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Simultaneous determination of volatile and non-volatile nitrosamines in processed meat products by liquid chromatography tandem mass spectrometry using atmospheric pressure chemical ionisation and electrospray ionisation

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ABSTRACT

A sensitive, selective and generic method has been developed for the simultaneous determination of the contents ($\mu g\,kg^{-1}$ range) of both volatile nitrosamines (VNA) and non-volatile nitrosamines (NVNA) in processed meat products. The extraction procedure only requires basic laboratory equipment and a small volume of organic solvent. Separation and quantification were performed by the developed LC–(APCI/ESI)MS/MS method. The method was validated using spiked samples of three different processed meat products. Satisfactory recoveries (50–130%) and precisions (2–23%) were obtained for eight VNA and six NVNAs with LODs generally between 0.2 and 1 $\mu g\,kg^{-1}$, though for a few analyte/matrix combinations higher LODs were obtained (3 to 18 $\mu g\,kg^{-1}$). The validation results show that results obtained for one meat product is not always valid for other meat products. We were not able to obtain satisfactory results for N-nitrosohydroxyproline (NHPRO), N-nitrosodibenzylamine (NDBzA) and N-nitrosodiphenylamine (NDPhA). Application of the APCI interface improved the sensitivity of the method, because of less matrix interference, and gave the method a wider scope, as some NAs were ionisable only by APCI. However, it was only possible to ionize N-nitroso-thiazolidine-4-carboxylic acid (NTCA) and N-nitroso-2-methyl-thiazolidine-4-carboxylic acid (NMTCA) by ESI. The validated method was applied for the analysis of processed meat products and contents of N-nitrosodimethylamine (NDMA), N-nitrosopyrrolidine (NPYR), N-nitrosomethylaniline (NMA), N-nitrosoproline (NPRO), NTCA, and NMTCA were found in one or several nitrite cured meat products, whereas none were detected in non-nitrite cured bacon.

1. Introduction

Nitrite (E 249–E 250) has been used for preservation of meat products for decades and is still a widely used preservative for products as e.g. bacon, sausages and luncheon meats. Nitrite curing provides efficient inhibition of the growth of *Clostridium botulinum* [1], and it thereby lowers the risk of botulism, and in addition it results in the development of unique colour, flavours and aromas [2]. However, nitrite can react with secondary amines present in the meat to form N-nitrosamines (NAs), many of which are genotoxic [3]. Several studies and reviews (e.g. [4,5]) conclude that there is an association between dietary intake of processed meat and increased risk of cancer, especially gastric cancer. Recently a large study revealed an association between increased mortality and a daily intake of processed meat above only 20 g [6]. Based on a review of all published cohort and case-control studies from 1985–2005, Jakszyn & Gonzalez 2006 [7] concluded that the available evidence supports a positive association between nitrite and nitrosamine intake and gastric cancer, as well as between the intake of meat including processed meat and gastric cancer and oesophageal cancer [7].

A literature study reveals that about 20 different NAs have been identified in processed meat products. In general the NAs are defined as either volatile NAs (VNA) or non-volatile NAs (NVNA) where the VNAs are applicable to extraction by steam distillation and/or to analysis by gas chromatography and the NVNA are not. The levels of NAs in nitrite preserved meat products vary greatly, from below detectability to levels in the order of thousand $\mu g\,kg^{-1}$, depending on the type of NA and the type of meat products. Of the most commonly found NAs in meat, N-nitrosodiethylamine (NDEA) has been evaluated as the most potent carcinogen, whereas N-nitrosodimethylamine

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(NDMA) has lower potency and N-nitrosopyrrolidine (NPYR) and N-nitrosopiperidine (NPIP) even lower [8]. N-nitrosoproline (NPRO) and N-nitrosohydroxyproline (NHPRO) have been shown to be noncarcinogenic [9]. Knowledge is however still needed regarding the genotoxicity and/or carcinogenicity of some NAs, such as N-nitroso-thiazolidine-4-carboxylic acid (NTCA) and N-nitroso-2-methyl-thiazolidine-4-carboxylic acid (NMTCA) [9]. In general the occurrence and toxicological profiles of the NVNA are less studied.

The VNAs, which are frequently found but in relatively low amounts of $\leq 5\ \mu g\ kg^{-1}$ [10–12], have been the subject of the majority of the available literature. Most of these studies were conducted in the 1970s and early 1980s. Furthermore, most of these early studies only investigated one to five NAs. NDMA and NPYR are the most frequently found VNA in nitrite/nitrate treated meat products. For example Spiegelhalder et al. [13] reported 32% and 11% of such products ($n = 395$) to contain detectable levels ($>0.5\ \mu g\ kg^{-1}$) of NDMA and NPYR, respectively. NDMA and NPYR at levels above $5\ \mu g\ kg^{-1}$ occur only in 2% and 6% of the samples, respectively [13]. Other types of VNA were only detected occasionally. The analytical procedures for the analysis of these volatile compounds in foods were well established at that time. The methods employed in the majority of these earlier studies as well as many recent studies [14,15] used gas chromatography for separation and detection by thermal energy analyser (TEA) [16,17]. The TEA detector provided higher sensitivity compared to GC–MS systems available at that time. In comparison with today's MS systems the TEA detector and UV detector have relatively lower specificity and therefore also a higher risk of false positive findings. Furthermore, because the TEA detector has limited versatility and is of relatively high cost this detector is not available in most laboratories. In previous studies dichloromethane, has been widely used as extraction solvent and concentration procedure using a Kuderna-Danish apparatus for VNA analysis [14,18,19]. Dichloromethane in many cases is an efficient extraction solvent; however because of its possible carcinogenic effect it has not been used in the present work.

On the other hand, knowledge on the occurrence and nature of NVNA in food is limited. The reported NVNA are primarily N-nitrosamino acids [14], which occur in processed meat products at significantly higher levels than the VNA [10]. For example N-nitroso-2-hydroxymethyl-thiazolidine-4-carboxylic acid (NHMTCA), NTCA and NMTCA were found in smoked cured meats at concentrations up to $2100\ \mu g\ kg^{-1}$ [20], $1600\ \mu g\ kg^{-1}$ and $98\ \mu g\ kg^{-1}$ [21], respectively. Publications describing methods for the determination of N-nitrosamino acids in meat products are available [18,22,23]. These studies are based on LC–TEA and extraction procedure consuming large amounts of organic solvent. More recently publications on methods for determination of N-nitrosamino acids using LC–MS/MS are available, but in matrices as tobacco [24] and smokeless tobacco products [25].

Since the 1970s and 1980s, the technology used by the industry to process meat and the consumption patterns of processed meats has changed. For example, it has become common procedure during production of most nitrite cured meat to add ascorbate or erythorbate as an antioxidant; hygiene and maintenance of the cool chain has been improved; fat contents, sodium chloride and nitrite levels have been reduced for most products. All these factors can affect the NA levels and the levels of NAs in processed meat products may therefore have changed since the majority of the studies in this field were performed.

In order to perform a well-founded and updated assessment of the risk associated with the human exposure to NAs via the consumption of nitrite preserved meats, data on the occurrence and nature of NAs, including both VNA and NVNA, in a wide range of processed meat products available on the market, is needed. For that purpose an analytical method allowing simultaneous extraction and determination of both VNA and NVNA in meat products is an important tool. A method based on LC will allow for the determination of the NVNA and highly polar NAs, i.e. NHPRO, N-nitrososarcosine (NSAR) and NDMA. Several recent publications describe separation and detection of several VNAs by LC–MS or LC–MS/MS [26–28]. Application of tandem mass spectrometry would further provide high sensitivity and specificity. A method for the simultaneous determination of both VNA and NVNA by LC–MS/MS has, to our knowledge, not been published so far.

The aim of the present study was to develop and validate a method for the simultaneous determination of NVNA and VNA in processed meat products. It was chosen to base the assay on high performance liquid chromatography combined with atmospheric pressure chemical ionisation (APCI) and/or electro spray ionisation (ESI) with detection by tandem mass spectrometry (MS/MS) since this would provide high sensitivity and specificity as well as have the possibility to include both groups of NAs in the same assay. The extraction procedure should be a common procedure, as simple as possible and preferably without the use of dichloromethane. The target NAs have previously been reported to occur in processed meat products and they were commercially available as pure standards (chemical structures of the target NAs are presented in Fig. 1). The method was applied to ten samples of processed meat products purchased at local supermarkets.

2. Experimental

2.1. Chemicals

Acetonitrile (extraction solvent) was of HPLC grade (Rathburn Chemicals Ltd, Walkerburn, Scotland). Formic acid (purity 98–100% for analysis), pipecolic acid (PIC) (purity 98%), and the methanol used as LC eluent (purity ≥99.9%, Fluka-Analytical) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). The pure standards of N-nitrososarcosine (NSAR), N-nitrosohydroxyproline (NHPRO), N-nitrosodibenzylamine (NDBzA), N-nitrosoproline (NPRO), N-nitrosomethylaniline (NMA), N-nitroso-2-methyl-thiazolidine 4-carboxylic acid (NMTCA) and N-nitroso-thiazolidine-4-carboxylic acid (NTCA) were purchased from Toronto research chemicals (Toronto, Canada), whereas the standards of N-nitrosodiethylamine (NDEA), N-nitrosodipropylamine (NDPA), N-nitrosomorpholine (NMOR), N-nitrosodimethylamine (NDMA) and N-nitrosodiphenylamine (NDPhA) were purchased from Sigma-Aldrich Co. N-nitrosomethylethylamine (NMEA), N-nitrosopyrrolidine (NPYR), N-nitrosodibutylamine (NDBA), N-nitrosopiperidine (NPIP) were purchased from Dr. Ehrenstorfer (Ausburg, Germany). The internal standards N-nitrosodimethylamine-d_6 (NDMA-d_6) and N-nitrosopyrrolidine-d_8 (NPYR-d_8) were purchased from Sigma-Aldrich Co. and CDN isotopes (Quebec, Canada), respectively. The purity of all the NA standards was ≥98% except for NMA which were of 95% purity.

Stock solutions in methanol of $1\ mg\ mL^{-1}$ were prepared for each of the NAs. A mixture of all the NA standards (standard solution) was then prepared by volumetrically dilution of the stock solutions with acetonitrile to $10\ \mu g\ mL^{-1}$. The stock solutions were stored at −60 °C and the standard solution was stored at −18 °C.

2.2. Extraction procedure

A fractional factorial design was set up to systematically evaluate the influence of different factors on the extraction efficiencies. A seven factor design was constructed using the software Minitab (Version 16.24). The seven factors were sample size, volume of extraction solvent, introducing a second extraction, acidifying the extraction solvent, clean-up by liquid/liquid extraction with hexane and dilution of the extract with water. The effect of the seven