos entre niños y niñas. Un niño en cada grupo de edad excedió el IDA. En dos de estos tres niños, la principal fuente de ingesta de nitritos fue la salchicha de pollo, esto es, el producto con mayor contenido de nitrito. En el tercer niño el paté fue el alimento que más contribuyo a la alta ingesta de nitritos. Los tres niños tenían un peso corporal inferior al promedio de sus respectivas edades.

Ingesta de nitratos de verduras, frutas, carne curada y agua potable

La ingesta media de nitratos de verduras, frutas, carnes curadas y agua potable fue 0.84, 0.68 y 0.45 mg/kg de peso corporal y día para los niños de 4, 8-9 y 11-12 años, respectivamente. Ningún niño excedió el IDA de 3.7 mg/kg de peso corporal y día. Basándose en los datos del contenido de nitrato en los hortalizas suecas y en la carne

curada, y aproximaciones del contenido en frutas y agua, se calculó la ingesta total estimada de nitratos. La ingesta diaria por kilogramo de peso corporal disminuyó con el aumento de la edad. Ningún individuo excedió el IDA. El agua potable contribuyó 21-26 % de la ingesta total de nitratos. De la ingesta de nitratos de los alimentos, excluyendo el agua, aproximadamente el 98% se originó de frutas y hortalizas, mientras que el 2 % restante provenía de productos cárnicos curados. Aproximadamente el 59 % de la ingesta total de nitratos de frutas y hortalizas provenía de hortalizas (excluyendo patatas), 34 % de patatas y 7 % de frutas.

Aspecto a considerar en la evaluación de la exposición

Al evaluar la exposición de nitritos, un problema pendiente es la armonización del procedimiento de cálculo. Debido a que todos los seres humanos están expuestos permanentemente a nitratos y nitritos, es lógico que la IDA de nitrito incluya tanto el consumo directo como la conversión endógena del nitrato dietético a nitrito. La ingesta media de nitrito de los productos cárnicos curados varió entre el 10 – 19 % de la IDA para los grupos de edad estudiados, y sólo 3 de los 2259 niños superaron este valor. Por otro lado, la ingesta media de nitratos de los alimentos y agua potable fue de 12-23 % del IDA y ningún individuo superó este límite. Sin embargo, cuando se considera el 5% de conversión endógena de nitrato dietético a nitrito, se encontró que el 12% de los niños del grupo de edad más temprana estudiado superaba el IDA de nitritos (0.06 mg/kg de peso corporal y día). Por lo tanto, la ingesta de nitritos en los niños puede ser motivo de preocupación para los grupos de niños de edad temprana cuando la conversión de nitrito endógeno es incluída en las estimaciones de la ingesta.

4.2.3 –*Disminución dependiente del tiempo de nitritos en productos cárnicos de cerdo, ternera y pollo que afecta a la estimación de la ingesta de nitritos.*

Fueron evaluados los efectos del procesamiento, almacenamiento, calentamiento (por ejemplo, hervir a fuego lento en agua salada) y freído sobre el contenido inicial de nitritos añadidos y su relación con el cálculo de ingesta. El decrecimiento del contenido de nitritos en el tiempo de cuatro productos cárnicos consumidos frecuentemente por los niños suecos: la salchicha de cerdo, paté y dos tipos de salchicha de pollo, confirmaron la pronunciada disminución del contenido de nitritos entre el tiempo de adición y fecha de caducidad. El decrecimiento de los nitritos depende del tiempo y del tipo de embutido, encontrándose mayores contenidos de nitrito residual en los productos de pollo curados que en los productos de carne de cerdo/ternera curados.

En diversos escenarios de la relación de decrecimiento de nitritos *vs* tiempo, en las que se consideró a los embutidos como representativos de productos cárnicos curados y se incluyó la conversión de nitrato dietético a nitrito, se encontró que el IDA fue excedido en todos los escenarios calculados (niños de 4 años). Esto sugiere que el procedimiento para estimar el IDA en el cual no se incluye la conversión endógena de nitrato a nitrito causa una subestimación de la ingesta real de nitritos.

La disminución del contenido de nitritos *vs* tiempo en 4 productos cárnicos se muestra en la Fig. 9. Despues de 24 h el contenido de nitritos en salchicha de pollo cocida, salchicha de pollo a la parrilla, embutido de cerdo y paté disminuyó aproximadamente 55%, 45%, 35% y 15% del nivel inicial, respectivamente. La disminución de nitritos es menor en las salchichas de pollo que en los embutidos de cerdo y paté, de modo que el contenido de nitritos durante el periodo de consumo autorizado fue aproximadamente de 40% en salchichas de pollo cocida y 15% salchicha de pollo a la parrilla.

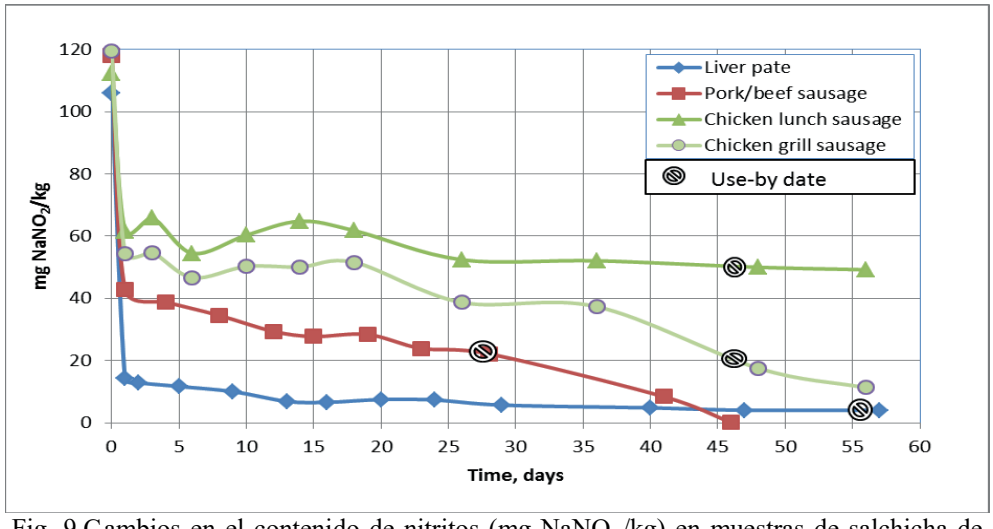

Fig. 9 Cambios en el contenido de nitritos (mg $NaNO_2$/kg) en muestras de salchicha de cerdo/carne, salchichas de pollo y paté desde la fecha de producción hasta la fecha de caducidad. El día 0 es el día de adición por el fabricante. El comienzo del período de almacenamiento se designa como día 1. La temperatura de almacenamiento fue de 2-3ºC

Influencia del agotamiento dependiente del tiempo del nitrito en la evaluación de la exposición

Los escenarios del cálculo de ingesta de nitrito en niños suecos de 4 años de edad fueron calculados aplicando el enfoque de "estimaciones puntuales" o modelo determinístico para integrar el consumo de alimentos y su correspondiente contenido químico. Por lo tanto, los datos de consumo basal de un estudio de ingesta anterior (Enghardt et al., 2003, Larsson et al., 2011) fueron combinados con el contenido de nitritos de carne de cerdo/ternera y salchichas de pollo registrados durante el estudio.

Tabla 6. Los contenidos de nitrito son obtenidos de datos que representan muestras analizadas o extrapoladas de valores adyacentes

Producto	**Tiempo**	**$NaNO_2$** (mg/kg)	**NO_2^-** (mg/kg)
Embutidos de cerdo/res	Formulación comercial	120	80
	A la mitad del tiempo de almacenamiento	30	20
	Fecha de caducidad	22	15
Salchicha de pollo	Formulación comercial	115	77
	A la mitad del tiempo de almacenamiento	63	42
	Fecha de caducidad	52	35

Para los cálculos de los escenarios de ingesta se utilizó la mediana, percentil 95, un factor de 5% de conversión nitrato-nitrito y tres tiempos diferentes de almacenamiento (día 0, la mitad del período de almacenamiento aceptado y la fecha de caducidad). Aplicando este procedimiento la mayoría de escenarios dieron ingestas de nitritos mayores al IDA de 0.07 mg/kgb.w. (Tabla 7). Sin embargo, debe señalarse que en general los cambios en el nitrito residual durante el almacenamiento de productos cárnicos curados pueden tener escasas consecuencias para la estimación final de la ingesta dietética.

Tabla 7. Escenarios de ingesta de nitrito en niños (4-años), basado en un estudio sueco del consumo de alimentos (Enghardt Barbieri et al., 2003).

Embutidos cerdo/ternera (mediana/95th percentil)			
Factor (%) de conversión de nitrato a nitrito	Formulación comercial	A la mitad del tiempo de almacenamiento	fecha de uso
0	**0.15/0.46**	0.04/**0.12**	0.03/**0.08**
5	**0.19/0.51**	**0.08/0.17**	0.07/**0.1**
Salchicha de pollo (mediana/95th percentil)			
0	**0.14/0.44**	**0.08/0.24**	0.06/**0.20**
5	**0.18/0.49**	**0.12/0.29**	**0.11/0.25**

Nota: En los escenarios, el consumo reportado de los productos cárnicos procesados representa el contenido de nitritos en carne de cerdo/res o salchichas de pollo en este estudio. Las negritas indican que la ingesta excede el IDA de nitritos (0.07 mg/kg b.w.)

Influencia de la conversión endógena de nitratos en nitritos en la evaluación de la exposición de nitritos en niños

La ingesta dietética total calculada de nitritos entre los niños de 4 años mostró claramente que la conversión de nitrato a nitrito a partir de vegetales era la principal fuente de exposición a nitritos (59%), esto puede tener una influencia significativa en

la exposición total de nitritos. Sin embargo, una cantidad significativa se debe también a los embutidos de cerdo/res (36%). Aún cuando la ingesta de salchicha de pollo es actualmente baja, los contenidos de nitrito residuales considerablemente más altos en salchichas de pollo en comparación con embutidos de cerdo/ternera sugiere que un cambio en el patrón de consumo de productos de embutidos de pollo podría resultar en un aumento significativo en la exposición total a nitritos.

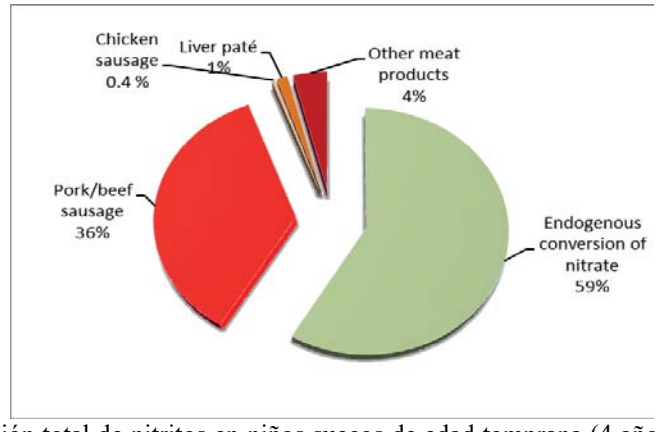

Fig. 10 Exposición total de nitritos en niños suecos de edad temprana (4 años). Se incluye la ingesta de productos cárnicos curados y el 5% de conversión de nitratos en otros productos alimenticios en nitritos.

Evaluación de la exposición de nitratos y nitritos en adultos

La Tabla 8 resume la evaluación de la exposición de nitratos y nitritos en adultos suecos realizado en la Administración Sueca de Alimentación, NFA (Darnerud et al., 2014). El cálculo se basó en datos de consumo de alimentos de 4 días de carnes procesadas, verduras y frutas de 1778 adultos seleccionados al azar (18-80 años). La ingesta de nitratos se basa en vegetales seleccionados con una cantidad

Tabla 8. Exposición dietética de nitratos y nitritos procedentes de productos alimenticios, y nitrito total (incluida la conversión endógena del 5% de nitrato en nitrito) (mg/kg peso corporal. y día).

	NO_3-ingesta (verduras o frutas)	NO_2-ingesta (cárnicos)	Total NO_2-ingesta despues de NO_3-conversion (5%)
Participants, n	1778	1778	1778
mediana	0.44	0.0009	0.02
Media	0.56	0.0022	0.03
95^{th} percentil	1.50	0.0081	***0.08***
Mayor ADI, n (%)	2 (ca 0.1%)	0	123 (7 %)

Nota: La exposición total de nitritos superó en un 7% el ADI (0,07 mg/kg y día) cuando se incluye una conversión endógena de 5% de nitratos a nitritos

particularmente alta de nitrato y valores medios representando los contenidos del resto de hortalizas. La media del nitrato proveniente de hortalizas y frutas es de 15% del IDA. La ingesta de nitritos se calcula utilizando niveles de nitritos de productos cárnicos específicos, algunos de los cuales han sido extrapolados en base a los niveles de productos similares. Para calcular la exposición total de nitritos se ha asumido una conversión endógena del 5% de nitrato a nitrito. De acuerdo con este cálculo, la contribución de los productos cárnicos representa sólo alrededor del 10% de la exposición total a nitritos entre adultos, mientras que la exposición principalmente de verduras, después de la conversión de nitratos, representa la mayor parte de la ingesta.

5. Conclusions

5. Conclusiones

Los principios teóricos del trabajo analítico práctico muestran que las dificultades de analizar nitrito/nitrato están influídas por diversos factores como los niveles de concentración, la reactividad e interacción de los iones nitrito y nitrato, y la complejidad de las matrices analizadas. Estos factores fueron considerados para:

- Establecer y proponer requerimientos metrológicos-analíticos de calidad que pueden ser usados para obtener resultados fiables. Valores númericos de la Máxima Incertidumbre Aceptable ("target uncertainty"), en orden creciente de concentración, son propuestos para su discusión.
- Seleccionar y normalizar un método de HPLC-ión cromatografía para el análisis de alimentos (verduras) que, además, ha sido aprobado como método Europeo (CEN-NMKL)
- Desarrollar y validar un método de Zn-espectrofotometría para el análisis de alimentos (productos cárnicos) que, además, ha sido aprobado como método Nórdico (NMKL)
- Cálcular l a ingesta de nitrito/nitrato en alimentos y agua potable de la población infantil y de adultos estudiados que resultó ser inferior a la ingesta diaria admisible (IDA).
- Cuantificar y evaluar la influencia de la disminución del contenido de nitrito y la conversión endógena de nitrato en nitrito en la evaluación de la ingesta total de nitrito.

En general, es importante señalar que la evaluación actual del riesgo de nitrato/nitrito se basa principalmente en la premisa de que estos iones representan un peligro para la salud, por lo que los consumidores deben ser adecuadamente protegidos a traves de advertencias, recomendaciones y regulaciones. Este punto de vista tiene un enorme impacto en la discusión y prácticas con respecto a l a utilización, tecnología, factibilidad de los controles, caracterización de riesgos (consumo agudo o crónico) y cálculo de ingesta de estos iones. Actualmente, las evidencias disponibles de los efectos beneficiosos del nitrito/nitrato y las contradictorias e insuficientes evidencias asociándolos con el riesgo de cáncer, demandan de más investigaciones de tipo analítico para poder cuantificar los efectos de las diversas matrices en la calidad del resultado analítico, como de evaluación toxicológica al objeto de realizar un nuevo balance ponderado de los posibles beneficios de su ingesta *vs* sus eventuales riesgos.

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Annex 1. Discussion (In English)

Annex 1. Discussion (In English)

4. Discussion

4.1 Work related to the standardization of an analytical method: Monitoring of nitrate in vegetables (Papers II-III)

4.1.1 Paper II - *Liquid chromatographic determination of residual nitrite/nitrate in foods: NMKL collaborative study*

The analytical method that is widely used involves the use of toxic cadmium. Upon a request from the Nordic Committee on F ood Analysis (NMKL) a study was performed to obtain an alternative chromatographic method to analyse residual nitrite and nitrate in meat products. The study was performed in three stages: a) comparative evaluation of the performance of three liquid chromatographic methods, b) validation of the selected ion chromatographic method and c) a collaborative study in which 17 laboratories from European countries participated.

a) Comparative evaluation and selection of the method

Assays were carried out to comparatively assess the type of column (weak and strong anionic columns), sample preparation (extraction, clarification) and mobile phases of the selected methods.

Selectivity.- The resolution of the nitrate and nitrite peaks showed poor selectivity for the a weak anionic column (Fig. 6). All trial-and-error experiments with the intention of separating the nitrate and nitrite peaks on standard solutions and the nitrate peaks from other interferences for carrot, onion, cucumber and potato samples were unsuccessful. It was concluded that the weak anionic column is not reliable for analysis of low concentrations of nitrate (<100 mg/kg).

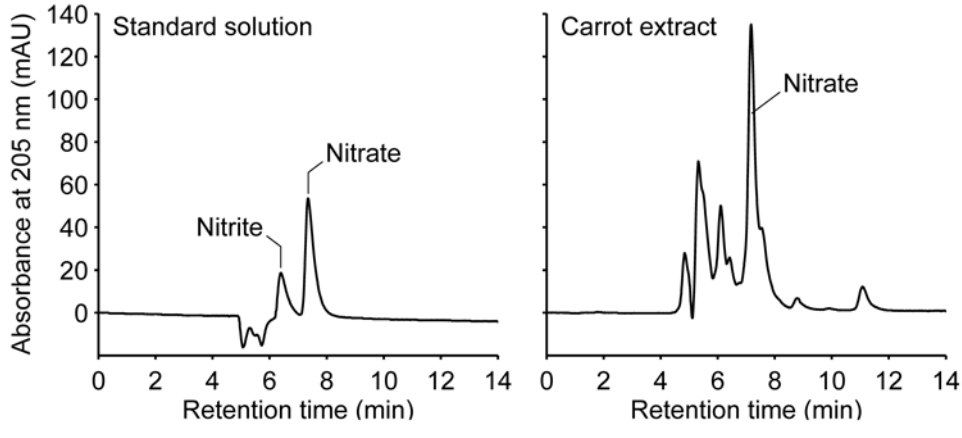

Fig. 6 Separation of nitrate/nitrite in standard solutions and nitrate in carrot extract on a weak anionic column.

On the other hand, good separation of nitrate and nitrite peaks was obtained with standard solutions and extracts of onion, carrot, cucumber and potato using a strong anionic column (Fig. 7).

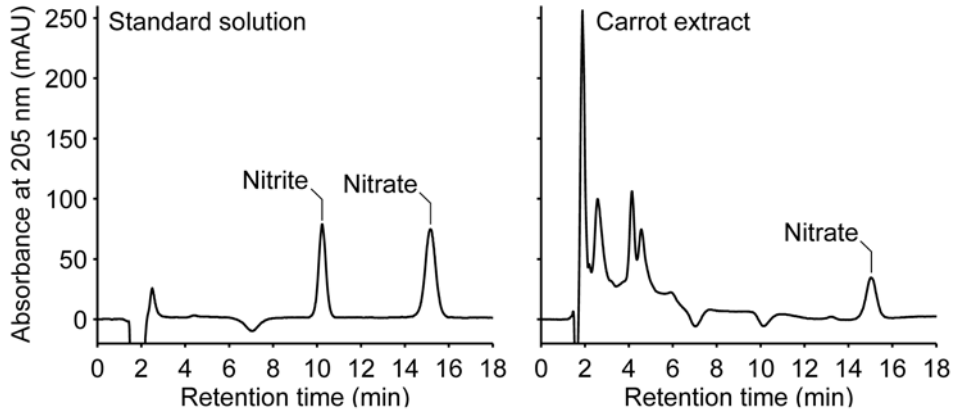

Fig. 7 Separation of nitrate/nitrite in standard solution and nitrate in carrot extract on a strong anionic column.

Clarification.- The assay results showed that Carrez solution, which is a suitable clarifying solution in spectrophotometry methods based on t he Griess reaction (see Paper III), does not work well with the ion chromatography method.

Effect of pH.- The effect of pH on the stability of the analytes and their corresponding recoveries was investigated. It was found that the higher the initial pH of the matrix, the higher the recovery of the fortified sample. This finding proved to be crucial to ensure the stability of nitrite/nitrate ions throughout the collaborative study allowing the correct evaluation of the performance of the analytical method.

b) Single-validation study

An experimental design based on analysis of variance (ANOVA) was applied to examine the applicability of the chosen method for several foodstuffs. Meat products (sausage, corned beef, ham, and chorizo), vegetable (potato), dairy product (cheese) and baby food were included in the study.

Recovery.- The recovery range of nitrite in fortified samples of sausage, corned beef, ham, baby food and cheese was 96-108%. The corresponding recovery range for nitrate was 96-107%.

Precision.- The duplicates analysed on the day of preparation showed good repeatability. However, the instability of the samples did not allow the intermediate precision to be determined in the single-validation study.

Limit of detection.- The limit of detection (LOD) for nitrite and nitrate was 0.06 and 0.36 mg/L in solution, respectively. This amount corresponding to 1 mg NO_2^-/kg and 10 mg NO_3^-/kg expressed as mass fraction.

In general, the performance of the method differed when nitrite and nitrate were analysed, with better performance being obtained in nitrite analyses.

c) Collaborative study

The collaborative study demonstrated that the HPLC-ion chromatography method can be applied for analysing foodstuffs other than meat and meat products, *e.g.* baby food, vegetables and cheese. The precision (repeatability and reproducibility) for nitrite and nitrate was satisfactory.

A proposal presented by the Swedish Working Group to the CEN to approve this method as European horizontal method (i.e. to be able analysing several matrices) was not accepted. It was supported by France (French Working Group, 1999) but opposed by Germany (German Working Group, 1999), which argued that the collaborative study did not include sufficient matrices and concentration levels. However, a final compromise was reached and an informative annex stating that the method is also applicable for the determination of nitrate in matrices other than meat products was added (CEN European Standard N 12014-part 4, 2005).

4.1.2 Paper III - *Levels of nitrate in Swedish lettuce and spinach over the past 10 years*

Monitoring of nitrate in Swedish-produced lettuce and spinach over the past twenty years (1995-2016) showed that more than 98 % of the samples were below the maximum levels established by the European Commission in 1997. The Good Agricultural Practices (GAP) used by Swedish farmers may partly explain these results.

Monitoring results of lettuce

The reliability of the analytical method used was demonstrated by internal and external validation studies (see Paper I).

All median values for the amount of nitrate in lettuce and spinach were well below the maximum levels established by the European Commission (Fig. 8).

The fresh lettuce was cultivated under cover during winter and summer and the iceberg lettuce on uncovered fields in the summer. Some differences in levels were observed during the 20-year observation period, but no clear-cut time trend emerged.

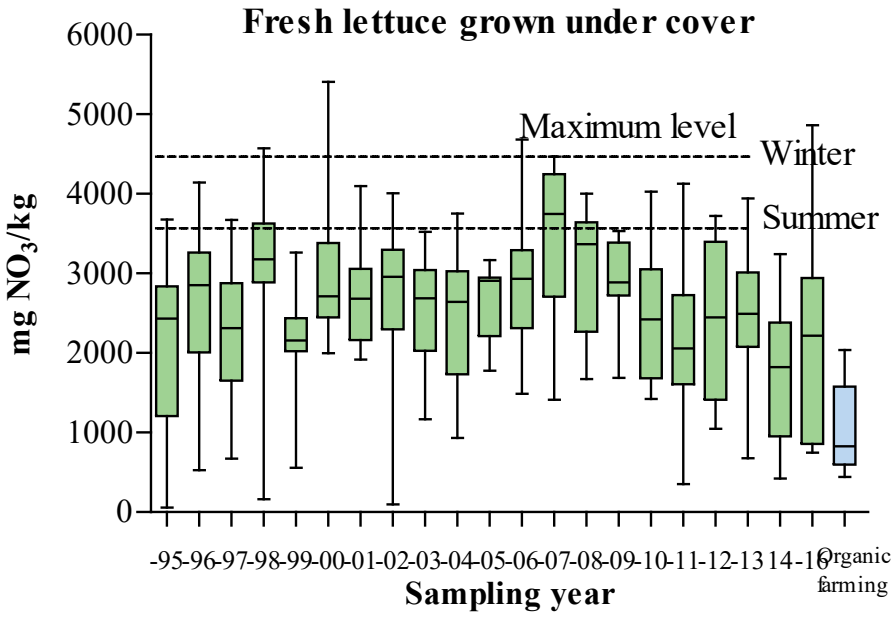

Fig. 8. Nitrate content of fresh Swedish-grown lettuce during the period 1995-2016. Four samples exceeded the maximum limit, one in summer 1996, one in summer 1998, two in winter 2000, one in summer 2016. Organically grown lettuce showed lower nitrate content.

The results from organic farming showed that the median nitrate level in fresh lettuce was 826 mg NO_3/kg^{-1}. Although only comparing nitrate data from one year (2000), the comparison of organically and conventionally grown lettuce showed that organic growing could lead a substantial decrease in the nitrate content.

In theory, the adverse climate conditions in Sweden, with low light intensity, may promote high levels of nitrates in plants. However, nitrate concentrations were below the maximum permissible level in 98% of the samples analysed during the 20 years of the Swedish monitoring programme. Good agriculture practice, which in Sweden is implemented as Integrated Production (IP) (Green Production, 2005) or organic farming (http:/www.krav.se), could be the reason why Swedish farmers are able to produce lettuce and spinach fulfilling the European regulations.

Protection from nitrates

As mentioned above (see section 1) there are changing and controversial opinions about the hazard posed by nitrate to human health. Therefore, it is difficult to draw definitive conclusions on this issue. However, regarding the other question of how much harm excess nitrate does to the environment, there is less disagreement and controversy in the face of mounting evidence of eutrophication of waters.

It is generally accepted that all forms of farming activity lose nitrate and that application of fertiliser, which has been continuously increasing for decades, is a factor in nitrate pollution. It is estimated that on average, 33% of nitrogen fertiliser applied to arable land brings no benefit, since it is not absorbed by the crops and stored in the soil as organic residues. More than half of this surplus leaches out into surrounding waters (Naturvårdsverket, 2008). Since our dependence on fertiliser for the production of food is basically unavoidable, optimising the ratio of food produced to nitrate released into the environment through the judicious and efficient use of fertiliser (GAP), instead of just aiming to maximise yield, could substantially reduce the amounts of nitrate entering the environment.

A number of actions are being taken in many European countries to reduce nitrate pollution. Although new surveys show that the total anthropogenic load of nutrients – including nutrients leaching from agricultural land in Sweden and certain other parts of Europe – is falling (Naturvårdsverket, 2017), new actions and commitments on a n international level are necessary to continue the downward trend.

However, it must be recognised that there is no single solution for the multi-faceted problem of eutrophication. The political, economic and social aspects involved make resolution of this problem difficult.

4.2 Work related to develop of an analytical method: Control and exposure estimate of nitrite in meat products (Papers IV-VI)

4.2.1 Paper IV - *Development and Validation of a Method for Determination of Residual Nitrite/Nitrate in Foodstuffs and Water after Zinc Reduction.*

An environmentally friendly and cost-effective spectrophotometric method to analyse nitrate and/or nitrite was developed. The method is based on reduction of nitrate with zinc powder (instead of the cadmium or enzymes used in the standard available methods approved by ISO/CEN). The initial nitrite concentration and total nitrite after reduction are determined by the very sensitive and widely used diazotisation-coupling Griess reaction.

Single-validation study

A single-laboratory validation was performed with five different matrices (vegetable, meat product, baby food, dairy product and surface water). The results show that the new method fulfils the international criteria for precision and recovery.

Calibration using fortified samples (matrix effect).- To evaluate the matrix effect, calibration curves were prepared using fortified blank materials. The bias arising from the effect of the matrix was measured throughout the comparison of the slope of nitrite and nitrate for the calibration curves of standard solutions and fortified minced meat. The observed slope of the calibration curve for nitrite falls into the confidence interval of the calculated slope for the fortified sample. Therefore, there is not definitive criterion for judging the presence of a bias caused by the matrix in the analysis of nitrite. On the other hand, the bias for the analysis of

nitrate calculated by the difference between the slopes is approximately 12%. This value is suggested to be used as recovery factor in the analysis of nitrate in meat products.

Limit of detection.- The limit of detection for several matrices ranged from 3 to 5 mg/kg of nitrite and nitrate, respectively. They were calculated from the intercept of the calibration curves using fortified samples.

Comparison with standard methods.- *The* results obtained were in good agreement with those obtained using a Cd reduction method and HPLC method. The paired t-test showed no statistical differences between the Zn method *vs* ISO method (Cd) and the Zn method *vs* CEN method (HPLC) for the analysis of nitrite and nitrate.

Precision.- All samples gave Horrat values less than 2, which provide reasonable evidence that the factors affecting the analytical system were sufficiently well controlled.

Recovery.- The range of recovery of residual nitrite for the six samples was 70-110 %. The range of recovery for nitrate was 73-105 %, which fulfils the recommended criteria established by the EU Commission for the official control of nitrate in foodstuffs, i.e. for a concentration range <500 mg/kg the recommended recovery value is 60-120 %, while for ≥500 mg/kg the recommended recovery value is 90-110 % .

Uncertainty.- The estimation of uncertainty in measurements was based on the random effect (measured in terms of within-laboratory reproducibility) and systematic effect (measured as the uncertainty associated with the recovery test carried out along the validation study). The ranges of uncertainty values obtained in the validation study fulfil the maximum standard uncertainty to expect (target uncertainty) proposed as criteria of acceptability of an analytical method (see above, section 1.3.3.1).

4.2.2 Paper V - *Estimated dietary intake of nitrite and nitrate in Swedish children*

Because data on children are sparse the intake of nitrate and nitrite in Swedish children were examined. Daily intake estimates were based on a nationwide food consumption survey and nitrite/nitrate content in various foodstuffs.

Intake of nitrite from cured meat

The mean intake of nitrite from cured meat among 2259 children in age groups 4, 8-9 and 11-12 were studied. Amount of nitrite and nitrate in the meat products most frequently consumed by Swedish children was collected. Using these data, the mean intake of nitrite from cured meat products was estimated at 0.013, 0.010 and 0.007 mg kg^{-1} body weight day^{-1} in children aged 4, 8-9 and 11-12 years, respectively. There was no considerable difference in nitrite intake between boys and girls. One child in each age group exceeded the ADI. In two of these three children, the major source of nitrite intake was chicken sausage, i.e. the product with the highest nitrite content, which was consumed at a rate of 35 and 90 g per day in the younger and

older child, respectively. In the third child who exceeded the ADI, liver pâté was the main contributor to the high nitrite intake. All three children had a lower body weight than average for their respective age.

Intake of nitrate from vegetables, fruit, cured meat and drinking water

The mean intake of nitrate from vegetables, fruit, cured meat and water was 0.84, 0.68 and 0.45 mg kg^{-1} body weight day^{-1} for children aged 4, 8-9 and 11-12 years, respectively. No individual exceeded the ADI of 3.7 mg nitrate kg^{-1} body weight day^{-1}. Based on data of the contents of nitrate in Swedish vegetables and cured meat, and previous presumptions on fruit and water levels, the total estimated intake of nitrate from these sources was calculated. The daily intake per kilogram body weight decreased with increasing age. No individual exceeded the ADI of 3.7 mg kg^{-1} body weight day^{-1}. Drinking water contributed 21-26% of the total nitrate intake. Of the nitrate intake from food excluding water, approximately 98% originated from fruit and vegetables, while the remaining 2% came from cured meat products.

Approximately 59% of the total nitrate intake from fruit and vegetables came from vegetables (excluding potatoes), 34% from potatoes and 7% from fruit.

Total intake of nitrite

The results of total nitrite intake, including 5% conversion of dietary nitrate from vegetables, fruit, water and cured meat and direct nitrite intake from cured meat show that approximately 12, 3 and 1% of the children in age groups 4, 8-9 and 11-12, respectively, exceeded the ADI. The estimated contribution from the conversion of dietary nitrate was approximately 70% of the total nitrite intake.

Point to be considered in exposure estimate

When evaluating the risk of nitrite exposure, the issue is how to estimate intake correctly. Because all humans are concomitantly exposed to nitrate and nitrite, it seems logical that the ADI for nitrite should include both direct exposure to nitrite and endogenous conversion from dietary nitrate. The mean intake of nitrite from cured meat products alone ranged between 10 and 19% of the ADI for the age groups studied here, and only 3 of 2259 children exceeded the ADI for nitrite. The average intake of nitrate from food and water was 12-23% of the ADI and no individual exceeded the ADI for nitrate. However, when 5% endogenous conversion of dietary nitrate to nitrite was added to the nitrite intake from cured meat, it was found that 12% of the children in the youngest age group studied exceeded the ADI for nitrite (see Paper VI, below). Thus, the intake of nitrite in Swedish children may be a concern for young age groups when endogenous nitrate conversion is included in the intake estimates.

4.2.3 Paper VI - *Time-dependent depletion of nitrite in pork-beef and chicken meat products affects nitrite intake estimation.*

The effects of processing, storage, boiling (e.g. simmering in salted water) and frying on the initial added nitrite level in relation to the outcome of exposure assessment are described. Time-dependent changes in nitrite levels in four Swedish meat products frequently eaten by children: pork-beef sausage, liver paté and two types of chicken sausage confirmed a steep decrease in nitrite level between the points of addition to the product until the recommended use-by date. The depletion of nitrite depended on time and the type of cured meat product, with higher residual nitrite levels in cured chicken products than in cured pork/beef products.

In scenarios of time-dependent depletion of nitrite using the data obtained for sausages to represent all cured meat products and including conversion from dietary nitrate, led to the ADI being exceeded in all scenarios calculated in 4 year-old children. This suggests that an approach to estimating the ADI not accounting for conversion of dietary nitrate causes an underestimation of the real nitrate intake.

The decrease in nitrite levels as a function of time after addition of $NaNO_2$ to the meat products studied is shown in Figure 9. Already at 24 h after addition of nitrite to lunch chicken sausage, grill chicken sausage, pork-beef sausage and liver paté, the nitrite levels had decreased to approximately 55%, 45%, 35% and 15% of the initial level, respectively. It is of interest that the decline in added nitrite was less pronounced in chicken sausage than in pork-beef sausage and liver paté, so that at the use-by date the nitrite levels were still approximately 40% in chicken lunch sausage and 15% in chicken grill sausage (Figure 9). A noteworthy feature was that

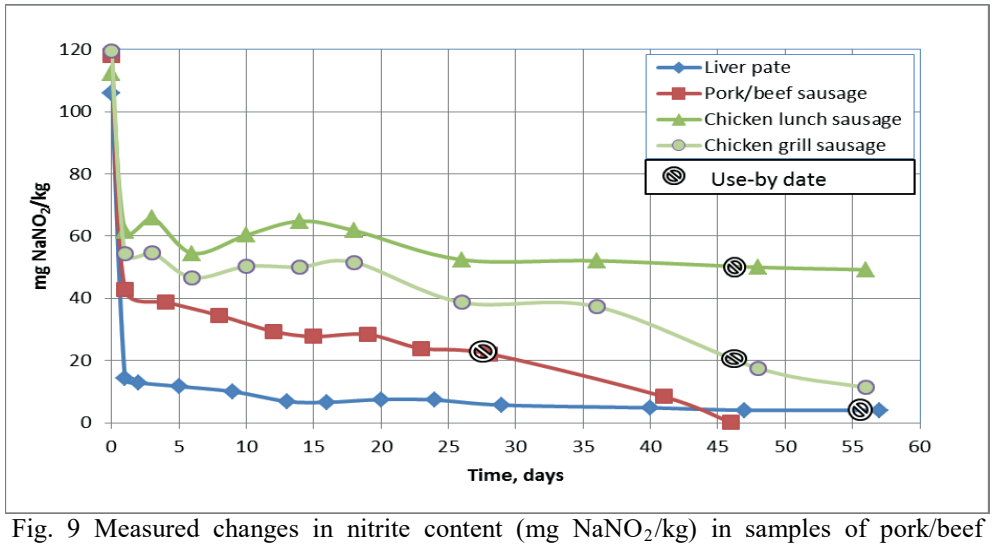

Fig. 9 Measured changes in nitrite content (mg $NaNO_2$/kg) in samples of pork/beef sausage, chicken sausages and liver paté from production date to use-by date. Day 0 is the day on which a calculated initial amount of nitrite was added by the manufacturer. The beginning of the storage period is designated day 1. The storage temperature was 2-3 °C.

the accepted storage period (until use-by date is reached) was almost twice as long for chicken sausage (48 days) as for pork-beef sausage (28 days). In fact, on applying the accepted period for chicken sausage, there was almost no nitrite left in the pork-beef sausage after 48 days.

Influence of the time-dependent depletion of nitrite in the exposure estimate

Intake scenarios on the calculated nitrite intake in 4-year-old Swedish children were formulated applying the 'point estimates' or deterministic modelling approach for integrating food consumption and chemical concentration. Hence, basal consumption data from an earlier intake study (Enghardt et al. 2003; Larsson et al. 2011) were combining with the nitrite levels determined in pork/beef and chicken sausages registered in the present survey (Table 6).

Table 6. The nitrite levels are taken from data points representing samples analysed or extrapolated from adjacent data points

Type of product	**Timeline**	**as $NaNO_2$** (mg kg^{-1})	**As NO_2^-** (mg kg^{-1})
Pork/beef sausage	At commercial formulation	120	80
	At half the accepted storage time	30	20
	At use-by date	22	15
Chicken sausage	At commercial formulation	115	77
	At half the accepted storage time	63	42
	At use-by date	52	35

The intake scenarios calculations used median and 95th percentile values, two different nitrate-nitrite conversion factors (5% and 20%) and three storage times (day 0, half the accepted storage period and use-by date). With this method of calculation not many alternatives resulted in nitrite intake below the ADI of 0.07 mg kg^{-1}b.w. (Table 7). However, it is worth notice that in general the changes in residual nitrite during storage of cured meat products may be of little consequences for the final estimation of the dietary intake.

Table 7. Intake scenarios for nitrite ion in 4-year-old Swedish children, based on a Swedish consumption survey (Enghardt Barbieri et al., 2003).

Factor (%) converting dietary nitrate to nitrite	At commercial formulation	At half the accepted storage time	At use-by date
Pork/beef sausage (median/95th percentile)			
0	**0.15/0.46**	0.04/**0.12**	0.03/**0.08**
5	**0.19/0.51**	**0.08/0.17**	0.07/**0.1**
Chicken sausage (median/95th percentile)			
0	**0.14/0.44**	**0.08/0.24**	0.06/**0.20**
5	**0.18/0.49**	**0.12/0.29**	**0.11/0.25**

Note: In the scenarios, reported consumption of all processed meat products was assigned the nitrite levels found in pork/beef or chicken sausages in this study. Bold-italic entries indicate intake exceeding acceptable daily intake (ADI) for nitrite (0.07 mg kg^{-1}b.w.)

Influence of the endogenous conversion of nitrate to nitrite in the exposure estimate of nitrite in children

The calculated total dietary intake of nitrite among 4-year-old children (Fig. 10) clearly showed that nitrate to nitrite conversion from vegetables was the major source of nitrite exposure (59%), which may have a significant influence on total nitrite exposure. However, a significant amount also originated from beef/pork sausage (36%). Even if intake from chicken sausage is currently low, our finding of considerably higher residual nitrite levels in chicken sausage compared with beef/pork sausage suggests that a switch in consumption pattern to cured chicken products may result in a significant increase in total nitrite exposure.

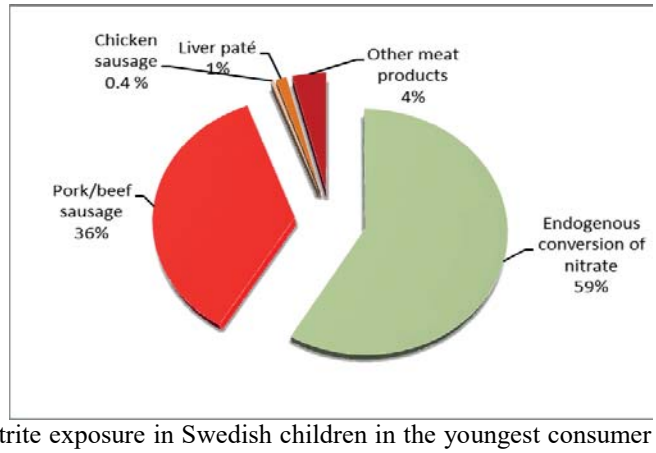

Fig. 10 Total nitrite exposure in Swedish children in the youngest consumer group (4 years), including intake from cured meat products and 5% conversion of nitrate in other foodstuffs to nitrite.

Exposure estimate of nitrate and nitrite in Swedish adults

Table 8 summarizes a new intake calculation of nitrate and nitrite for Swedish adults performed at the NFA (Darnerud et. al., 2014). The calculation of the intake of nitrite and nitrate were based on 4-day food consumption data of processed meats, vegetables and fruit of 1778 randomly selected adults (18-80 years). Nitrate intake is based on selected vegetables with particularly high amount of nitrate and the mean values to represent the levels for other vegetables. The mean adult daily intake of exogenous nitrate from vegetables and fruit is 15% of the ADI.

Nitrite intake is calculated using nitrite levels of specific meat products, some amount have been extrapolated based on levels in similar products. To calculate the total exposure of nitrite a 5% conversion of nitrate to nitrite in the body has been assumed. According to this calculation the contribution of meat products represents

only about 10% of the total nitrite exposure among adults, while exposure primarily from vegetables, after conversion from nitrate, accounts for the largest portion.

Table 8. Median and percentile dietary exposure estimates for Swedish adults to nitrate and nitrite from foodstuffs, and total nitrite (including the endogenous 5% conversion of nitrate to nitrite from other foodstuffs (mg/kg^{-1} body weight day^{-1}).

	NO_3-intake (vegetables or fruit)	NO_2-intake (meat products)	Total NO_2-intake after NO_3-conversion (5%)
Participants, n	1778	1778	1778
median	0.44	0.0009	0.02
Mean	0.56	0.0022	0.03
95th percentile	1.50	0.0081	***0.08***
Higher ADI, n (%)	2 (ca 0.1%)	0	123 (7 %)

Note: The total exposure to nitrite exceeded by 7% the ADI (0.07 mg/kg^{-1} bw day^{-1}) when an estimated 5% conversion of nitrate to nitrite is included.

Annex 2. Conclusion (In English)

Annex 2. Conclusions (In English)

5. Conclusions

The theoretical principles of the practical analytical work show that the difficulties of analyzing nitrite/nitrate are influenced by several factors such as concentration levels, reactivity and interaction of nitrite and nitrate ions, and the complexity of the matrices analyzed. These factors were considered in this thesis to:

- Establish and propose metrological-analytical requirements of quality that can be used to obtain reliable results. Hence, numerical values of the maximum acceptable uncertainty ("target uncertainty"), at increasing order of concentration, are proposed for discusion.
- Select and standardize an HPLC-ion chromatography method for analysis of foodtuffs (vegetables). The method has been approved as European method (CEN-NMKL).
- Develop and validate a Zn-spectrophotometry method for aalysis of foodstuffs (meat products). The method has been approved as Nordic method (NMKL).
- Calculate intake of nitrite/nitrate from food and drinking water in the children and adults studied.
- Quantify and evaluate the influence of the reduction of residual nitrite/nitrate content and the endogenous conversion of nitrate to nitrite in the evaluation of total nitrite intake.

In general, it is worth noting that the current assessment of nitrate/nitrite risk is based primarily on the premise that these ions are hazardous to health; therefore consumers should be adequately protected through warnings, recommendations and regulations. This view has a huge impact on the discussion and practices regarding the use, technology, feasibility of controls, risk characterization (acute or chronic consumption) and calculation of intake of these ions. At present, there is available evidence of the beneficial effects of nitrite/nitrate and contradictory and insufficient evidence of a causal association with cancer risk. This facts demand more analytical research to quantify the effects of diverse matrices on the quality of the analytical result, as well their toxicological evaluation in order to carry out a new weighing balance of the possible benefits of nitrite/nitrate ingestion *vs* their possible risks.