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# Establishment of analytical methods for the determination of new foodprocessed contaminants

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# ESTABLISHMENT OF ANALYTICAL METHODS FOR THE DETERMINATION OF NEW FOOD-PROCESSED CONTAMINANTS

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# ESTABLISHMENT OF ANALYTICAL METHODS FOR THE DETERMINATION OF NEW FOOD-PROCESSED CONTAMINANTS

Memòria presentada per Mohammed-Soubhi Altaki per optar al grau de doctor per la Universitat de Barcelona

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La Dra. Maria Teresa Galcerán Huguet catedràtica del Departament de Química Analítica de la Universitat de Barcelona, i el Dr. Francisco Javier Santos professor titular del mateix Departament,

FAN CONSTAR,

Que el present treball d'investigació titulat "Establishment of analytical methods for the determination of new food-processed contaminants" ha estat realitzat sota la nostra direcció pel Sr. Mohammed-Soubhi Altaki I que tots els resultats presentats són fruit de les experiències realitzades pel citat doctorant.

I per a que així consti, expedim i signem el present certificat.

Barcelona, 5 de Abril de 2017

Dra. Maria Teresa Galcerán Huguet                    Dr. Francisco Javier Santos

*THIS THESIS IS DEDICATED TO:*

*MY WOUNDED CITY "ALEPPO-SYRIA"*

*MY PARENTS*

*BARCELONA; THE CITY OF MY LOVED SONS*

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# TABLE OF CONTENTS

# ABSTRACT

**ABSTRACT**

Furan and volatile *N*-nitrosamines (VNAs) are induced food toxicants that are unwantedly formed during food processing. Due to their potential harmful effects on human health, they have been classified as probable and/or possible carcinogens to humans by the International Agency for Research on Cancer (IARC). Since these compounds are formed in foods at low concentration levels (ng $g^{-1}$), there is a need to dispose of reliable analytical methods to evaluate their occurrence in processed foods and beverages and to estimate the human exposure risk to these compounds through food consumption.

In this context, the main objective of the thesis is to establish fully automated headspace solid-phase microextraction (HS-SPME) gas chromatography coupled to ion-trap mass spectrometry (GC-ITMS) methods to evaluate the presence of these contaminants in food products and to estimate the real exposure of the population to these compounds. Parameters affecting the HS-SPME performance including extraction and desorption conditions were experimentally optimised in order to obtain the maximum extraction efficiencies. In addition, GC-ITMS methods using electron ionization and full scan MS mode for furan analysis and selected reaction monitoring tandem mass spectrometry with chemical ionization (CI-IT MS/MS) for VNAs analysis were developed.

For HS-SPME analysis of furan, several fibres of different characteristics were evaluated and the highest extraction efficiency was obtained using a Carboxen/polydimethylsiloxane (CAR/PDMS, 75 μm) fibre. The best conditions for temperature (30 ºC) extraction time (20 min), headspace/sample phase volume ratio (1.5) and ion strength (20% NaCl) were established. A comparison between the performance of the proposed HS-SPME method and the direct headspace method, proposed by the US Food and Drugs Administration (US FDA) was performed, and the results shown that the accuracy is similar, but better precisions (RSD, 5–8%) and lower LOQs (0.05–0.40 ng $g^{-1}$) were obtained than those given by HS FDA method (RSD, 9–12% and LODs from 1.39 to 2.86 ng $g^{-1}$). Moreover, furan formation during analysis is

avoided and a higher sample throughput was obtained since it was demonstrated that furan quantification by isotope dilution can be performed instead of the standard addition method proposed by the FDA. To examine the feasibility of the developed method, it was applied to the analysis of furan in several Spanish food commodities and concentrations ranging from 0.1 ng g$^{-1}$ to 1.1 μg g$^{-1}$ were found. These results are consistent with data published in the literature for similar food samples, being baby foods (0.5–40 ng g$^{-1}$), brewed coffee (20–70 ng g$^{-1}$), and powdered instant coffee (up to 1.1 μg g$^{-1}$), the food categories with the highest furan content.

For the analysis of volatile *N*-nitrosamines in food, a method that combines HS-SPME with GC-CI-MS/MS was developed. With regard to the fibres tested for HS-SPME the best results were also achieved using a 75 μm-CAR/PDMS fibre working at 70 ºC for 45 min and with a headspace/sample phase volume ration of 0.6 in a 20-mLvial. To improve the extraction and transport of VNAs from the aqueous phase to headspace the addition of salt (Na$_2$SO$_4$) and NaOH was required. In order to improve sensitivity, methanol was used as CI reagent and MS/MS conditions were thoroughly optimised allowing achieving a limit of quantification (0.10–0.38 μg kg$^{-1}$) lower than the maximum levels established by international regulations. In addition, the method showed good trueness (recoveries: 90–106%) and precision (RSD% < 12%) and adequate decision limits (CC$_α$: 0.40–0.44 ng g$^{-1}$) and detection capabilities (CC$_β$: 0.44–0.50 ng g$^{-1}$). The developed method was applied to the determination of VNAs in commercialized beers and processed meat products from the Spanish market. VNAs were not detected in any of the analysed beer samples and were only found in 3 out 18 samples (one cured sausage and two Spanish chorizo) at low levels (2.4- 3.7 ng g$^{-1}$). These results confirm the effectiveness of the actions applied by the manufactures for reducing the VNA formation during the food processing.

The second part of the thesis is devoted to assess the occurrence of furan in coffee and baby foods to estimate the exposure of the Spanish population and the risk associated with their consumption. The presence of furan in these food categories constitutes a potential risk for human health because they contain a high furan content

and are highly consumed. This study was not performed for VNA because of the low frequency and levels found in the samples analysed in this thesis. First, a survey on furan content in coffee brews and baby foods was conducted using the HS-SPME GC-MS method developed. The highest level of furan was found in coffee brews (12–244 ng ml$^{-1}$) and meat- and fish-based baby foods (7.9–84 ng g$^{-1}$). For coffee, the influence of the coffee type and brewing procedure was evaluated and the results shown that brews prepared from an espresso coffee machine contain higher levels of furan (14–146 ng ml$^{-1}$) than those obtained from a home drip coffee maker (20–78 ng ml$^{-1}$). However, the highest furan content in coffee brews was found for Nesspresso (117–244 ng/ml), mainly due to the hermetic seal of the capsules that prevents furan losses during handling and the higher pressure applied by the coffee machine. The influence of roasting process of coffee beans in furan formation was also studied and the roasting temperature and time were shown to be the most important factors. Therefore, a low temperature and long-time (140 ºC, 20 min) are recommended to decrease (up to 50%) the amount of furan in brewed coffee.

Regarding baby foods, several types of products were evaluated and furan was detected in all canned and/or jarred baby food (0.7–84 ng g$^{-1}$) and baby cereal (0.15–2.1 ng g$^{-1}$) samples, while it was only found in 50% of infant formula samples (0.08–2.1 ng g$^{-1}$). The highest furan concentrations were found in fish (19.1–84.2 ng g$^{-1}$) and meat based baby foods (7.9–64 ng g$^{-1}$), which can be attributed to the presence of unsaturated fatty acids. In contrast, fruit-based (0.7–2.68 ng g-1) and vegetable-based baby foods (1.7–28.8 ng g$^{-1}$) were the samples with lower furan content as a result of a higher stability of the precursors and the lower temperature used during industrial processing. The influence of baby food preparation on furan content was also evaluated. As a result, it was found that furan concentrations were reduced by up to 35% when samples were heated in a dish using microwave oven and by up to 53% when a hot water bath was used. Moreover, a standing time of 5 min after heating promoted the loss of furan by evaporation to about 50%. Therefore, we recommend the use of a gently heating process using a hot water bath followed by a standing time of 5 min with stirring to reduce significantly the furan content.

Finally, the furan exposure and risk assessment from baby food and brewed coffee consumption were estimated. The average daily intake of furan from coffee consumption ranged from 0.25 µg kg$^{-1}$ body weight (bw) day$^{-1}$ for male to 0.18 µg kg$^{-1}$ bw day$^{-1}$ for females. In addition, the average furan intake from baby food consumption was estimated to be 0.44 µg kg$^{-1}$ bw day$^{-1}$. The margin of exposure (MoE) was used to evaluate the health risks associated to furan intake. MoE values from baby food consumption showed a potential public health concern for fish- and meat-based baby foods, although the handling time that favours the loss of furan by evaporation reduces the furan intake for these matrices. In contrast, the consumption of infant formula and jarred cereal baby foods presents a low public health concern and, therefore, a low priority for risk management actions is required.

# OBJECTIVES AND STRUCTURE OF THE THESIS

**OBJECTIVES AND STRUCTURE**

Heat processed and cooking food induce the generation of a plethora of chemicals which contribute to their aroma and flavour and ensure their microbiological safety. However, some of these products may not be beneficial or may even be toxic to humans. Among these, there are furan and volatile *N*-nitrosamines, which are the focus of this thesis. The occurrence of furan and *N*-nitrosamines in processed foods and beverages has been known for a few decades and is a cause for concern because they exhibit carcinogenic and cytotoxic activities on animals and can cause harmful effects on human health. For this reason, these toxicants have been classified as possible or probably human carcinogens by the International Agency for Research on Cancer (IARC). Since these food toxicants are usually found in processed foods and beverages at low concentration levels (ng g$^{-1}$), there is a need to dispose sensitive and selective methods for their determination. In addition, more information on the occurrence of these compounds in processed food samples is required to assess the exposure of humans to these compounds through food consumption.

In this context, the main objective of this thesis is to establish analytical methods to evaluate the occurrence of these contaminants in commercial food products from Spain and to estimate the exposure risk of the population to these compounds.

This general objective is structured around several specific objectives:

• To develop and validate sensitive and selective methods for the analysis of both groups of compounds in food samples. To this purpose, gas chromatography coupled to ion-trap mass spectrometry (GC-ITMS) and headspace solid-phase microextraction (HS-SPME) methods, able to avoid losses and/or formation of target compounds during analysis, will be established.

• To provide data on the occurrence of furan and volatile *N*-nitrosamines (VNAs) in processed food and beverages commercialized in the Spanish market.

• To study the effect of different factors on the formation of the studied toxicants in industrial processing foods and domestic preparation procedures before consumption and to propose strategies to reduce their presence in food products.

• To estimate the daily intake and risk assessment resulting from food consumption by Spanish population.

This thesis is divided in four chapters:

The first chapter provides an introduction about the motivation for this research project and the reason to select and study furan and volatile *N*-nitrosamines in processed food products. The general and relevant background information found in the literature, including chemistry, toxicity and formation pathways of furan and volatile *N*-nitrosamines in food is included.

The second chapter includes the studies performed for the development and validation of analytical methodologies for the determination furan and volatile *N*-nitrosamines in processed food using methods based on headspace solid-phase microextraction (HS-SPME) coupled to gas chromatography and ion-trap mass spectrometry (GC-MS). After a brief overview of the state-of-the art of the analysis of furan and volatile *N*-nitrosamines, the experimental results obtained in these studies are presented in three scientific papers, two devoted to furan determination, *Analysis of furan in foods by headspace solid-phase microextraction–gas chromatography–ion trap mass spectrometry* (Journal of Chromatography A, 2007) and *Automated headspace solid-phase microextraction versus headspace for the analysis of furan in foods by gas chromatography–mass spectrometry* (Talanta, 2009); and one to the analysis of volatile *N*-nitrosamines, *Automated headspace solid phase microextraction combined with gas chromatography - chemical ionization - tandem mass spectrometry for the analysis of volatile N-nitrosamines in food matrices* (sent to Journal of Chromatography A, 2017). Finally, a discussion of the results obtained is included.

The third chapter of the thesis is devoted to examine the presence of furan in selected food samples of particular concern, coffee and baby foods, in which furan has

been found at relatively high concentrations. The evaluation of furan levels found in these food products are included in two scientific articles, *Occurrence of furan in coffee from Spanish market: contribution of brewing and roasting* (Food Chemistry, 2011) and *Furan in commercial baby food from the Spanish market: estimation of daily intake and risk assessment* (Food Additives & Contaminants: Part A, 2017). In addition, the effect food preparation, heating and handling, on furan content and an estimation of risk assessment of furan intake from food consumption are also included.

Finally, the conclusions derived from the work performed in this thesis and the references cited along the text are included.

# 1. INTRODUCTION

## 1.1. PROCESS-INDUCED FOOD CARCINOGENS

Food is a fundamental prerequisite for human survival as it is the unique source of essential nourishment required for vital processes in human body. Nowadays, food composition is well established being carbohydrates, proteins, fats, vitamins, minerals and water the main chemical components of food which account a nutritional value of more than 99.9% of the total mass of food (Janssen and Voragen, 1997). However, the development of sensitive analytical instrumentation has allowed to identify non-nutrient chemical substances in food which may impact the human health and since the end of the last century, they have been recognised as food chemical contaminants (de Man, 1999; Long and Chase, 2003). Some of these contaminants are originated from food processing and several studies have demonstrated that some of them can induce tumours in animals (Janssen, 1996; Knize et al., 1999; Watson, 2001). Moreover, nutritional epidemiological studies have lent some support to the hypothesis that some of these compounds might represent a risk of causing food-related cancer in humans (Doll and Perto, 1981; Skog and Alexander, 2006).

Process-induced food carcinogens are defined as those undesirable chemicals unintentionally present in food as a result of processing and/or preparation of the raw material. These compounds may exert adverse physiological (toxicological) effects on humans creating a potential or real risk to human health. Food processing includes any deliberate change and/or array of technological actions performed on food such as curing, crushing, addition of food additives and thermal treatment including cooking (Stadler and Lineback, 2009). The use of heat through food processing operations or for cooking is considered the most used processing technique in modern society (Arnoldi, 2001) and in fact, most of our foods, if not all, are thermally treated at certain point during processing operations (e.g. the pasteurization, sterilization, smoking and cooking). These practices were originated due to the need to ensure the safety of diets, to improve the edibility of food and to maintain food supplies for longer periods of time.

Food processing includes several chemical reactions which lead to the formation of a plethora of new compounds via multiple mechanisms. The natural raw components of food are excellent reactants whereas the formed products will mainly depend on the process type and the conditions used (Shahidi and Ho, 1998). These reactions can involve only the natural raw components of the food or they may take place between exogenous compounds intentionally added to the food or outside agents (gases into contact with food) and inherent components of the food. In addition, chemical reactions in foods are strongly dependent on temperature and time of the process because foods are thermodynamically unstable (Earle and Earle, 2003).

Although processing of foods has enormous advantages in relation to food safety, edibility and sensory characteristics, process-induced food carcinogens are also produced during these processing operations and/or cooking. It is now well known (but no completely understood) that the formation of these carcinogens is due to chemical reactions that occur during food processing (Abnet, 2007; Friedman and Levin, 2008). Unfortunately, the generation of these carcinogens in human food is almost impossible to avoid and must be considered as an undesirable outcome of the undoubtedly beneficial effects of food processing. Well known examples of these carcinogens are polycyclic aromatic hydrocarbons (PAHs) in barbecued meat, heterocyclic aromatic amines (HAs) in overheated meat and fish and N-nitroso compounds (NOC) in many processed foodstuffs.

The discovery of acrylamide in processed carbohydrate rich food in 2002 sparked renewed interest on some other known process-induced food carcinogens such as volatile N-nitrosamines (VNAs) and has also led to the discovery of some others that were previously unknown like chloropropanols, ethyl-carbamate and furan (Tareke, 2002; Hellenäs et al., 2005).

The risk of developing cancer in humans from carcinogens occurring in food is of widespread interest to scientific research and food surveillance institutions (Lachenmeier, 2009). In the past, this interest was often focused on process-induced food carcinogens classified by the International Agency for Research on Cancer (IARC)

as carcinogenic to humans (group 1) such as benzo[a] pyrene. Probable carcinogens such as acrylamide and heterocyclic amines have also been studied (Hellenäs et al., 2005) but other agents with high level of evidence of their possible carcinogenicity such as furan and volatile N-nitrosamines, already classified by IARC as probable or possible carcinogens to humans must be also taken into account.

## 1.2. RATIONALE AND SIGNIFICANCE OF THE PROPOSED RESEARCH

Furan has been known as a flavour of food for many years. It was listed as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer at the end of the last century (IARC, 1995) and it has been included as a possible human carcinogen in the US Department of Health and Human Service Report on carcinogens National Toxicology Program (NTP, 2011a). Up to our knowledge, concern over furan in food began on May 2004 when scientists of US Food and Drug Administration (FDA) identified furan at a relative high concentration level in a large number of heat treated foods (US FDA, 2004a). Subsequently, the European Food Safety Authority (EFSA) issued a call for more information on furan in foods (EFSA, 2004; 2006). Furthermore, a workshop on furan in food was organized in May 2006 by the Directorate-General for Health and Consumers of the European Commission, the EFSA and the European Commission Joint Research Centre in order to gather information about furan in food. Five years later, EFSA published a report on the status of analytical methods for furan and on data needs for risk assessment (EFSA, 2011) concluding that it would be beneficial future testing of furan in coffee and baby food products since limited values were available. Moreover, they recommended including samples analysed as purchased and also as consumed. In parallel, a meeting of the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA) was held in February 2010 and concluded that health concerns can arise from the presence of furan in food and identified furan as a priority compound for research.

The study of volatile N-nitrosamines (VNAs) as process-induced food carcinogens is of interest due to their high carcinogenicity even at low-dose exposure (Sung, 2004). Most of VNAs have been classified by IARC as probable or possible carcinogens to humans (IARC, 1978). In the past, it was thought that the use of nitrite as curing agent for meat derived foods was the single source of VNAs in the diet. However, today it is accepted that other food processing procedures specially heat-treatment enhance the formation of VNAs in foods containing a residual amount of nitrosating agents (Drabik-Markiewicz et al., 2009). The major dietary sources of VNAs include cooked meat and fish products and beer which are followed by cheese and non-fat dry milk (Yurchenko and Mölder, 2005; 2006; 2007). Levels of VNAs have been declining during the past decades because of several modifications of production process, concurrent with a decrease of the use of nitrite in food processing and a better control of thermal treatment conditions. However the small amounts of VNAs in food can be significant because of the possibility, even likelihood, that humans are more sensitive to these carcinogens than the laboratory animals (EFSA, 2010).

Recently, researches on furan and volatile N-nitrosamines have been focused on the formation pathways and carcinogenicity mechanisms and less attention has been paid to the analysis of these toxicants in food (Wenzl, 2007, Crews, 2010). So, in this thesis, methods for the analysis of these compounds have been established and also an evaluation of their levels in food has been performed. The analysis of such process-induced food carcinogens is requesting the development of cost-effective analytical methodologies to guarantee the safety and traceability of foods in compliance with legislation (Jackson, 2009). Challenges facing the analysis of these carcinogens in food are minimizing sample treatment and obtaining high selectivity and sensitivity of detection, since they commonly occurred at very low concentration levels.

## 1.3. FURAN IN FOOD: CHEMISTRY, TOXICITY AND FORMATION

## 1.3.1. FURAN CHEMISTRY

Furan is a heterocyclic organic compound consisting of a five-membered aromatic ring with four carbon atoms and one oxygen atom (Figure 1.1). It is a reactive chemical that slowly decomposes by auto-oxidation to form explosive peroxides on air and forms resins by its contact with mineral acids. Furan is a highly volatile compound with a boiling point close to room temperature and it has a considerable low solubility in water, but is quite soluble in alcohol, ether and other common organic solvents. The most important chemical and physical properties of furan are included in Table 1.1.

The principal industrial use of furan is as organic solvent and as intermediate reagent or raw material for the synthesis of other organic compounds such as tetrahydrofuran (THF). Furan serves as an intermediate in the synthesis and preparation of numerous linear polymers used to prepare temperature-resistant structural laminates and copolymers and it is also used in the production of several lacquers, stabilizers, pharmaceuticals and agriculture chemicals such as insecticides (IARC, 1995).
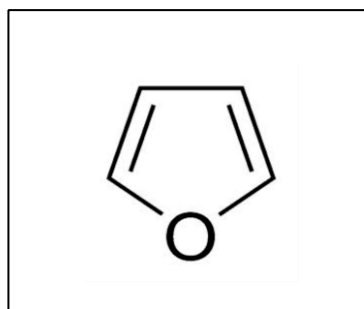


Figure 1.1: Chemical structure of furan.

Human exposure to furan from the environment is limited as it is conducted and handled in closed systems and containers during industrial processes although several processes such as oil refining, coal mining, and coal gasification have contributed to the releasing of furan into the environment.

Table 1.1: Chemical and physical properties of furan

| | |
|---|---|
| CAS registry number | 110-00-9 |
| Other names | Furfuran; Divinylene oxide; 1,4-Epoxy-1,3 butadiene |
| Molecular Formula | $C_4H_4O$ |
| Molecular mass | 68.074 g/mol |
| Exact mass | 68.026215 |
| Boiling point | 31.36 ºC at 760 mm Hg |
| Melting point | -85.6 ºC at 760 mm Hg |
| Density | 0.9371 g/ml |
| Log $K_{ow}$ | 1.34 |
| Water solubility | 10000 mg/L |
| Relative vapour density | 2.35 |
| Vapour pressure | 493 mm Hg at 20 ºC |

In addition, cigarette smoke and exhaust gas from diesel and gasoline engines also contribute to the release of furan into the air. Furan is not adsorbed on suspended solids or sediments when it is released into water but it is volatilized to the atmosphere where it is photo-chemically degraded producing hydroxyl radicals with an estimated half-life of about 9.5 hours. So, furan is not frequently found in environmental samples although it has been found in home indoor air (Jarke et al, 1981) and in surface water at low concentrations.

Furan and much of its derivatives have been known for long time as food constituents and they have been associated with the flavour that contributed to the sensory characteristics of food (Maga, 1979). Its presence in several foods such as coffee (Johnston and Frey, 1938), cooked chicken (Grey and Shrimpton, 1967), white bread (Mulders et al., 1972) and canned beef (Persson and von Sydow, 1973) was first reported more than 50 years ago. However, its concentration was usually very low and it could not be determined due to the lack of reliable quantification methods. As a

result, before 2004, furan in food was not considered an issue related to food safety. Despite the fact that furan was classified as a possible carcinogen to humans (group 2B) (IARC, 1995), and there were evidences about its capacity to cause serious health problems in laboratory animals (NTP, 1993), no attention on furan in food was paid till 2004 when researchers at US FDA found furan in several canned and jarred foods and confirmed that furan is produced as a result of food heat treatment (FDA, 2004a).

Since furan has been known as a volatile flavour for many years, it was not currently analysed and very limited data on furan in food were available. Therefore, several monitoring programs to survey furan content in food were launched by international food agencies such as FDA and EFSA. In addition, several European projects paid attention on furan in order to understand its formation and occurrence in food and also to re-evaluate its toxicity (Heatox, 2003; Furan-RA, 2007; Prometheus, 2011). In the next two sections of this introduction, a brief summary on the furan toxicity mechanism and its formation pathways in food is presented.

## 1.3.2. FURAN TOXICITY AND CARCINOGENICITY

Due to the widespread use of furan for industrial purposes, it was included by the US National Toxicology Program in the middle nineties in the list of compounds for carcinogenic and toxicity evaluation using male and female mice and also rats (NTP, 1993). Several studies using $[^{14}C]$-labelled furan demonstrated that it easily passes through biological membranes and is extensively absorbed from intestine (Burka et al. 1991). After a 2 year bioassay in rodents it was concluded that the critical toxicological effect of furan is carcinogenicity, and liver is the main target organ of furan-induced toxicity (NTP, 1993). Taking into account this information and since the human health effects of furan were unknown; it was listed as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer (IARC, 1995).

The metabolic activation for furan-induced toxicity and carcinogenicity was proposed in the past century (Burka, et al., 1991). Although the major metabolite of furan is $CO_2$, it was shown that Cytochrome P450 enzymes (CYP), predominately CYP2E1, mediates via oxidation the formation of *cis*-2-butene-1, 4-dial (BDA) from furan, directly or by intermediate 2, 3 epoxy formation (Chen et al., 1995) (Figure 1.2).



Furan

Cis-2-butene-1, 4-dial (BDA)
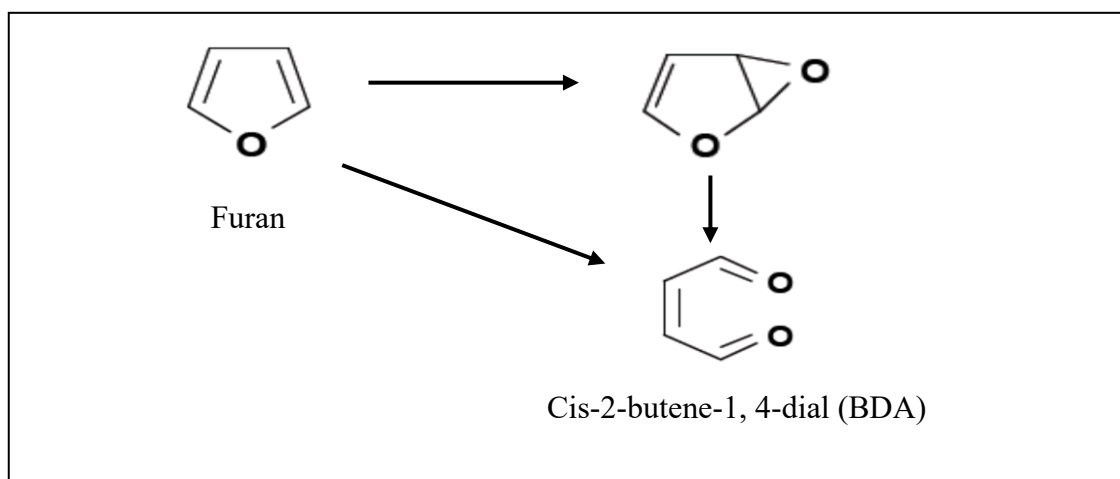
Figure 1.2: Metabolism of furan into cis-2 butene-1, 4-dial (BDA) by CYP. (Chen et al., 1995).

This furan metabolite (BDA) is a highly reactive compound that can react with cellular nucleophiles by addition reactions (Chen et al., 1997). More recently, several in vitro and in vivo studies have been conducted showing that BDA reacts with both thiol and amino groups of glutathione as well as with cysteine and/or lysine residues of proteins (Peterson et al., 2005, 2006; Lu et al., 2009; Lu and Peterson, 2010) and several metabolites of furan have been identified in urine and bile of treated rats (Kellert et al., 2008a; 2008b). BDA also forms adducts with nucleosides in vitro (Byrns et al., 2002) and DNA adducts in vivo (Hamberger et al., 2010; Neuwirth et al., 2012).

The mechanisms behind the carcinogenic effect of furan are yet unclear and both, genotoxicity (based on binding of reactive metabolites to DNA) and non-genotoxicity (based on protein modification with induction of cytotoxicity, chronic inflammation and compensatory cell proliferation) have been reported for furan carcinogenicity. For a review about this subject see Moro (Moro et al., 2012).

Regarding the genotoxicity mechanism of furan, inconsistent data in vitro and in vivo are available. For instance, no mutagenic effects of furan were reported in the Ames test (NTP, 1993) and in the micronucleus-test in mice (Durling et al., 2007) and, moreover, no genotoxic effects have been observed after treatment of L5178Y tk(+/-) mouse lymphoma cells with furan (Kellert et al., 2008a). However, inconsistently, furan was shown to induce chromosomal aberrations in hamster ovary cells (NTP, 1993) and more recently, a statistically significant increase of micro-nucleated cells in the spleen of furan-treated mice has been reported (Leopardi et al., 2010). Furthermore, DNA damage (Neuwirth et al., 2012 and McDaniel et al. 2012) has been observed in rats and in a Comet assay (turkey foetuses) DNA-DNA and DNA-protein crosslinks were found (Jeffrey et al., 2011). In contrast to furan, its metabolite BDA shows a definite genotoxicity effect and reactivity towards DNA. It has been found to be mutagenic in the Ames test (Peterson et al., 2000), to cause DNA damage (Marinari et al., 1984), to form adducts with 2-deoxyribonucleosides in vitro (Byrns et al., 2002) and to show a strong cytotoxic effect (Kellert et al., 2008a). In 2005 the EFSA Scientific Panel on Contaminants in the Food Chain (CONTAM) came to the conclusion that furan-induced carcinogenicity was probably linked to a genotoxic mechanism of action (EFSA, 2005), however, nowadays it seems that furan induced tumours are the result of both genotoxic and chronic cytotoxic mechanisms of action. The relative importance of these mechanisms would depend on the dose level and exposure pattern (Moser et al. 2009; Bakhiya and Appel, 2010).

Epidemiological studies or relevant experience with regard to toxic effects associated with human exposure to furan are still not available. To date, no limits have been established for furan in food as international regulatory information or industry standard. To set food safety priorities arising from the presence of genotoxics and carcinogens in food, risk assessors used the margin of exposure (MoE) approach which compares the dose causing a small but measurable adverse effect in experimental animals with the estimated exposure of humans to the studied carcinogen (EFSA, 2012). The dose corresponding to the lower 95% confidence limit benchmark response causing 10% increase in the background incidence of tumours (BMDL$_{10}$) is used as reference. In

order to calculate MoE, this reference value is divided by the estimation of the dietary exposure. A MoE value lower than 10,000 indicates a high food safety priority although a MoE of 10,000 or greater does not necessarily represents any risk and that further actions would not be needed. The Food and Agriculture Organization of the United Nations and the World Health Organization FAO/WHO expert committee on food additives (JECFA) have set the benchmark dose lower confident limit ($BMDL_{10}$) for furan at 0.96 mg/kg bw/day calculated from the development of hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC) in female mice (JECFA, 2010) while, it was set to 1.28 mg/kg bw/day by the US FDA and EPA (Carthew et al., 2010) using male rats. Using this last value Carthew et al., (2010) reported MoEs between 2133 and 4266 for adults in USA and from 731 to 1600 in Europe while for infants, the estimated MoE was between 1280 and 3200 in USA and from 1000 to 4266 in Europe. As can be note, these MOEs are lower than 10,000 indicating that furan carcinogenicity can be a matter of concern for human health. On the other hand, researchers of Health Canada suggested setting the non-observed adverse effect level (NOAEL), another approach used for many years in dose-response assessment, for hepatotoxicity at 0.03 mg/kg bw/day of furan in rat and at 0.12 mg/kg bw/day of furan in mice (Gill et al., 2010; 2011). As regards the acceptable daily intake (ADI), it is based on the evaluation of available toxicological data and it is established using the NOAEL value found in the most sensitive experiment among test animals. The ADI is usually extrapolated to humans by dividing the NOAEL with a safety factor which is normally set to 1000 for extra security of humans. Thus, from the data reported in the literature the ADI value of furan could be estimated to be in a range from 0.03 to 0.12 µg/kg bw/day.

## 1.3.3. FORMATION OF FURAN IN FOOD

As commented above, furan has been known for a long time as a flavour food constituent (Johnston and Frey, 1938). This compound has been identified as a result of the Maillard reaction in several food model studies containing reducing sugars and amino acids (Walter and Fagerson, 1968; Sugisawa, 1966) and lactose/casein

(Ferretti *et al.,* 1970). In a work published at the end of the seventies, Maga (Maga, 1979) proposed that thermal degradation of different compounds such as glucose, glyceraldehydes, D-erythrose, pentosans, hexoses and polysaccharides is the main mechanism pathway for furan formation in food. However, due to the lack of urgency at that time and also to the consideration of furan as a food constituent, further research on furan formation in foods was limited. Moreover, the study of the potential precursor compounds in the Maga's work was performed at high temperatures (300 °C), which do not represent habitual food processing conditions. However, the classification of furan as a possible carcinogen (IARC, 1995) and the survey about the relatively high levels of furan in foods of US Food and Drug Administration (FDA, 2004a) increased the interest in understanding the mechanism by which this chemical is formed in heat-treated foods.

Nowadays, it has been firmly established that furan in food can be formed from several precursors with multiple formation pathways. Some data indicate that polyunsaturated lipids and ascorbic acid are the major sources of furan which are followed by carotenoids, carbohydrates and certain amino acids. For a review see Crews and Castle (Crews and Castle, 2007) and Moro (Moro et al., 2012). However, there is only limited information about the mechanisms of furan formation under conditions simulating the industrial food processing or domestic cooking (EFSA, 2004). Most published data are based on model studies aiming the identification of potential precursors and elucidating the various formation mechanisms of furan although it must be mentioned that the conditions used in these food models are similar to those of commonly thermal treatment of food.

One of the attempts to identify precursors that yield to furan during heat treatment of food models was performed by the Health Canada (Becalski and Seaman, 2005a). In this case a food model system (10 mg of tested precursor and 0.5 ml of water) was heated at 118 ºC for 30 min and furan concentration in the mix was determined. In Figure 1.3 the results found for several precursors are given. These results confirmed the role of ascorbic acid and its derivatives, the polyunsaturated fatty acids (PUFAs) such as linoleic, linolenic and the corresponding triacylglycerides and

carotenoids in furan formation and suggest lipid oxidation as general mechanism for the formation of furan.
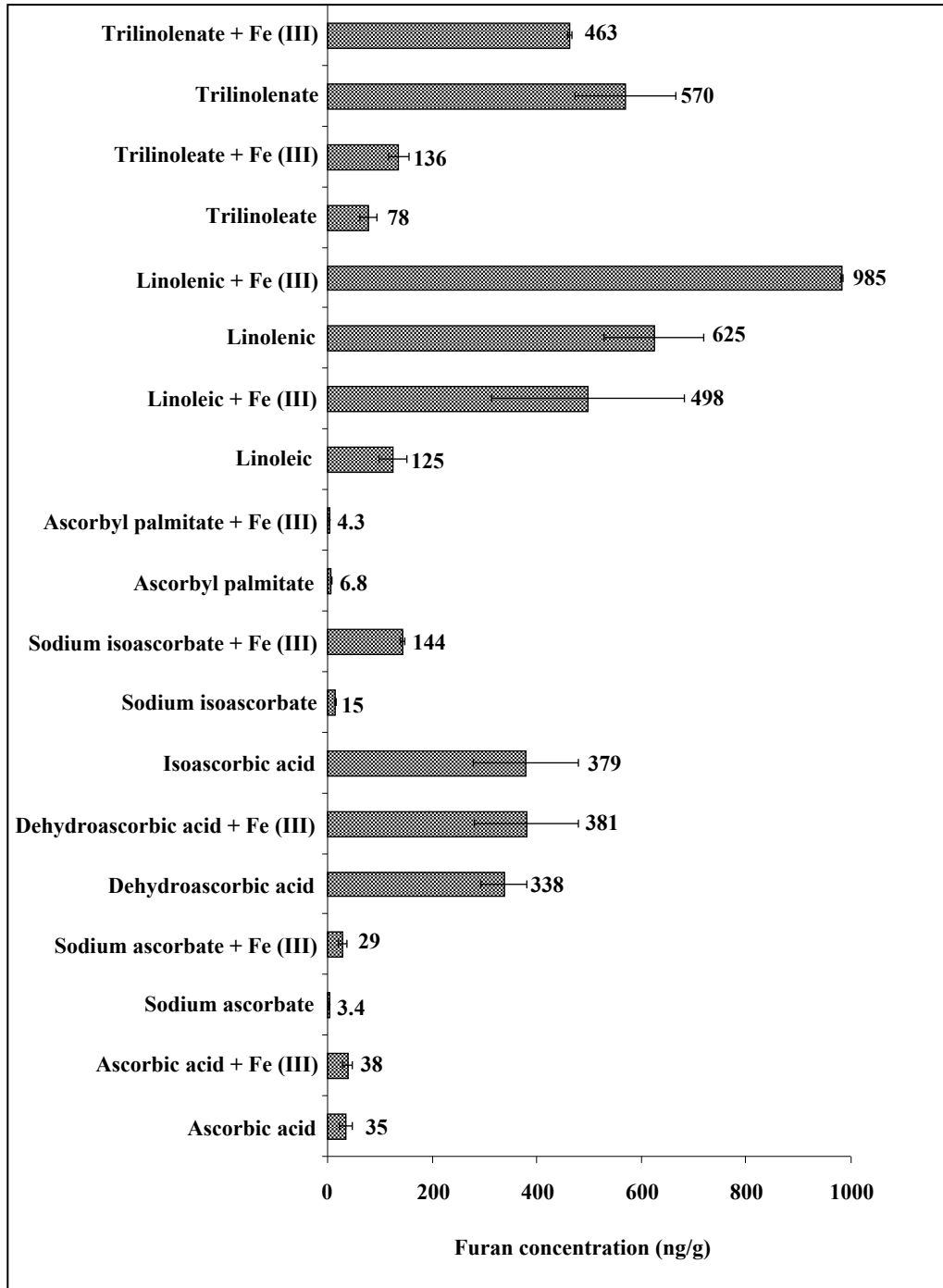


Figure 1.3: Formation of furan from different possible precursors. (Becalski and Seaman, 2005)

Perez et al. (Perez and Yaylayan, 2004) propose the formation of furan by the oxidative degradation of PUFAs producing lipid peroxides in a first step. These peroxides then yield to 4-hydroxy-2-alkenals (e.g. 4-hydroxy-2-butenal) through the hemolytic cleavage of PUFA hydroperoxides which by cyclization and dehydration produce furan (Figure 1.4).



Figure 1.4: Proposed mechanism pathway of furan formation from polyunsaturated Fatty Acids (PUFAs). (Perez and Yaylayan, 2004)

Furan formation from ascorbic acid and its derivatives is complex and its efficiency depends very much on the reaction conditions. For instance, Becalski and Seaman (2005) reported an efficiency for furan formation from dehydroascorbic acid about 10-fold higher than that of ascorbic acid (Figure 1.3). In contrast, Märk et al. (2006) found that ascorbic acid yielded concentration levels 30/35-fold higher than dehydroascorbic acid under the same heat treatment (220 ºC), although furan generated from ascorbic acid was reduced (- 46% to – 95%) when other precursors such as sugars, amino acids or lipids were present. Furthermore, Limacher et al. (2007) found that furan formation from ascorbic acid and its derivatives depends on the pH, and that a pH of 4 favours the furan formation.

In food systems, it is known that ascorbic acid quickly yields dehydroascorbic acid which after hydrolysis gives 2, 3-diketogluconic acid (DKG) in oxidative media while under non-oxidative conditions, ascorbic acid hydrolyses and undergoes beta-elimination and decarboxylation to produce 3-deoxypentosulose (DP). Furan can be

formed at both conditions, from DKG through the generation of aldotetrose in oxidative media and from DP in non-oxidative one (Figure 1.5). Since the 2-deoxyaldotetrose does not require a reduction step and directly produces furan, ascorbic acid is a more efficient source of furan than dehydroascorbic acid under non-oxidative conditions but under oxidative degradation conditions this order is reversed.
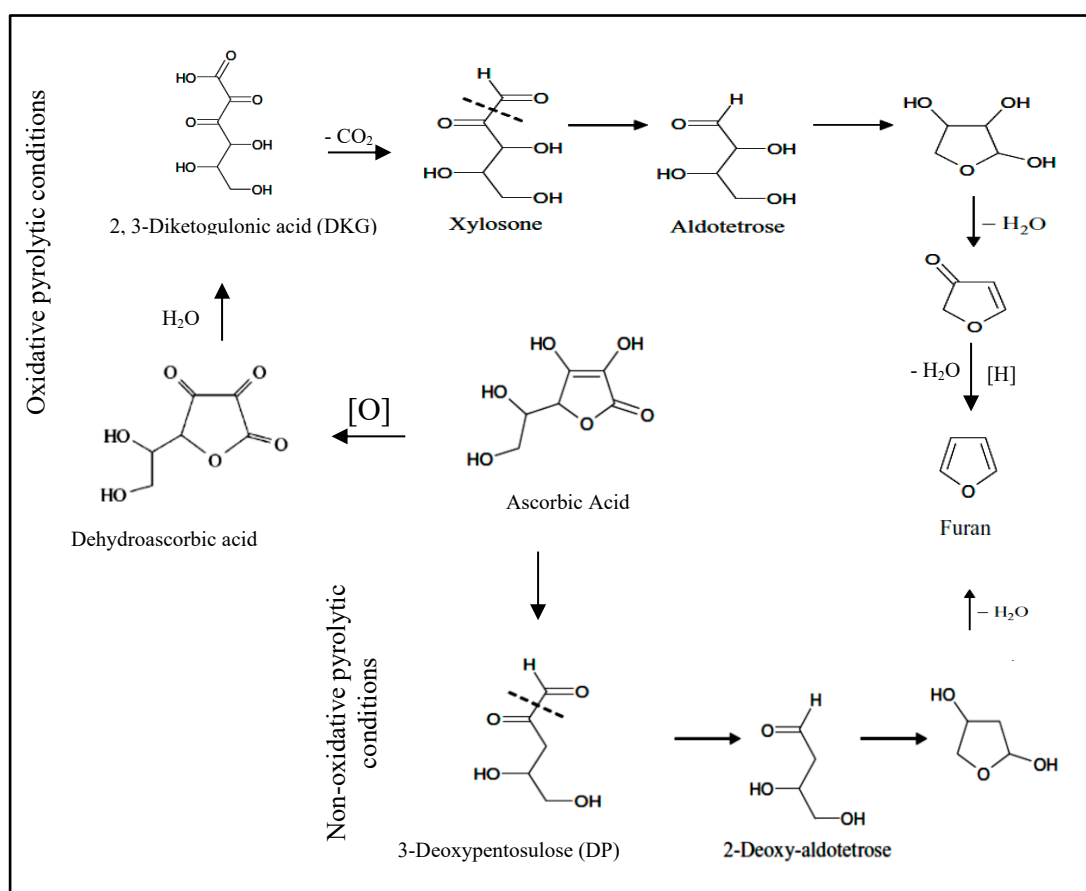


Figure 1.5: Proposed mechanisms of furan formation from ascorbic acid (from Perez and Yaylayan 2004 with some modifications).

Furan can also been formed from amino acids by the aldol condensation of acetaldehyde and glycolaldehyde and the cyclization of the 2-deoxyaldotetrose intermediate (Perez and Yaylayan, 2004). Figure 1.6 shows as an example, the reaction for serine. Ethanolamine obtained via decarboxylation can lose ammonia yielding

acetaldehyde. Alternatively, serine can also undergo dehydration and deamination reactions to form pyruvic acid which in turn by decarboxylation form acetaldehyde. Amino acids such as serine are also capable to form a glycolaldehyde either via Strecker reactions in the presence of sugars or through the interaction between pyruvic acid and ethanolamine to form the corresponding imine which is hydrolysed to form glycolaldehyde (Yaylayan, 2006). Other amino acids such as aspartic acid, alanine and threonine only generate acetaldehyde and, therefore, they need the presence of other components such as reducing sugars, serine or cysteine to furnish the glycolaldehyde.
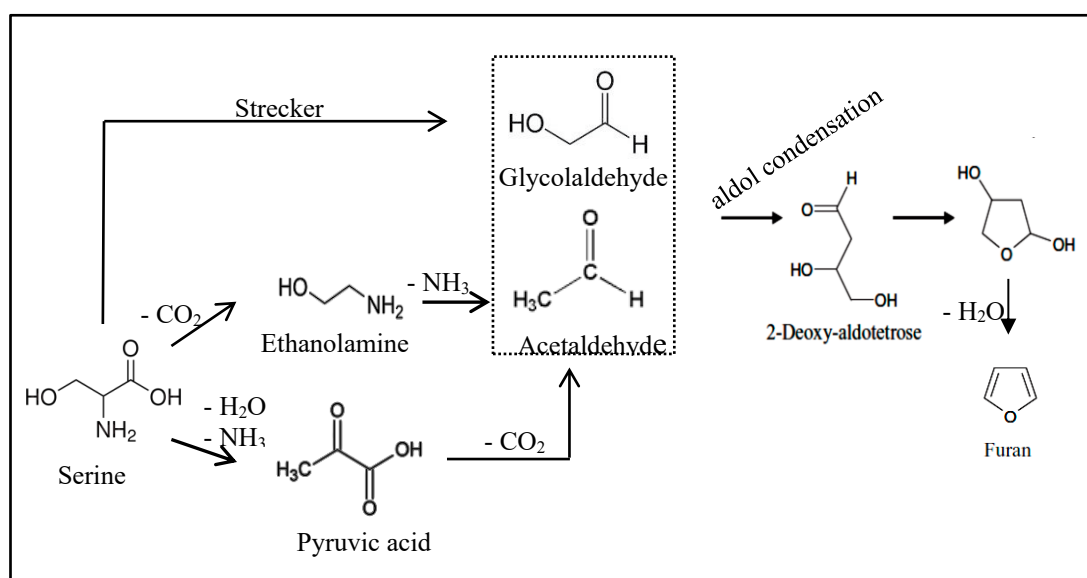


Figure 1.6: Pathway of furan formation from serine. (Yaylayan, 2006)

Furan formation efficiency from carbohydrates was also studied (Perez and Yaylayan, 2004) in mixtures of sugars and serine and it was found that the order of reactivity of the individual studied carbohydrates was: D-erythrose > D-ribose > D-sucrose > D-glucose = D-fructose. In addition, the MS results obtained from the binary mixtures of independently labeled D-glucose/ L-serine indicated that there are four possible pathways for the formation of furan from sugars (Figure 1.7). The major pathway (50%) (A and/or B) is initiated by the formation of 1-deoxyosone in the

presence of amino acids (A) which produce aldotetrose, although this last compound can be directly formed (B) through a retro-aldol cleavage (RA). In both pathways, this intermediate forms furan by cyclization, dehydration and reduction. Furan (10%) can also be generated (pathway C) after dehydration and a retro-aldol cleavage (RA) to form 2-deoxy-3-ketoaldotetrose.
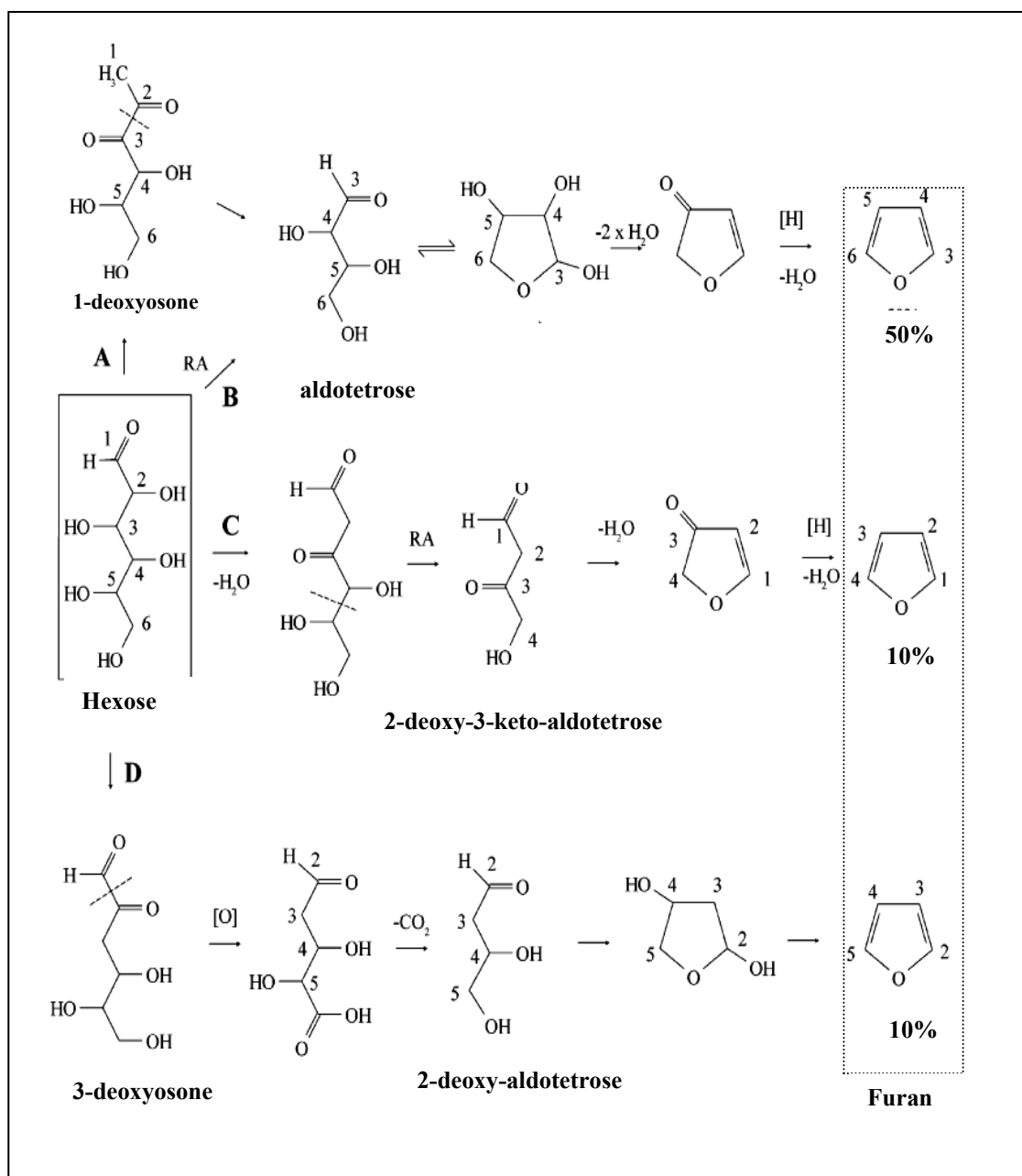


Figure 1.7: Pathway of furan formation from carbohydrates. (Perez and Yaylayan, 2004)

The last pathway (10%) arises from an R-dicarbonyl cleavage of the 3-deoxyosone intermediate followed by its oxidation to form the carboxylic acid moiety. Here it is important to mention that the remaining 30% of furan was formed from serine degradation.

As regards food samples, Limacher et al. (2007) studied the furan formation in food products containing fruits and vegetables spiked with ascorbic acid and heated under conditions that simulate the sterilization process (123°C, 22 min). The products studied were a vegetable puree (pumpkin), a vegetable (carrot) and a fruit (orange) juice. The concentration of furan was determined before and after sterilization and the furan level increased (+124%) for pumpkin vegetable puree and for the carrot juice (+38%), while a decrease was observed for the orange juice (-16%). These results indicated that there is not a direct correlation between the concentration of ascorbic acid in the samples and the furan amounts generated.

In summary, furan can be formed from a wide range of precursors, being ascorbic acid and polyunsaturated fatty acids the most important ones followed by carotenoids, sugars and amino acids, all of them intrinsic food constituents. However, furan formation is quite sensitive to reaction conditions and precursor composition. Furan amounts can be reduced to a great extent by favouring competing reactions and/or interacting in the redox system. Therefore, furan levels are generally much lower in complex systems such as foods, than one would expect from the data obtained from pure precursors. Furan levels found in foods are commented in the introduction of Chapter 3 of this thesis.

## 1.4. VOLATILE *N*-NITROSAMINES IN FOOD: CHEMISTRY, TOXICITY AND FORMATION

### 1.4.1. VOLATILE *N*-NITROSAMINES: CHEMISTRY

N-nitrosamines are nitrogen organic compounds containing a nitroso (-N = O) functional group. The chemical and physical properties of N-nitrosamines are related to the nitroso structure and $R_1$ and $R_2$ groups which may range from a simple methyl group to more complex chemical substituents (NTP, 2011b). There are more than 300 known N-nitrosamines including symmetrical and asymmetrical dialkyl-nitrosamines and cyclic-nitrosamines (Loeppky and Michejda, 1994). Depending on their relative vapour pressure, N-nitrosamines are categorized as steam-volatile, non-volatile (high molecular weight) and volatile (low molecular weight) (Rath and Reyes, 2008). The most studied volatile *N*-nitrosamines (VNAs) are shown in Figure 1.8.

Volatile N-nitrosamines (VNAs) are relative polar compounds which their solubility in water rapidly decreases as the molecular size increase (Pourazrang et al., 2002). The partition coefficients of VNAs in octanol/water are relatively low and therefore these compounds are difficult to be extracted with organic solvents. In addition, the Henry constants of VNAs are small, which means that VNAs cannot be easily extracted from aqueous solutions by aeration (Nawrocki and Andrzejewski, 2011). The most important physical and chemical properties of these VNAs are listed in Table 1.2.

VNAs are stable in dark as well as in neutral and strongly alkaline solutions. Even though, when exposed to ultra-violet light, they decompose to aldehydes, nitrogen and nitrous oxide and this decomposition is faster in strong acidic conditions (NTP, 2011). In general, VNAs are principally produced as research chemicals, they are intermediates in the synthesis of other organic compounds and used as copolymer softeners, lubricants additives and stabilizers in plastics (Hotchkiss, 1987).
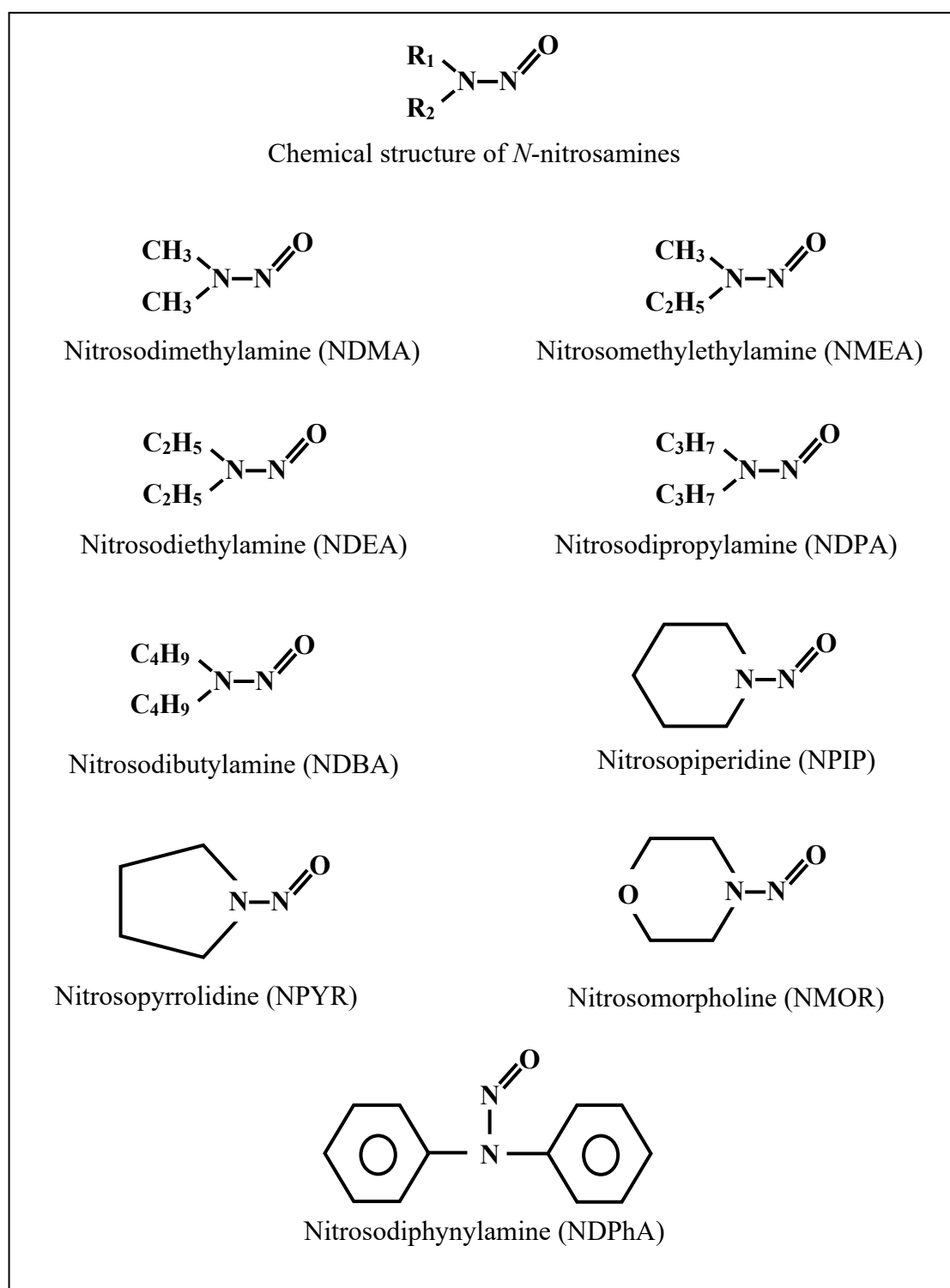
Figure 1.8: Chemical structure of most studied volatile N-nitrosamines (VNAs).

Table 1.2: Physical and chemical properties of volatile N-nitrosamines.

| Compound | NDMA | NMEA | NDEA | NDPA | NDBA | NPIP | NPYR | NMOR | NDPhA |
|---|---|---|---|---|---|---|---|---|---|
| CAS registry number | 62-75-9 | 10595-95-6 | 55-18-5 | 621-64-7 | 924-16-3 | 100-75-4 | 930-55-2 | 59-89-2 | 86-30-6 |
| Molecular weight (g/mol) | 74.1 | 88.1 | 102.1 | 130.2 | 158.2 | 114.2 | 100.1 | 116.1 | 198.2 |
| Exact mass (amu) | 74.04801 | 88.0637 | 102.0793 | 130.1106 | 158.1419 | 114.0793 | 100.0637 | 116.0586 | 198.0793 |
| Water solubility (g/l) at 24 °C | 1000 | 300 | 106 | 13 | 1.27 | 76.5 | 1000 | 1000 | 0.035 |
| Density (g/ml) at 18 °C | 1.01 | 0.94 | 0.95 | 0.93 | 0.91 | 1.06 | 1.24 | 0.84 | 1.23 |
| Vapor pressure (mm Hg) at 20 °C | 2.7 | 4.1 | 0.86 | 0.086 | 0.05 | 0.092 | 0.06 | 0.036 | 0.000057 |
| Log $K_{ow}$ | - 0.57 | - 0.15 | 0.48 | 1.36 | 2.63 | 0.36 | - 0.19 | - 0.44 | 3.13 |
| Boiling point (°C) | 151 to 153 | 163 | 175 to 177 | 206 | 116 | 219 | 214 | 224 | 268 |

The exposure to VNAs can be considered a health problem because most of these compounds are suspected to be human carcinogens (Magee, 1996) although an association between VNAs exposure and human cancer has not yet been firmly established. VNAs are potent animal toxicants and produce carcinogenic effects in laboratory animals even at low concentration doses (Lijinsky, 1999) and have been included in the lists of toxic compounds by international organizations. For instance, the International Agency for Research on Cancer (IARC) has classified NDMA and NDEA as Group 2A substances (probably carcinogenic to humans), while NMEA, NPYR, NMOR, NPIP, NDPA and NDBA are classified as possible carcinogens to humans (Group 2B) (IARC, 1978). In addition, the European Union categorizes NDMA and NDEA as 1B (presumed to have carcinogenic potential for humans; largely based on animal evidence) compounds (EU, 2007). Moreover, in the US, the Environmental Protection Agency classifies both NDMA and NDEA as probable human carcinogens (category B2) under its carcinogen assessment guidelines (EPA, 1980).

The main route of VNAs formation is nitrosation of amines or its derivatives by nitrosating agents such as nitrogen oxides (NOx) or nitrosyl chloride (NOCl) (Walker, 1990). Due to the wide occurrence of these precursors, VNAs have been found in the environment (air, wastewater and soil) as well as in drinking water and food (US EPA, 1977). In the environment, VNAs are formed due to the presence of nitrogen containing compounds originated from several industrial processes such as manufacture of amines, herbicides, pesticides, pharmaceuticals and rubber. Moreover, fertilization with mineral nitrogen helps the formation of N-nitrosamines in soils (Marano et al., 1982). Regarding drinking water, VNAs are formed as disinfection by products (DBPs) as a result of water treatment processes, for a review see Krasner et al., (Krasner et al., 2013). A source of particular interest is the formation of VNAs in a wide variety of food such as meat and fish products, cheese, milk as well as in malt beverages especially beer (Crews, 2010). For long time it has been known that the formation of VNAs in food is related to the use of nitrite salts in the processing or to nitrogen oxides that come to contact with food. Furthermore, it has been demonstrated that VNAs formation depends

on the temperature achieved in the preparation of food after the addition of nitrite salts (Drabik-Markiewicz, 2009; 2010; 2011). High temperature processing and protein degradation to secondary and tertiary amines increase the rate of VNAs formation. Although VNAs occur at low concentration level in food ($\mu g\ g^{-1}$), their carcinogenicity when they are administrated at low concentration for long periods makes their presence in food a human health problem.

In this introduction, the carcinogenicity mechanisms and the formation pathways of VNAs in food are summarized. In addition, the most important modifications implemented in food processing procedures in order to reduce the occurrence and concentration level of VNAs are also briefly described.

## 1.4.2. VOLATILE N-NITROSAMINES: TOXICITY AND CARCINOGENICITY

Nowadays, it is more than seven decades since the toxic effect of N-nitrosamines was first recognized by Freund (1937) who reported two cases of accidental poisoning from inhalation of NDMA. Later, Magee and Barnes (1956; 1959) demonstrated that the chronic dietary administration of NDMA produces liver and kidney tumours in rats. To date, no animal species tested with NAs have been found to be resistant to their carcinogenic actions. Numerous carcinogenicity studies have shown that NAs are a group of carcinogens which induce tumour formation in a high number of organs and in many animal species (Mirvish, 1995). In addition, N-nitrosamines induce tumours independently of the route of administration which is probably explained by the combination of their high stability and solubility in biological conditions (Preussmann and Stewart, 1984).

The carcinogenicity of NAs is explained by their ability to form strong electrophilic alkylating agents, through enzymatic metabolism pathways, which can react with the nucleophilic sites of DNA, RNA and proteins (Lijinsky et al., 1968; Yang et al., 1990). The carcinogenic and toxicity potencies of alkyl-nitrosamines are inversely proportional to the size of the substituents and to their number. For example, NDMA

and NDEA are more toxic than nitrosodiarylamines (Lijinsky, 1992). Among all known N-nitrosamines, the volatile N-nitrosamines, especially those found in foods, show higher toxic and carcinogenic activity than others. Moreover, volatile NAs are more effective as toxic and carcinogenic agents to experimental animals when administered at low repeated doses than in a high single dose. This is the exact situation of human low-dose exposure to volatile NAs from food indicating that their toxicity can be a matter of concern for human health (Järgested and Skog, 2005).

Early experiments by Magee and his co-workers (Magee and Faber, 1962) employing $^{14}$C-labelled NDMA showed that proteins and nucleic acids of rat liver contained products of NDMA metabolism. Further, in-vivo experiments led to the identification of $^{14}$C-labelled 7-methylguanine as an end product of NDMA, in DNA and RNA of liver of treated rats (Gombar et al., 1983) indicating that VNAs undergo complex metabolism in animals and several intermediate products are formed. These intermediates are biologically active and could induce carcinogenic effects in different organs which can explain the variety of organ-specific effects (Lijinsky, 1992). Also, biochemical studies with human liver in vitro have produced evidence that N-nitrosamines are metabolized and interact with nucleic acids (Gruengerich and Shimadal, 1991) suggesting that human metabolism of N-nitrosamines may also produce carcinogens similar to those found in animal studies.

Regarding the mechanism of carcinogenicity, N-nitrosamines are absorbed from the gastrointestinal tract, distributed by the bloodstream and rapidly metabolized in the liver. The first essential step of metabolic activation in the mechanism of N-nitrosamines carcinogenicity (Figure 1.9) is the oxidation by the mixed function oxidase system inducing hydroxylation at the α-carbon atom (Preussmann and Stewart, 1984; Shibamoto and Bjeldanes, 2009). The metabolic reaction is catalyzed by members of the enzymatic family cytochrome P450 which are present in liver, kidney and in other organs. The resultant α-hydroxyalkylnitrosamine is unstable and undergoes hydrolysis and fragmentation to give the corresponding aldehyde and an unstable monoalkylnitrosamine compound which gives a diazoalkane that decomposes to an alkylcarbonium ion. This ion is the electrophilic alkylating agent considered as the

ultimate carcinogen (Shuker and Bartsch, 1994). For long chain alkylnitrosamines, oxidation at the terminal carbon atom or β-oxidation can occur giving as a result stable non-α-hydroxylated N-nitrosamines that might be directly excreted (Teiber et al., 2001). However, at the same time, consecutive enzymatic oxidation reactions could occur on non α-carbon atom which followed by enzymatic α-oxidation yield to diazomethane ($H_2C = N^+ = N^-$) the final electrophilic alkylating agent. The second and third steps of NAs carcinogenicity mechanism are the alkylation of biological macromolecules and their replication. Alkylation of DNA by diazonium and carbonium ions mainly takes place at the ring-nitrogen positions of the base being N-7 alkylguanine the major DNA bases adduct of N-nitrosamines (Archer et al., 1991; Georgiadis et al., 1991). These alkylated bases in DNA are responsible for initiating cancer and act by miss-pairing during DNA replication although DNA damage could be repaired by error-correcting mechanisms in the cell.

The clear evidences of carcinogenicity from animal experiments provide a reasonable basis to conclude that volatile N-nitrosamines have potential level of carcinogenic risk to humans even though they occur at low level in the food. Nevertheless, there is a lack of directives for the maximum allowable amounts of NAs in food although in several countries regulations that limit the presence of VNAs in foods have been established. For instance, in Germany, Italy and Switzerland the maximum limit of NDMA in beer is 0.5 µg kg$^{-1}$ while in United State it is ten times higher 5 µg kg$^{-1}$ (Sung, 2004). In Canada, it has been set a maximum level of 10 µg kg$^{-1}$ for each compound (NDMA, NDEA, NDBA, NPIP and NMOR) and 15 µg kg$^{-1}$ for NPYR in meat products while in United State it is 10 µg kg$^{-1}$ for all NAs (Rath and Reyes, 2008). Moreover, in Russia the allowed content of NDMA and NDEA in different foodstuffs has been established between 2 and 15 µg kg$^{-1}$ (Rath and Reyes, 2008), while in Estonia the maximum permitted levels of all volatile NAs in meat and fish food products is 3 µg kg$^{-1}$ (Yurchenko and Mölder, 2005; 2006; 2007).
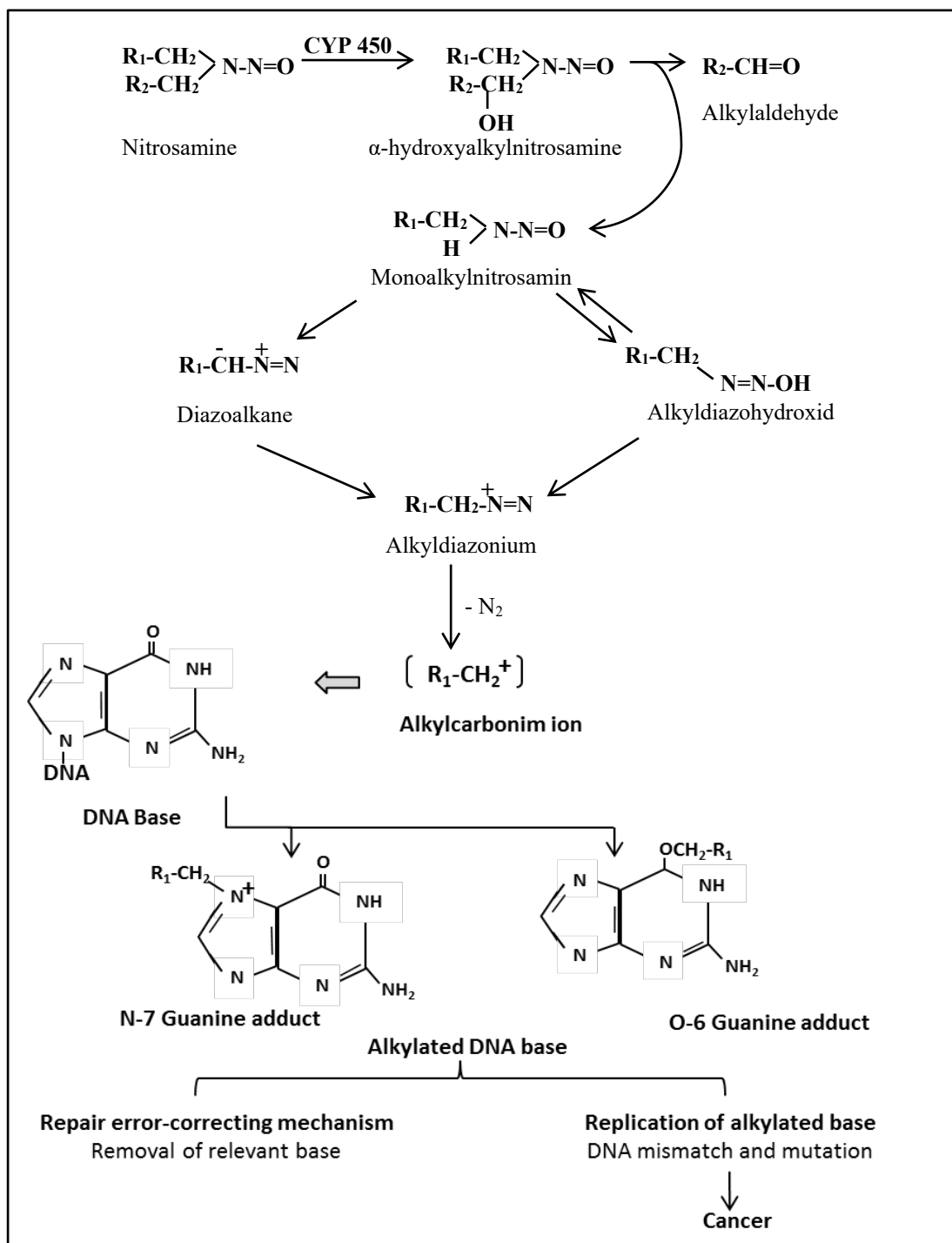
Figure 1.9: N-nitrosamines carcinogenicity mechanism (according to Shibamoto and Bjeldanes 2009).

## 1.4.3. FORMATION OF VOLATILE N-NITROSAMINES IN FOOD

Nowadays, it is well known that contamination of food with VNAs is mainly caused by their formation through preparation processes. N-nitrosamine compounds are formed when nitrosatable compounds (amines and their precursors) and nitrosating agents such as nitrite or its derivatives such as dinitrogen trioxide ($N_2O_3$) and dinitrogen tetroxide ($N_2O_4$) come in contact (Scanlan, 1983). Foods preserved with addition of nitrates or nitrites, smoked, dried by combustion gas, pickled or salt-preserved and also stored or grown in high humid conditions have been associated with VNAs (Stadler and Lineback, 2009). Nitrite salts are used in food processes mainly to prevent the growth of the bacterial spores of *Clostridium botulinum* and its transformation into toxins thus eliminating the possibility of food poisoning. The nitrosatable organic compounds in food such as amines and also amino acids, proteins and biogenic amines are transformed into VNAs during thermal processing (Tricker and Preussmann, 1991). The amount of N-nitrosamines generated during thermal processing of food depends on temperature, duration of the treatment, distance from the source of heating, fat content, and the type of combustible used. Moreover, several thermally catalysed reactions such as the generation of nitrite from nitrate or nitrite esters which are thermally formed from the reaction between nitrogen oxides and unsaturated fatty acids or cholesterol enhanced the formation of VNAs (Ross et al., 1987). Smoking and drying food are important sources of nitrogen oxides which play an important role in VNAs formation. For instance, the formation of VNAs in beer has been related to the thermal treatment of moist germinated barley. When the barley is brought into contact with a stream of heated gas for drying, its components can react with the nitrogen oxides formed in the flame. NDMA concentrations between 15 and 80 ng $L^{-1}$ in pale malt and from 80 to 320 ng $L^{-1}$ in dark malt have been reported when green malt is dried in direct-fired kilns (Yurchenko and Mölder, 2005).

The first and rate-limiting step of N-nitrosamines formation in food is an electrophilic attack by the nitrosating agent to the free electron pair of the amine nitrogen; so, the amine is preferentially attacked in its unprotonated form. Amines with

a low $pK_a$ have high nitrosation rates and the type of amine is important in determining the formation pathway of NAs in food (Tricker, 1997). Secondary amines yield stable N-nitrosamines (Figure 1.10-a) while diazonium salts, unstable for aliphatic amines and stable for aromatic ones, are obtained from primary amines. However, it has been demonstrated that N-nitrosamines can also be formed from primary amines through the formation of secondary amines during nitrosation (Figure 1.10-b) (Scanlan, 1983). The generation of secondary amines by dealkylation is the first step in the formation of N-nitrosamines from tertiary amines (Figure 1.10-c) as happens for instance, in NDMA generation from trimethylamine or trimethylamine precursors found in meat and fish foodstuffs.

In fact, the formation of VNAs in food is a complex process and several factors such as the presence of nitrosating catalysts and inhibitors and cooking and preparation methods can contribute to their formation. For instance, the presence of certain carbonyl compounds can catalyse the nitrosation of amines present in foods at neutral or alkaline pH as has been shown for formaldehyde (Figure 1.11-a), an effective catalyst for the nitrosation of dialkylamines (Keefer et al., 1973). The formation of the iminium ion $(R_2N=CH_2)^+$ from the formaldehyde and the amine is the initial step of VNAs formation (Casado, et al., 1984). Moreover, nucleophilic anions such as halides also catalyse amine nitrosation and catalysis by chloride is of particular interest due to its occurrence in most foods. In addition, polyphenolic compounds present in many foods may play an important role in VNAs formation and can act as inhibitors or catalysts of nitrosation depending on their structure. It has been reported that phenol is nitrosated 1000 times faster than dialkylamines and as a consequence, it should be an inhibitor, but, Davies et al. (1978) showed that the resulting nitrosophenol can catalyze the nitrosation and in systems where both dialkylamines and phenol are present the overall result is an increase in the rate of NAs formation (Figure 1-11.b).

A wide variety of amines from methylamine to pyrrolidine or piperidine is found in foods and an important number of amino acids such as tyramine, putrescine, proline

or sarcosine are potential VNAs precursors in food since amines are produced by their thermal decarboxylation (Shephard et al., 1987).
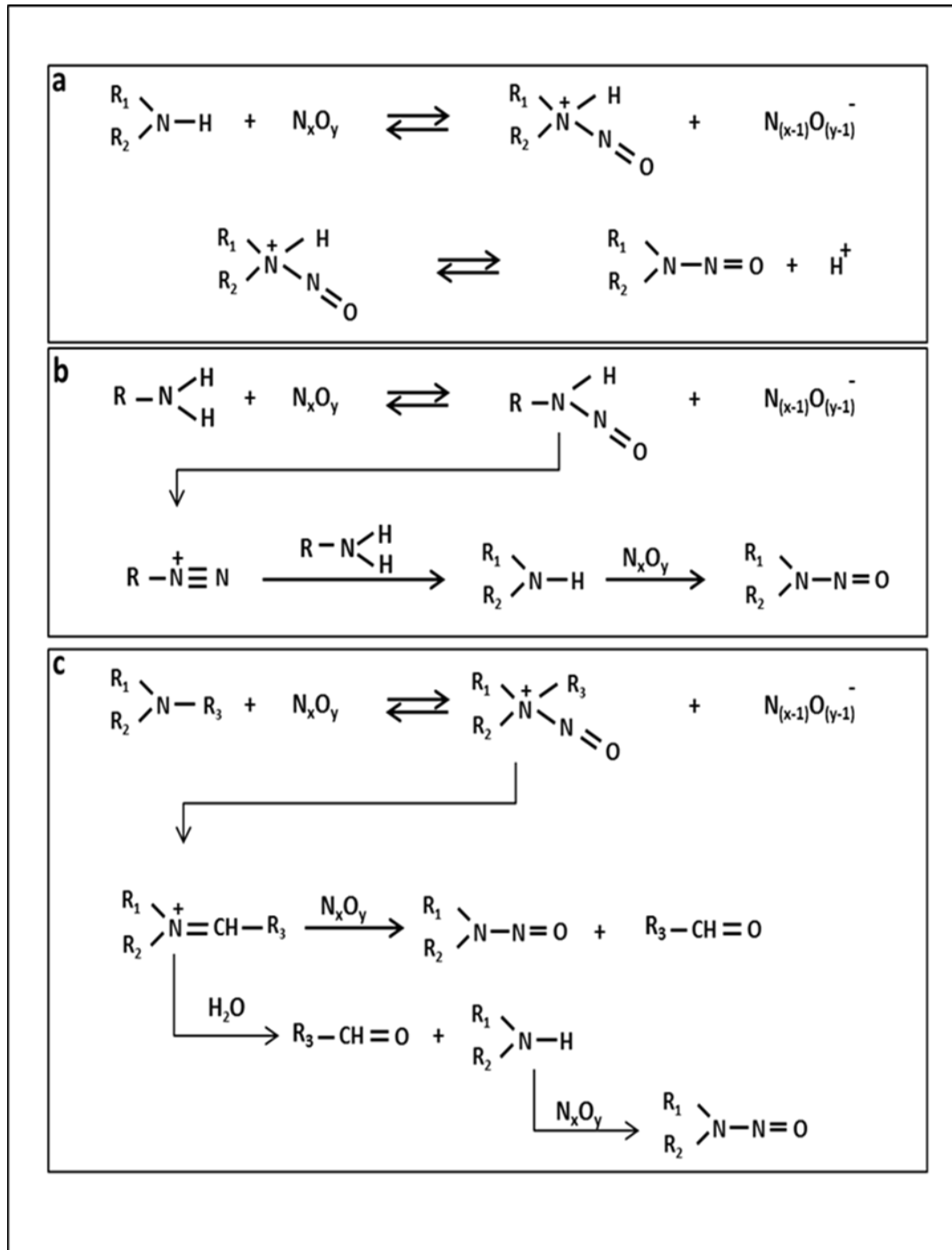


Figure 1.10: Formation pathways of N-nitrosamines from different amines.
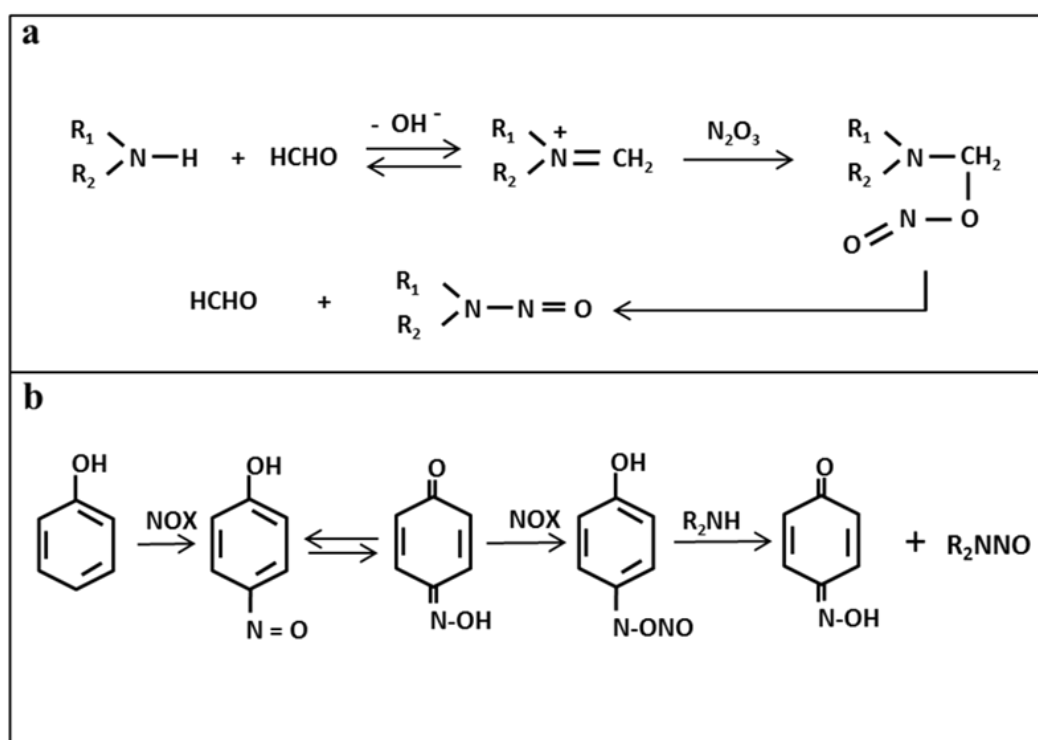
Figure 1.11: Catalysed nitrosation of secondary amines by (a) formaldehyde, (b) phenols.

Sarcosine found in malt, meat and fish food products which is generated form creatine during thermal processing of food is the most likely precursor of NDMA in meat products, especially bacon (Gray et al. 1978). Sarcosine can undergo a nitrosating reaction forming nitrososarcosine which later thermally decarboxylates to give NDMA (Figure 1.12). In addition, sarcosine can liberate dimethylamine which is later transformed to NDMA. Lecithin, a phospholipid present in meat and fish, is an important precursor not only for NDMA but also for NDEA and NDPA (Pensabene et al., 1975) and hordenine which occurs in germinated barley is the main precursor of NDMA in beer (Figure 1.13). However, heat is required to decompose the nitrosated hordenine intermediates (Wainright, 1986). As a matter of fact, experience shows that additional heating during ale malt kilning increases NDMA formation in beer in agreement with the increase of the conversion rate of hordeine to NDMA.
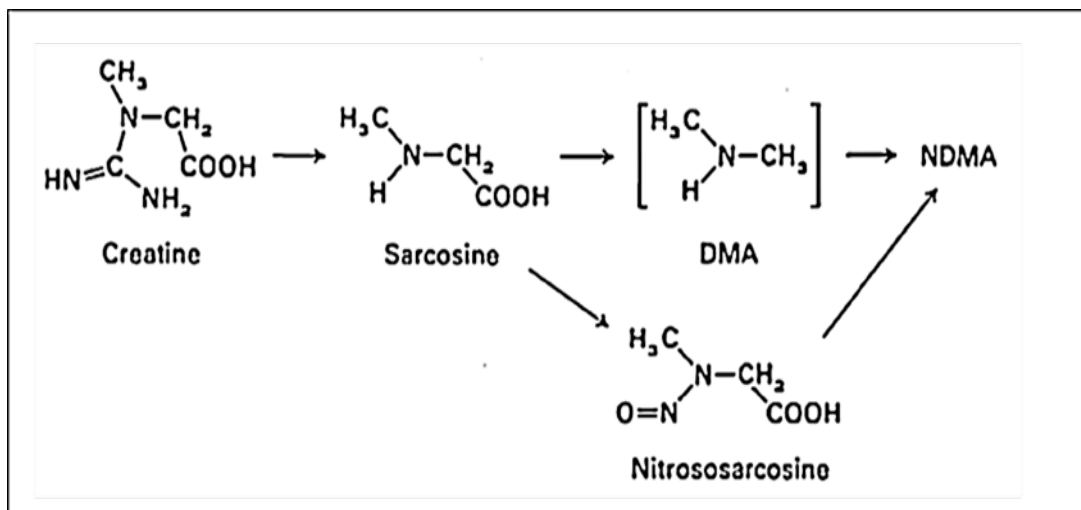
Figure 1.12: NDMA formation pathway from creatine and sarcosine.
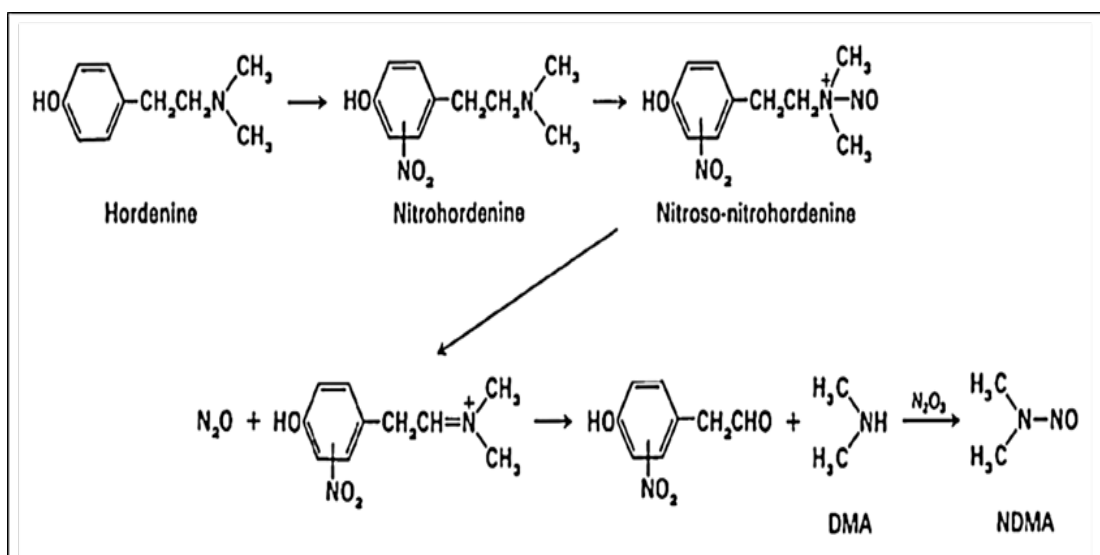


Figure 1.13: Mechanism of NDMA formation from hordenine. (Wainright, 1986)

Other VNAs such as NPIP and NPYR are also found in beer but less frequently than NDMA and commonly at smaller concentration. These VNAs are more common in processed meat and fish products. In fact, most VNAs that are consistently detected in meat and fish products are NDMA, NPYR and NPIP while other VNAs such as NDEA, NMOR and NDBA are only occasionally detected and at lower concentration levels.

The concentration of volatile N-nitrosamines in these foodstuffs grows during processing at elevated temperatures and time. Therefore, heat treatment of cured meat (like sterilization, cooking, and baking) can be responsible for the formation of N-nitrosamines.

Cyclic N-nitrosamines such as NPIP and NPYR are formed from compounds with two amine groups separated by four or five carbon atoms such as lysine and cadaverine and NPYR is also formed from putrescine and proline. The formation mechanism of NPYR has been intensively studied in cooked meat and especially in fried bacon as it is one of the most abundant VNAs in such a food. NPYR is generated from putrescine, obtained by decarboxylation of ornithine that at high temperature gives pyrrolidine which can be transformed into NPYR by nitrosation (Figure 1.14a). However, the greatest sources of pyrrolidine ring compounds in food are the two imine amino acids, proline and hydroxyproline which are abundant in the protein collagen (Drabik-Markiewicz, 2009, 2010). NPYR can be formed by the reaction between nitrite and proline either present as the free amino acid or produced by the hydrolysis of a protein. Two mechanisms for NPYR formation have been proposed depending on the order of both nitrosation and decarboxylation reactions (Figure 1.14-b).

The better understanding of VNAs formation mechanisms lead in the 1970s to the introduction of food processing modifications which have resulted in an important reduction of VNAs concentration in food. Before these modifications the concentration of VNAs in meat and fish products were up to 100 ng g$^{-1}$ while the concentration of NDMA in beer ranged from 5 to 20 ng g$^{-1}$ (Hecht, 2007) although higher concentrations ( 78 ng g$^{-1}$) were reported in some cases (Lachenmeier and Fügel, 2007). As an example of the important reduction of NDMA concentration after the introduction of new processing practices in food industries, in Table 1.3 the concentration of NMDA in several foods in 1997 and in 2000-2006 are included.

The use of indirect fire kilns in beer factories and dried dairy products industries preventing the incorporation of the combustion nitrosating agents into the drying air has led to a high reduction of VNAs concentration.
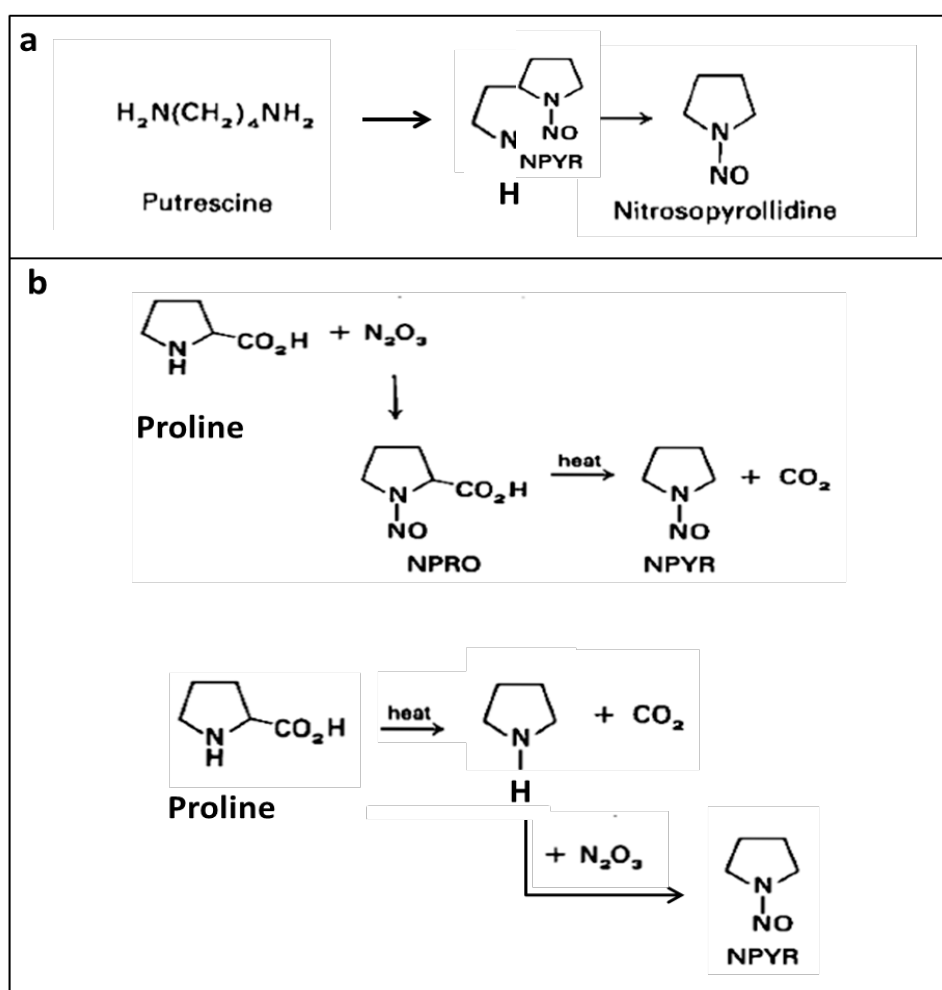
Figure 1.14 Formation pathways of NPYR from (a) putrescine and (b) proline

In addition, the incorporation of sulphur dioxide ($SO_2$) into the combustion gases produced an important decrease of NDMA in beer since $SO_2$ prevents the formation of intermediate products during the early stage of the killing process which are responsible for NDMA formation in later stages. For instance, the concentration of NDMA in beer was 100 times lower in 2007 than 30 years before (Lachemeier and Fügel, 2007). In fact, in a study performed in 2007, Baxter et al. (Baxter et al., 2007) found that 79% of the analysed beer samples (138 samples from 42 countries) did not contain NDMA (LOQ = 0.1 µg $L^{-1}$) and only three samples exceeded 0.5µg $L^{-1}$.

Nowadays, the small amount of NDMA in beer is generally attributed to the presence of nitrogen oxides in air and of VNAs in the used water.

Table 1.3: NDMA concentration in different food categories and years.

| Food | Year | NDMA concentration range (ng g$^{-1}$) | References |
|---|---|---|---|
| Fish products | 1971 | n.d. - 26 | Fazio et al., 1971 |
| | 2001 – 2005 | 0.54 – 1.99 | Yurchenko, U. Mölder (2007) |
| Meat products | 1974 | n.d. - 35 | Sen et al., 1974 |
| | 2004 | n.d. – 7.3 | Byun et al., 2004 |
| Bacon | 1973 | n.d. - 30 | Sen et al., 1973 |
| | 1993 – 1994 | n.d. - 3 | Glória et al., 1997 |
| Beer | 1978 – 1979 | n.d. - 78 | Lachenmeier and Fügel, 2007 |
| | 2000 - 2006 | n.d. – 0.66 | Lachenmeier and Fügel, 2007 |

In Spain, little information on NDMA in beer is available. Even though, Ruiz-Lopez et al., 1988 reported an average level of NDMA in Spanish beer of 0.27 ng g$^{-1}$, concentrations up to 0.55 ng g$^{-1}$ (Izquierdo et al., 1996) and 1.05 ng g$^{-1}$ (Càrdenes et al., 2002) have been found. More recently, Jurado-Sánchez et al., 2007 found NDMA and NPIP in beer at concentrations ranging from 0.14 to 0.56 ng g$^{-1}$ and from 0.15 to 0.39 ng g$^{-1}$, respectively.

Total VNAs in meat products has also been reduced to few ng g$^{-1}$. For instance maximum levels of 2.5 ng g$^{-1}$ in Germany (Tricker and Preussmann , 1991), 20.2 ng g$^{-1}$ in USA (Haorah et al., 2001), 1.1 ng g$^{-1}$ in Italy (Sannino and Bolzoni, 2013), 9.3 ng g$^{-1}$ in France (Biaudet et al., 1994), 23.85 ng g$^{-1}$ in Turkey (Ozel et al., 2010; Kocak et al., 2012), 7.7 ng g$^{-1}$ in Estonia (Yurchenko and Molder, 2006; 2007) and 12 ng g$^{-1}$ in Spain (Campillo et al., 2011) have been published. This reduction can also be observed in Table 1.3 for NDMA in meat and fish products where values 3 to 10 times lower in 2005 than in 1974 can be observed. However, total elimination of VNAs in food, especially in meat products, is not possible because of the naturally occurring precursors in the foods. For instance, Drabik-Markiewicz et al. (et al., 2011) indicated that the

formation of N-nitrosamines in meat and meat products containing biogenic amines such as spermidine causes a significant increase in the NDMA yields in meat products and its concentration increases significantly at higher processing temperatures. In addition, for meat food products, until now there are no real alternatives to nitrite and especially the antioxidative and curing flavour forming effects of nitrite cannot be substituted by other additives (Lücke, 2003). For this reason the use and amount of nitrite and nitrate in meat products is regulated. For instance, the European Union (Directive 2006/52/EC) has established a maximum amount for potassium and sodium nitrite (E249 and E250) of 150 mg/kg for meat products in general and of 100 mg/kg for heat treated meat products. For potassium (E251) and sodium nitrate (E252) a general maximum ingoing amount of 150 mg/kg has been established for non-heat treated meat products while heat treated meat products are no longer permitted to contain nitrates. In USA, the Code of Federal Regulations (USA, 2005) limits the amount of sodium nitrite to not more than 200 ppm in the finished meat product, and the amount of sodium nitrate at not more than 500 ppm. The key point of these regulations is to find a balance between ensuring the microbiological safety of meat products by the use of nitrate and nitrite as preservatives keeping as low as possible the level of N-nitrosamines in the final product (EFSA, 2010). Another important practice in meat processing is the use of VNAs inhibitors which favours the reduction of nitrite to inactive products and thus the inhibition of VNAs formation. Ascorbic acid, sodium ascorbate and erythorbate have been used for suppressing the N-nitrosation reaction in food. Consequently, in the United States the addition of at least 550 ppm of ascorbic acid in manufactured meats is permitted. Additionally, manufactures may include alpha-tocopherol (vitamin E) to further inhibit VNAs production.

This effort to reduce the VNAs level in food resulted in a reduction of human exposure to these carcinogenic compounds. For instance, the daily NDMA exposure from beer was 0.74 µg/day in 1979/1980 in Germany (Spiegelhalder et al., 1980) and it was reduced to 0.1 µg/day in 1987 (Frommberger, 1989). Similar low exposures have been also reported for other countries. As examples, the average daily intake of NDMA from food was 0.12 µg/day per person in Sweden (Osterdahl, 1988), 0.10 µg in the

Netherlands (Ellen et al., 1990), 0.5 µg in Japan (Yamamoto et al., 1984), 0.22 µg NDMA in Germany (Tricker, 1997) and in France, it was reduced to 0.19 µg per person and day, from 1987 to 1992 (Biaudet et al., 1994). In general, the decrease in concentration level of VNAs in foodstuffs and as a consequence the daily exposure to these compounds indicates the considerable success of the modification of food processing.

# 2. ANALYSIS OF PROCESS-INDUCED FOOD CARCINOGENS

## 2.1. INTRODUCTION

To guarantee food safety, identification and quantification of food contaminants as well as, the evaluation of their occurrence and risk assessment are needed. An indispensable prerequisite to fulfil these objectives is to have available reliable analytical methodologies. Since the acrylamide incident in 2002, the development of rapid, automated, selective and sensitive analytical methods to determinate process-induced food carcinogens has become a subject of interest in the research in food analysis.

As has been commented in the first chapter of this thesis, in 2004, furan in food appeared as a significant issue for several national and international food safety agencies such as US FDA and EFSA, due to the discovery of its occurrence at ppb concentration levels in highly consumed foodstuffs (US FDA, 2004a). Similarly, volatile N-nitrosamines in food was and still occupy, an area of analytical chemistry and food safety researches in order to propose simple and sensitive methods for food control, especially after the recent attempts to reduce or eliminate their presence in food, and also to implement additional legislation (Crews et al., 2010). The analysis of furan and volatile N-nitrosamines is challenging due to their physicochemical properties, relatively low concentration levels and the complexity of the food matrices and therefore, there is interest to explore new analytical methodologies able to enhance sensitivity, selectivity, adaptability and also to meet the criteria of European Commission (2002/657/EC) regarding the validation and establishing the performance of analytical methods.

This chapter is devoted to the development and validation of rapid and sensitive analytical methods to detect and quantify furan and VNAs in foodstuffs at sub-parts-per-billion levels. It includes a review of the literature about analytical methods proposed for the analysis of furan and volatile N-nitrosamines in food, the results of our studies (**Papers I, II, III**) and the discussion of the obtained results.

## 2.2. METHODS FOR THE ANALYSIS OF FURAN AND VOLATILE N-NITROSAMINES IN FOOD

### 2.2.1. FURAN

Furan is currently found in food that undergo to thermal treatment including liquid, semi-solid and solid foods such as juices, baby food and ground coffee. Due to its high volatility and low molecular weight the analysis of furan in food is not easy. The low boiling point of furan (31.36 ºC with a log $P_{ow}$ of 1.34) makes headspace techniques such as direct headspace (HS) and headspace solid phase microextraction (HS-SPME) coupled to gas chromatography the main methods of choice.

Direct static headspace is a solvent-less sample preparation technique in which a sample is heated in a sealed vial (with a headspace volume) and the analyte diffuses into the gas phase (headspace) until, usually, the system reaches a state of equilibrium. The distribution of the analytes between phases is determined by their partition coefficients between the sample phase and the gas phase (headspace) at the equilibrium conditions. For the analysis, an aliquot is taken from the headspace, manually or automatically, and it is transferred to the gas chromatography (GC) system. Three commercially available direct headspace techniques are used to transfer the headspace sample to the GC system, namely gas-tight syringe, balanced pressure, and pressure loop headspace. Using the gas-tight syringe system, an aliquot (commonly 1000 µl) is taken from the headspace (normally after reaching the equilibrium) and is injected into the GC system using a split/splitless injector. In the balanced-pressure system, a heated transfer-line equipped with a valve is used to transfer the headspace sample to the GC system. During the incubation step the needle of the transfer-line of the headspace system is inserted into the vial which is pressurized with the same carrier gas used in the GC system. When the equilibrium has been reached, the valve is switched for a specific amount of time to redirect the sample into the transfer line. However, since this technique uses a fixed time for sample injection, the absolute sample injected volume is not exactly known. To solve this problem, pressure-loop systems with a several-port valve and a sample loop of known volume are used. In this case, after pressurization and equilibrium, the valve

is turned and the loop is filled with the sample and then, the valve is turned again to redirect the gas flow and flush the sample into the transfer line.

The US FDA in June 2004 published the first analytical method based on direct headspace coupled to gas chromatography and mass spectrometry for the analysis of furan in food (US FDA, 2004b). In this method, both, balanced-pressure (Turbo Matrix 40 headspace autosampler, Perkin Elmer) and pressure-loop (7694 Headspace Autosampler, Agilent) systems are recommended to transfer the headspace aliquot from the vial to GC-MS system. Sample sizes between 5 and 10 g depending on the sample nature and a vial of 20 ml are used. The optimum operating conditions for each headspace method and GC-MS parameters are summarized in Table 2.1.

Table 2.1: Optimal HS-GC-MS conditions of the US FDA analytical methods for furan analysis in food (US FDA, 2004b).

| Headspace system and conditions | | GC-MS conditions |
|---|---|---|
| Balanced-pressure | Pressure-loop | |
| **Temperature (ºC)** <br> - Needle: 100. <br> - Transfer Line: 130. <br> - Oven: 80. | **Temperature (ºC)** <br> - Transfer Line: 150. <br> - Oven: 80. <br> - Valve: 100. | **Gas chromatography conditions** <br> **Column:** HP-Plot-Q <br> (15m × 0.32mm I.D.× 20-µm film thickness). <br> **Oven:** Initial temperature 50 ºC (0 min) rising to 200 ºC (10 ºC / min) and hold for (10 min). |
| **Time (min)** <br> - Injection: 0.2. <br> - Pressurization: 0.5. <br> - Equilibration: 30. <br> - Shaking: on. | **Time (min)** <br> - Injection: 2. <br> - Pressurization: 0.5. <br> - Equilibration: 30. <br> - Loop filling: 0.2. <br> - Loop equilibration: 0.05. <br> - Shaking: on. | **Injector:** <br> - Temperature 200 ºC. <br> - Split ratio (2: 1). <br> - Flow-rate (2.0 ml min$^{-1}$) (He). <br><br> **Mass spectrometry conditions** <br> - Quadrupole temperature (150 ºC). <br> - Transfer-line temperature (225 ºC). <br> - Ion source temperature (230 ºC). <br> - Electron impact (70 eV). <br> - Full scan mode (*m/z* 30 to *m/z* 150). <br> - Scan speed (5.56 scans sec$^{-1}$). |

In this method, standard addition calibration at seven levels (requiring seven extractions per sample) with labelled-deuterium furan as internal standard is used for furan quantification. The estimated limit of quantification in food is sample depended and ranged from 2 to 5 ng g$^{-1}$; no data about precision are reported. This analytical method has been continuously modified and updated (June 2005 and October 2006) in order to improve its trueness and robustness and also to reduce the interferences originated from the matrix (Nyman et al., 2006; 2008, Morehouse et al., 2008). It is considered as reference for furan analysis in food and it is frequently used (Crews et al., 2009, Mesías et al., 2013, Pye and Crews 2014 and Mesías and Morales 2014).

Since 2004 and as the occurrence of furan in food has become an important issue in USA and in Europe, the commission recommendation 2007/196/EC of March 2007 advised EU member states to perform monitoring of the presence of furan in foodstuffs that have undergone heat treatment. Therefore, several authors have proposed methods based on direct headspace extraction for furan analysis in food. In Table 2.2 the HS-GC-MS methods published during the period 2004– 2016 are summarized. As can be seen in this table, sample preparation needs a special attention, since furan might be lost during this step. In all published works it is mentioned that samples need to be chilled between 0 ºC and 10 ºC in a closed container and the thawing of frozen samples must be performed at low temperature as recommended the US FDA method. In addition, homogenisation of samples must be done at low temperature using chilled ultra-turrax or simply shaking the cold sample (depending on the nature of the sample). It is essential to perform the manipulation of the sample as quickly as possible after opening the sample container. Similar considerations must be also taken into account in the preparation of the standard solutions. Moreover, it is clear from Table 2.2 that gas-tight syringe HS technique is more frequently used than pressure-loop and/or balanced-pressure for furan analysis in food which is probably due to its simple manipulation, easy coupling to any GC system without requiring any special system configuration, and to the high robustness of the nowadays available autosamplers (e.g. CTC Combi-Pal). Regarding the sample amount, a high range, between 0.5 and 10 g, is proposed depending on the amount of furan in the food but in general, a test portion size of 5 g as proposed by the US FDA methods is used (Table 2.2). Water is also added to the sample (between 5 and 10 ml) to reduce the viscosity of the solid and semi-solid

samples. The volume of the HS vial is 20 ml although in some cases a vial of lower volume was used (Becalski et al., 2005b). The equilibrium conditions of the published HS methods varied between 30 ºC and 80 ºC for the temperature and between 10 min and 60 min for the time (Table 2.2) although in general, slightly lower values than those reported in the first version of the US FDA method (FDA, 2004b) are used to improve sensitivity and trueness.

Occasionally, dynamic headspace methods have also been proposed for furan analysis in food in order to improve sensitivity since the amount of compound extracted in dynamic headspace techniques is higher than in direct static headspace methods. Lachenmeier et al., 2009, used an Eclipse 4660 HS purge-and-trap sample concentrator system coupled to a GC-MS for the analysis of furan in home prepared baby food samples. A mixed three sorbent trap (Tenax, silica-gel, carbon molecular sieve) was used, the sample was purged at 40 ºC for 5 min and the desorption was performed for 4 min at 190 ºC. Using this method, the obtained LOQ for furan in baby food was 0.44 ng g$^{-1}$ which is 5-10 fold lower than that reported using the US FDA HS method. More recently, Huault et al., 2016 used a new HS-trap analyser (Perkin Elmer) to analyse furan in bread crust samples. The extraction conditions were similar to those indicated in Table 2.2; equilibration at 50°C for 30 min with constant shaking and the furan is transferred to a trap (CarboTrap and Carbosieve SIII) using 4 cycles of 2 min and a temperature of 28 ºC. The adsorbed furan on the trap was desorbed by a gas flow of helium and the obtained LOQ was 0.1 ng g$^{-1}$ which is 50-fold lower than LOQ of US FDA method.

Headspace solid-phase microextraction (HS-SPME) has also been used for the analysis of furan in food. Several researchers, we were among the forerunners, put interest in developing analytical methods based on HS-SPME for the determination of furan in food since it is a more selective than HS and integrates in one step the HS extraction and furan pre-concentration on SPME-fibre increasing the sensitivity.

Table 2.2: Headspace methods used for furan analysis in food (2004- 2016).

| Code | Sample/preparation | Headspace extraction | | Optimal conditions | | LOQ (ng g$^{-1}$) | Reference |
|---|---|---|---|---|---|---|---|
| | | $V_{vial}$ / $V_{sample}$ (ml) | System | Incubation | Transfer/injection | | |
| 1 | Ground green coffee, tomato and orange juices/sample treatment at 4 °C /(3g) | 20 / 5 | Pressure-loop | (40 °C) (30 min). Low shaking. Salt addition (N.R. [b]) | Loop: (1 ml); (100 °C). Transfer-line: (120 °C). Pressurization: (14 psi) (0.15 min). Injection time: (0.5 min). | N.R. | Şenyuva and Gökmen (2005) |
| 2 | Baby food, coffee and other/ homogenisation at cold/ (0.5g) | 2 / 1 | Gas-tight syringe | (30 °C) (60 min). No shaking. Na$_2$SO$_4$ (50%). | Syringe Temp. (25 - 30 °C). Volume (50 µl). | 1 | Becalski et al., 2005b |
| 3 | Baby food, coffee, canned or jarred foodstuffs/ homogenisation at 4 °C for 1 min/ (5-10 g) | 20 / 10 | Gas-tight syringe | (80 °C) (10 min) Vigorous shaking. NaCl (20%). | Syringe Temp. (70 °C). Volume (1ml). | 0.5 - 2 | Zoller et al., 2007 |
| 4 | Baby food, coffee and other/homogenisation in ice-bath for 20 sec/ (5g) | 20 / 10 | Gas-tight syringe | (50 °C) (30 min) Maximum shaking. Salt addition (N.R.). | Syringe Temp. (80 °C). Volume (1000 µl). | 6.6 | Hasnip et al., 2006. Crew, et al., 2009 |
| 5 | Baby food/ homogenization with chilled water in ice-bath for 1 min/ (1-10g) | 20 / 10 | Pressure-loop | (60 °C) (30 min). Vigorous shaking. NaCl (4 g) | Loop:3 ml;100 °C. Transfer-line (130 °C). Pressurization (15 psi) (0.3 min). Injection time (2 min). | 0.5 - 2 | Yoshida et al., 2007 |
| 6 | Coffee/homogenisation at low temperature (liquid N$_2$)/ (0.5g) | 20 / 5 | Gas-tight syringe | (50 °C) (30 min). Salt addition (N.R.) Agitation (N.R.). | Volume (1ml). Syringe Temp. (N.R.). | N.R. | Guenther et al., 2010 |
| 7 | Baby food/ homogenisation in ice-bath/ (5g) | 20 / 10 | Pressure-loop | (80 °C) (30 min). Salt addition (N.R.) Agitation (N.R.). | Loop Temp. (95 °C). Transfer-line (110 °C). Injection time (1 min). | 4 | Ruiz et al., 2010 |

N.R: Not reported

Continued table 2.2:

| Code | Sample/preparation/ | Headspace extraction | | Optimal conditions | Transfer/injection | LOQ (ng g$^{-1}$) | Reference |
|---|---|---|---|---|---|---|---|
| | | $V_{vial}$ / $V_{sample}$ (ml) | System | Incubation | | | |
| 8 | Baby food, coffee, fruit, vegetable and others/ homogenisation at cold for 1 min/ (12.5ml). | 20 / 12.5 | Gas-tight syringe | (35 °C) (60 min). Agitation (500 rpm). Na$_2$SO$_4$ (50%). | Syringe Temp. (80 °C). Volume (250 µl). | 0.17 - 10 | Becalski et al., 2010; 2016 |
| 9 | Vegetables juice, nutrition drink/ sample treatment at 4 °C/ (15g). | 20 / 15 | Gas-tight syringe | (40 °C) (10 min). NaCl (30%). Agitation (N.R.) | Volume (1000 µl). Syringe Temp. (N.R.). | 1 - 50 | Wegener and López-Sánchez (2010) |
| 10 | Coffee/ homogenisation by shaking at 4 °C/ (5 ml) | 20 / 5 | Gas-tight syringe | (50 °C) (30 min). KOH (pH = 8). Salt addition (N.R.). | Volume (1ml). Syringe Temp. (N.R.). | 1.2 | Waizenegger et al, 2012 |
| 11 | Baby food, canned and jarred foodstuffs/ homogenisation at 4 °C/ (0.5 – 5g) | 20 / 5 | Gas-tight syringe | (60 °C) (20 min). Maximum shaking. NaCl (5M). | Syringe Temp. (70 °C). Volume (1ml). | 0.02 - 2 | Mariotti et al., 2013 |
| 12 | Infant formula, coffee, nutrition drink, others/ homogenisation at 4 °C for 3 min / (3-10 g) | 20 / 10 | Pressure-loop | (70 °C) (30 min). Low shaking. NaCl (10%). | Loop Temp. (110 °C). Transfer-line (130 °C). Pressurization (10psi) (0.5min). Injection time (1 min) | 0.5 – 1.5 | Nie et al., 2013 |
| 13 | Baby food/ homogenisation under cold conditions/ (5g) | 20 / 15 | Gas-tight syringe | (70 °C) (10 min). Maximum shaking. NaCl (2 g). | Syringe Temp. (N.R.). Volume (1.5 ml). | 0.07 | Pugajeva et al., 2016 |
| 14 | Varity of foodstuffs/ homogenisation at 4 °C (3 – 7g) | 20 / 6 | Pressure-loop | (60 °C) (15 min). NaCl (1 g) | Loop Temp. (110 °C). Transfer-line (130 °C). Pressurization (10psi) (0.5 min). Injection time (1 min). | 0.5 - 10 | Shen et al., 2016 |

HS-SPME includes two main steps (i) extraction of volatile and semi-volatile compounds from the HS of the sample by a fibre and (ii) thermal desorption of the extracted analytes from the fibre into the hot injection port of the GC system. The used fibre in SPME is coated with a stationary phase (polymer), a solid (sorbent), or a combination of both that allow to concentrate analytes via equilibrium-based absorptive and/or adsorptive mechanisms. So, two equilibrations take place during HS-SPME extraction: one between food sample matrix and its HS and the second between the HS and the fibre, and the extracted amount of analyte is determined by its partitioning between these three phases. Kinetics of the HS-SPME is very sensitive to experimental conditions and its performance depends on a number of parameters (e.g. fibre type and its thickness, temperature, time, sample size, HS volume, agitation, sample matrix modification such as pH and salting out) affecting the distribution of analytes between the phases and thus the extraction efficiency. Therefore, these parameters must be optimized in order to achieve high sensitivity, reliable quantification and good reproducibility.

When we started our study, very little was known about the use of HS-SPME for the analysis of furan in food, however, nowadays several applications have been reported. The scientific works and the optimal operating HS-SPME conditions published in the literature are summarized in Table 2.3.

Among the commercially available SPME fibres, the most frequently used for the extraction of furan from food samples is the mixed carboxen/polydimethylsiloxane (CAR/PDMS) of 75μm phase-thickness although some authors employed the highly crosslinked phase of 85μm because it has a longer life time than the 75μm CAR/PDMS (Ho et al., 2005 and Palmers et al., 2014). Some new fibres synthesized in the laboratory have also been tested for the HS-SPME of furan. For instance, Yazdi et al., 2012 compared a poly-(ethylene glycol) (PEG) fibre and a PEG one reinforced with multi-walled carbon nanotubes (PEG/CNTs) in order to demonstrate the advantage of using carbon nanotubes to increase the retention.

Table 2.3: HS-SPME methods used for furan analysis in food (2004 and 2016).

| Code | Sample / preparation | HS-SPME parameters $V_{vial}$ / $V_{sample}$ (ml) | Optimal conditions Fibre / Extraction parameters | Desorption | LOQ (ng g$^{-1}$) | Reference |
|---|---|---|---|---|---|---|
| 1 | Baby food, orange juice, brewed coffee/ sample preparation and homogenisation at low temperature /(0.05-0.5 g) | 10 / 1 | CAR-PDMS (75μm). 50 °C for 20 min with constant agitation 250 rpm, salt addition 0.2 g of NaCl. | 300 °C for 1 min | 0.09 | Goldmann et al., 2005 |
| 2 | Brewed coffee/ sample preparation at low temperature / (1g) | 20 / 6 | CAR-PDMS (85μm). 30 °C for 20 min with constant agitation 250 rpm, salt addition 4 g of NaCl. | 235 °C for 1.5 min | 0.8 | Ho et al., 2005 |
| 3 | Baby food / sample preparation at low temperature / (4 g) | 10 / 6 | CAR-PDMS (75μm). 30 °C for 10 min with constant magnetic stirring, without salt addition. | 230 °C for 3 min | 0.04 | Bianchi et al., 2006 |
| 4 | Balsamic vinegars / manual shaking / (1 g) | 20 / 5 | CAR-PDMS (75μm). 25 °C for 30 min with low constant magnetic stirring, salt addition 2g of NaCl. | 220 °C for 10 min | 1.5 | Bononi et al., 2009 |
| 5 | Brewed coffee and solid coffee beans / extracted by magnetic-assisted and ultrasound extraction at 20 °C / (20 μl) | 4 / 1.5 | CAR-PDMS (75μm). 25 °C for 15 min with constant magnetic stirring, without salt addition. | 290 °C for 5 min | 0.006 | La Pera et al., 2009 |
| 6 | Jarred and canned food, baby food/ homogenisation in ice/water bath for 3 min /(5g) | 20 / 10 | CAR-PDMS (75μm). 50 °C for 20 min agitation or stirring is not reported, no salt addition. | 250 °C for 5 min. | 0.12 | Kim et al., 2009; 2010 |

Continued Table 2.3.

| Code | Sample / preparation / | HS-SPME parameters V$_{vial}$ / V$_{sample}$ (ml) | Optimal conditions Fibre / Extraction parameters | Desorption | LOQ (ng g$^{-1}$) | Reference |
|---|---|---|---|---|---|---|
| 7 | Baby food/ homogenisation in ice-bath / (2.5g) | 40 / 5 | CAR-PDMS (75μm). 45 °C for 20 min with constant magnetic stirring, with salt addition 2.5 g of ice-cold 20% NaCl, 250μl of MeOH | 300 °C, time not specified. | 4 | Jestoi et al., 2009 |
| 8 | Baby food / Homogenisation in ice bath / (1g) | 40 / 2 | CAR-PDMS (75μm). 25 °C for 30 min, magnetic stirring at 1200 rpm, without salt addition. | 230 °C for 6 min | 2.4 | Arisseto et al., 2010 |
| 9 | Jarred and canned food, coffee and baby food/ homogenisation in ice bath for 2 min/ (1g for solid and 15 g for liquid samples) | 40 / 15 | CAR-PDMS (75μm). 25 °C for 15 min, magnetic stirring at 1200 rpm, without salt addition | 275 °C for 2 min | 1.2 | Liu and Tasi, 2010 |
| 10 | Several food products/ homogenisation in cold ambient / (1 g) | 20 / 2 | CAR-PDMS (75μm). Sub-room temperature (4 °C) for 26 min, salt addition 0.4g NaCl. | 230 °C for 5 min | 1 | Scholl et al., 2012a |
| 11 | Deep-fried fish samples/ homogenisation in cold ambient (4°C)/ (2g) | 50 / 10 | CAR-PDMS (75μm). 37 °C for 40 min, salt addition 3g of NaCl, magnetic stirring at 600 rpm | 280 °C for 10 min. | 2.2 | Pérez-Palacios et al., 2012 |
| 12 | Apple and orange juices, baby food / homogenisation in ice bath / (0.5g for solid and 15 g for liquid samples) | 25 / 15 | home-synthesized PEC fiber 25 °C for 30 min, magnetic stirring at 600, salt addition 20% of NaCl home-synthesized PEG/CNTs fiber 30 °C for 10 min, magnetic stirring at 600, salt addition 20% of NaCl | 230 °C for 15 s. | 0.005 0.0005 | Yazdi et al., 2012 |

Continued Table 2.3.

| Code | Sample / preparation | HS-SPME parameters | | | LOQ (ng g$^{-1}$) | Reference |
|---|---|---|---|---|---|---|
| | | $V_{vial}$ / $V_{sample}$ (ml) | Optimal conditions | | | |
| | | | Fibre / Extraction parameters | Desorption | | |
| 13 | Vegetables puree, fruit juices/ homogenisation at low temperature/ (2.5g) | 10 / 6 | CAR-PDMS (85μm). 30 °C for 15 min, 2.5 ml of saturated solution of NaCl, stirring is not reported | 200 °C for 1 min | 1.86 | Palmers et al., 2014 |
| 14 | Grain products, coffee, infant formula, liquor, canned food/homogenisation in ice bath for 2 min / (5g) | 15 / 5 | CAR-PDMS (75μm). 60 °C for 20 min, salt addition 1g of NaCl, stirring is not reported | 250 °C for 3 min | 0.18 | Sijia et al., 2014 |
| 15 | Fruit juices | | CAR-PDMS (75μm). 32 °C for 20 min, salt addition 15% of NaCl, stirring at 600 rpm | | 0.76 | Hu et al., 2015 |
| 16 | Bread-coated frozen foods/ homogenisation in ice bath / (2g) | 20 / 10 | CAR-PDMS (75μm). 37 °C for 40 min, stirring is not reported, salt addition 3 g of NaCl. | 280 °C for 10 min. | 2.3 | Juaniz et al., 2016 |

Accordingly, the obtained LOQ using the PEG/CNTs fibre (0.0005 ng g$^{-1}$) was 10-fold lower than that obtained using PEG fibre which is particularly due to the high surface area of CNT. However, in this study a GC-FID system was used and a comparison between these fibres and the 75µm CAR/PDMS fibre was not performed. Regarding equilibration conditions (Table 2.3), an extraction temperature between 25 ºC and 35 ºC and an extraction time between 10 and 30 min are used in most of the methods, although in some cases a higher temperature, up to 60 ºC was applied. Scholl et al., 2012, proposed using 4 ºC as the optimum extraction temperature of furan by HS-SPME although the LOQ reported in this study is not better than those obtained using higher extraction temperatures (Table 2.3). Generally water is added to solid and semi-solid samples to facilitate stirring, moreover, the addition of salt (NaCl), is proposed by several authors in order to increase extraction efficiency. The thermal desorption of the extracted furan from the fibre in HS-SPME was achieved by exposing the fibre in the GC-injector port at temperatures between 200 ºC and 300 ºC, in accordance with fibre manufacturer recommendations, for 1 to 10 min. In general, the fibre is kept in the GC-injector port for an additional time (3- 5 min) to ensure that no carryover on the fibre occurs. Only Yazdi et al., 2012 when studying the behaviour of a fibre with CNTs used desorption time considerably lower (15 s).

Regarding the chromatographic conditions and in accordance with the US FDA method, porous layer open tubular (PLOT) GC columns, namely PLOT-Q or CP Pora-Bond-Q with a bonded polystyrene-divinylbenzene based phase, are the most frequently used for the GC analysis in the HS methods (codes: 3, 4, 6, 7, 9-14, Table 2.2) since it is resistant to the presence of low amounts of water. In contrast when using HS-SPME this column is only occasionally utilized (codes: 1, 6, 7, 9, 12, Table 2.3). The column length of the PLOT columns is typically 15 or 30 meters while the thickness generally is 20-µm. In HS-SPME capillary columns with stationary phases of different polarities have been used. For instance, some authors proposed using a polar wax column with a bonded polyethylene glycol phase (codes: 3, 4, 8, Table 2.3) or the semi-polar column namely, BPX-Volatile with a bonded cyanopropyl-phenyl dimethylpolysiloxane phase

(La Pera et al., 2009). A stationary phase of phenyl-methylpolisiloxane has also been used for the determination of furan in food (Pérez-Palacios et al., 2012 and Palmers et al., 2014). In relation to the injection mode, split injection is mainly used in static HS, (split ratio between 2:1 and 10:1) since a large volume is injected, 1-3 ml, while for HS-SPME, splitless injection is the most popular injection mode. However, Becalski et al., 2005b used splitless injection with a HS method as the total injected volume was 50 µl and Ho et al., 2005 employed split injection (split ratio of 10:1) with HS-SPME, probably to reduce band broadening of chromatographic furan peak. In order to achieve an effective band focussing and obtain a sharp furan peak shape within splitless injection mode, Goldmann et al., 2005, proposed to employ a cryofocuser with liquid nitrogen (-20 ºC).

Mass spectrometers used for furan analysis are mainly single quadrupoles operating in electron ionization mode and selected ion monitoring (SIM) scanning mode (*m/z* 39 and 68) in order to obtain high sensitivity. Full-scan mode has been occasionally used with quadrupole analysers (codes; 1, 9, Table 2.2 and 5, 11, Table 2.3) and by most of the authors that work with ion-trap analysers (codes; 3, 6, Table 2.2) since with this late analyser similar sensitivity can be obtained with both working modes. In the recent years and due to the implementation of high-medium cost and new advanced MS systems in food analysis laboratories, tandem mass spectrometry using triple quadrupole mass spectrometers have begun to be used for the analysis of furan (Pugajeva et al., 2016).

Other sampling methods such as headspace liquid phase microextraction (HS-LPME) using single drop microextraction (HS-SDME) and stir bar sorptive extraction (SBSE) have been occasionally reported for furan analysis in food. In the HS-SDME technique, the analytes are partitioned between a stirring sample, the HS phase and a drop of extracting solvent which is suspended from the tip of a conventional micro-syringe needle by surface tension to the HS phase in a closed vial. After extraction for a set period of time, the drop is retracted into the syringe and injected into a GC chromatographic system. The difference between HS-SDME and HS-SPME is the

extracting phase (solvent or sorbent) and therefore the effectiveness of extraction in SDME process is also influenced by factors such as physical and chemical properties of the solvent, drop volume, extraction time and temperature, stirring rate and the ionic strength of the sample solution. Habibi et al., 2013, developed a HS-SDME method for the analysis of furanic compounds, including furan, in baby food samples. This author propose using n-dodecane as extraction solvent, the dilution of the baby food sample with 10 ml of water, salt addition and an extraction temperature (40 °C) and time (15 min) similar to those proposed for HS-SPME. This HS-SDME-GC-MS method was also used by Chaichi et al., 2015 for the analysis of furan in coffee samples obtaining a LOQ at 0.06 ng g$^{-1}$. Stir bar sorptive extraction (SBSE) has also been applied for the analysis of furan in coffee brews and baby food samples (Ridgway et al., 2010). In this technique a glass-coated magnetic stirring bar (Twister) is used for the extraction from the sample, the stir-bar is removed, and the analytes are either desorbed thermally in a GC system or back extracted with an organic solvent. In this study the extraction from sample (0.5g diluted by 5 ml water) is performed at room temperature (20 °C) during 1h using a polydimethylsiloxane (PDMS) twister and thermal desorption (20 °C for 0.5 min ramped at 60 °C min$^{-1}$ to 200 °C) was used. The reported LOQ (10 ng g$^{-1}$) for coffee sample is comparable with that obtained using HS although uncertainty for the HS method was slightly better. Compared to the HS-SPME, the extraction time is longer for SBSE (1h), the desorption process is slower (10 min) and is less selectivity and more prone to matrix effects. No more works based on these late techniques for furan analysis in food were found in the literature.

### 2.2.2. VOLATILE N-NITROSAMINES

The presence of N-nitrosamines in food is known since 1970 but the early analytical results are untrustworthy since no reliable and sensitive analytical methods were available at that time. Due to the different physicochemical properties of non-volatile and volatile N-nitrosamines (VNAs), only methods able to analyse one group of compounds, non-volatile N-nitrosamines or volatile ones (VNAs), are currently used. In

addition, it is well known that in foods the VNAs are the N-nitrosamines of main relevance and interest, and for this reason only these compounds have been studied in this thesis and are considered in this section.

In the past, intense research efforts were devoted to develop analytical methods for the analysis of VNAs in food. In 1975, the development of a chemiluminiscence device based on the generation of excited species that undergo photon emission, as a specific detector for gas chromatography led to the reliable detection of VNAs in food (Fine et al., 1975). In this case, the detection of N-nitrosamines is performed after their cleavage in a thermal energy analyser (TEA) and oxidation to nitric oxide, which reacts with ozone to form nitrogen dioxide in an excited state which experience photon emission around 1200 nm. As a consequence, a great number of scientific papers were published in the period 1975–1990 about the presence of VNAs in food, most of them based on this method. Liquid chromatography (LC) has been less applied for VNAs analysis since the attempts to combine LC with TEA detector gave inconsistent results with respect to both sensitivity and resolution (Bellec et al., 1996). Other detectors such as the nitrogen-phosphorus detector (NPD), the electron-capture detector (ECD) after the conversion of VNAs into the corresponding N-nitramine analogues, and the flame photometric detector (FPD) after the denitrosation and derivatization of VNAs have also been used for the determination of these compounds by GC. With regard mass spectrometry for VNAs analysis, it was seldom used till the end of the last century because at that time the TEA detector provided enough sensitivity and specificity. Even though, the International Agency for Research on Cancer (IARC) in 1994 recommended the use of GC-MS for the unequivocal confirmation of the presence of VNAs in food (Frassantio et al., 1994).

For VNAs extraction from food samples, steam distillation and the subsequent extraction by a low-boiling solvent such as dichloromethane (DCM) is the method most frequently used and it has been adopted by the Association of Official Analytical Chemists as Official Method 982.11, 982.12 984.16, 982.22, and 982.28 (AOAC, 2004). Other procedures, such as supercritical fluid extraction with carbon dioxide for the analysis of solid samples and extraction with DCM for liquid samples, such as beer, have also been used. In the literature there are several reviews (Hotchkiss, 1981; Sen,

1984; Scanlan and Reyes 1985) that compile the analytical methods used in the past for the VNAs analysis in food. Also a report about methods used for the analysis of VNAs has been published by the European Commission, Joint Research Center (Wiltschko et al., 1998).

Nowadays, new analytical methods are used for VNAs analysis in food improving selectivity, detectability, analysis time, and cost. The changes and evolution of the applied methodologies are strongly related to the development of mass spectrometry and the introduction of modern sample preparation techniques with minimum number of processing steps. Methods based on the coupling of mass spectrometry, with quadrupoles (single and triple quadrupoles) and ion traps, to gas or liquid chromatographic systems have been developed showing the potential of these techniques for the determination of N-nitrosamines in food, mainly due to their high detectability and selectivity capacities.

Regarding sample preparation, the efficient extraction, clean-up and concentration of VNAs from food matrices, using small amounts of organic solvents, has been the main purpose of all sample preparation methods. Table 2.4 summarises the reported analytical methods that have been proposed and used in the last 15 years for the analysis of VNAs in food.

As can be seen in the Table 2.4, most of the analytical methods developed for the analysis of VNAs in food are based on gas chromatography while liquid chromatography coupled to mass spectrometry (LC-MS) or fluorescence detection (LC-FLD) is only occasionally employed (codes 1, 17, 24, Table 2.4). When fluorescence detection is used, VNAs are previously denitrosated to produce secondary amines, commonly using a mixed solution of hydro-bromic acid-acetic acid (codes 1, 24). In addition, since the resulted amines do not have fluorescence properties, they must be derivatised with reagents able to introduce a fluorescent group. Since both denitrosation and derivatisation steps are relatively slow (commonly 30 min each), several approaches to reduce analysis time have been proposed.

Table 2.4: Analytical methods for VNAs analysis in food (2000-2016).

| Code | Sample | Sample preparation | Analytical method | Figures of merit | | Reference |
|---|---|---|---|---|---|---|
| | | | | LOQ(ng g⁻¹) | RSD (%) | |
| 1 | Beer | LLE (DCM), Denitrosation and derivatization (dansyl-chloride). | LC fluorescence detection and External Calibration | $0.3 - 2.5$ | $1 - 3$ % | Cárdenes et al., 2002 |
| 2 | Sausages | Steam distillation and LLE (DCM). | GC-TEA Internal calibration (NDPA, I.S.). | N.R. | N.R. | Byun et al., 2004 |
| 3 | Alcoholic-beverages | Steam distillation and LLE (DCM). | GC-TEA. Internal calibration (NDPA, I.S.). | 0.17 | N.R | Shin et al., 2005 |
| 4 | Sausages | HS-SPME (PDMS-DVB 65 µm). Equilibrium time 10 min, extraction at 45 °C for 25 min and no salt addition, constant stirring, $V_v/V_s = 24/12$ ml. Desorption (200 °C for 8 min). | GC-TEA Standard addition calibration | 10 | 5- 12 % | Andrade et al., 2005 |
| 5 | Meat patties | HS-SPME (PDMS-DVB 65 µm) using direct extraction device DED. Equilibrium time 15 min, extraction at 25 °C for 60 min Desorption (260 °C). | GC-EI-MS Quadrupole (SIM). External calibration | $0.14 - 9$ | $1 - 70$ % | Ventanas and Ruiz, 2006 |
| 6 | Beer, fish and meat products | Two steps SPE: Extrelut, elution with hexane/DCM (40:60) Florisil, elution with DCM/MeOH (95:5). | GC-CI-MS (Ammonia CI) Quadrupole (SIM). External calibration. | 0.24- 0.37 | $5 - 12$ % | Yurchenko and Mölder, 2005; 2006; 2007 |
| 7 | Several beverage | SPE: LiChrolut EN eluted with ethyl acetate–acetonitrile (9:1). | GC-NPD Internal calibration (Bromophos-methyl, I.S.) | 0.03- 0.1 | 4.3- 6 % | Jurado-Sánchez et al., 2007 |
| 8 | Beer samples | HS-SPME (PDMS-DVB 65 µm). Equilibrium 200h., extraction at 42 °C for 40 min, 20% of NaCl, $V_v/V_s = 15/10$ ml. Desorption (225 °C for 8 min) | GC-EI-MS. Quadrupole (SIM). Standard addition calibration. | 3 | N.R. | Pérez et al., 2008 |

Continued Table 2.4.

| Code | Sample | Sample preparation | Analytical method | Figures of merit LOQ(ng g⁻¹) | RSD (%) | Reference |
|---|---|---|---|---|---|---|
| 9 | Kebab and meat products | Two steps SPE: Extrelut, elution with hexane/DCM (40:60) Florisil, elution with DCM/MeOH (95:5) LLE (Me-OH) | GC × GC-TEA External calibration. | 7 - 17 | 7 - 21% | Ozel et al., 2010 |
| 10 | Heated cured pork meat | Vacuum distillation and LLE (DCM). | GC-TEA Internal calibration (NDPA, I.S.). | 0.38 – 0.41 | N.R. | Drabik-Markiewicz et al., 2010; 2011. |
| 11 | Meat products. | Microwave-assisted extraction (NaOH). Dispersive liquid–liquid microextraction MeOH / CCl₄ (1.5 ml: 20 µl). | GC-EI-MS Quadrupole (SIM). External calibration. | 0.4 – 1.9 | 6 – 10 % | Campillo et al., 2011 |
| 12 | Milk and dairy products | Protein precipitation with ACN. SPE: LiChrolut EN elution with ethyl acetate–acetonitrile (9:1). | GC-EI-MS Quadrupole (SIM). Matrix-matched calibration (2-tert-butyl-4-methylphenol, I.S.). | 0.03 – 0.43 | 3 – 7.5 % | Jurado-Sánchez et al., 2011 |
| 13 | Grilled lamb and vegetables | Two steps SPE: Extrelut, elution with hexane/DCM (40:60) Florisil, elutionwith DCM/Me-OH (95:5) LLE (MeOH) | GC × GC-TEA External calibration | N.R. | 5- 10 % | Kocak et al., 2012 |
| 14 | Meat products | Steam distillation and LLE using DCM. | GC-EI-MS. Quadrupole (Full-scan). External calibration | N.R. | N.R. | Li et al., 2012 |
| 15 | Meat products | LLE (NaOH/methanol). Two steps SPE: ChemElut, Elution with DCM. Florisil, elution with DCM/MeOH (95:5). | GC-CI-MS (Ammonia CI). Triple-Quadrupole (MRM). Internal calibration (5 deuterated VNAs, I.S.). | 0.3- 0.4 | 5- 11 % | Sannino and Bolzoni, 2013 |

Continued Table 2.4.

| Code | Sample | Sample preparation | Analytical method | Figures of merit | | Reference |
|------|--------|--------------------|--------------------|----------------|--------|-----------|
| | | | | LOQ(ng g$^{-1}$) | RSD (%) | |
| 16 | Meat products | Microwave-assisted extraction (NaOH). Dispersive micro solid extraction (100 mg Carboxen-1000 for 30 min), elution with 200 µl DCM. | GC-CI-MS (Me-OH CI) Ion-trap (SIM). Internal calibration ($^2H_8$-naphthalene, I.S.; NDMA-d$_6$ and NDEA-d$_{10}$ as surrogates). | 0.03- 0.36 | 8- 10 % | Huang et al., 2013 |
| 17 | Meat products | LLE with acetonitrile / 1% formic acid. Frozen at -60°C. | LC - (APCI/ESI)-MS Triple-Quadrupole (MRM) Internal calibration (NDMA-d$_6$, NPYR-d$_8$, I.S.). | 0.5- 34 | 1- 42 % | Herrman et al., 2014. |
| 18 | Fermented sausages | Vacuum distillation and LLE (DCM) | GC-TEA Internal calibration (NDPA, I.S.). | 0.6- 2.5 | N.R. | De Mey et al., 2014 |
| 19 | Frankfurter sausages | PLE with superheated water (120 °C, 5 min, 300 psi). SPE: Florisil, elution with diethyl ether/dichloromethane (60:40). | GC-EI-MS Quadrupole (SIM) Internal calibration (NDPA, IS) | 1.6 – 4.8 | 6– 7.2 % | Chienthavorn et al., 2014 |
| 20 | Sausage and salami | Microwave assisted extraction KOH/MeOH (80:20). Dispersive liquid–liquid microextraction MeOH/ Chloroform (0.5 ml: 110 µl). | GC-EI-MS. Quadrupole (SIM). Internal calibration (Biphenyl, I.S.) | 0.41- 1.45 | 3.5– 5.4% | Ramezani et al., 2015 |
| 21 | Meat products | LLE (DCM) | GC-CI-MS (Me-OH, CI) Ion-trap (SIM) External calibration | 0.5 – 1.24 | 3 – 28 % | Scheeren et al., 2015 |
| 22 | Soy sauce | LLE (ethyl-acetate) followed by QuEChERS (PSA, 1 C18, GCB). | GC-EI-MS. Quadrupole (SIM). Internal calibration (NDMA-d$_6$, NDPA-d$_{14}$, I.S.) | 1.2 - 3 | 2.7- 7 % | Zeng et al., 2016 |

Continued Table 2.4.

| Code | Sample | Sample preparation | Analytical method | Figures of merit | | Reference |
|------|--------|--------------------|-------------------|------------------|--|-----------|
| | | | | LOQ(ng g$^{-1}$) | RSD (%) | |
| 23 | Red wine | HS-SPME (PDMS-DVB 65 µm). Equilibrium 4h., extraction at 40 °C for 20 min., 0.5 g of NaCl, $V_v/V_s$ = 15/10 ml. Desorption at 225 °C. | GC-EI-MS Quadrupole (full-scan). Standard addition calibration | 0.4 - 1 | 1.2– 29 % | Lona-Ramirez et al., 2016 |
| 24 | Meat, fish, egg and other foodstuffs) | LLE (DCM) Denitrosation and derivatization (1,2-benzo-3,4-dihydrocarbazole-9-ethyl chloroformate, BCEOC) | LC fluorescence detection LC-APCI-MS for confirmation. External calibration | 4.3- 8.3 | 1.5 – 3% | Zhao et al., 2016 |
| 25 | Oil, cereals vegetables, beverages | Solid-supported (Extrelut NT), elution with DCM/hexane (9:1). SPE: Florisil, elution with DCM-MeOH (95:5). | GC-CI-MS (Ammonia, CI) Triple-Quadrupole (MRM) Isotope dilution | 0.3 – 0.74 | 1- 15% | Seo et al., 2016 |
| 26 | Meat products | Microwave-assisted extraction NaOH (0.05M). Dispersive liquid–liquid microextraction MeOH/CCl₄ (1.5ml: 50µl). | GC-EI-MS Quadrupole (full-scan ) External calibration | 0.4 – 1.2 | 10 % | Amelin and Lavrukhin, 2016 |

N.R: Not reported

For instance, Cárdenes et al., 2002 used a microwave to accelerate the dansyl derivatization reaction while, Zhao et al., 2016 proposed a new designed derivatisation reagent (code 24). Regarding liquid chromatography coupled to mass spectrometry, Hermman et al., 2014 developed a method for the simultaneous determination both of non-VNAs and VNAs. Atmospheric pressure chemical ionisation (APCI) was proposed because it provided higher sensitivity and lower ion suppression than electrospray ionisation (ESI). The authors proposed using tandem mass spectrometry (MRM in a triple quadrupole) where two product ions of the $[M + H]^+$ ions were used for the NAs included in the method except for NDMA as its product ion has a very low mass making it unspecific. In this work and at these conditions (code 17, Table 2.4), high limits of quantification were obtained (up to 34 ng g$^{-1}$ for NPYR) with poor repeatability (up to 42% for NDMA expressed as RSD) probably due to the attempts to establish a unique method for the simultaneous analysis of volatile and non-volatile N-nitrosamines. With respect to the columns used for the LC separation of the VNAs, a fused-core column with phenyl-hexyl as stationary phase (Poroshell, 150 × 2.1 mm I.D., 3 μm) (Hermman et al., 2014) and $C_{18}$ reversed phase columns such as a NovaPak $C_{18}$, (150 × 3.9 mm I.D., 4 μm) (Cárdenes et al., 2002) and Hypersil BDS $C_{18}$ (200 × 4.6 mm I.D., 5 μm) (Zhao et al., 2016) have been used. The mobile phases (isocratic or gradient elution) were mixtures of acetonitrile and water (codes, 1, 24) or methanol and water with formic acid (0.1%) that maximize the intensity of the protonated ions when APCI-MS was used (Hermman et al., 2014).

Regarding gas chromatography, capillary columns with stationary phases of low, moderate and high polarity including 5% diphenyl / 95% dimethylsiloxane such as DB-5 and DB-5 ms (codes 2, 3, 5, 7, 11, 15, 16, 20,21, 26), 14% cyanopropylphenyl / 86% dimethylploysiloxane such as DB-1701 ms (code 6), poly 90% biscyanopropyl / 10% cyanopropylphenylsiloxane such as SP-2380 (code 12) and polyethylene glycol such as HP-INNOWax (codes 4, 8, 14, 22, 23) have been used. In most of the methods, the length of the columns was 30 m but columns of 50 m or 60-m were sometimes employed (codes 4, 25) while the film thickness and internal diameter were between 0.25- 1.5 μm and 0.25- 0.53 mm, respectively. For the analysis of VNAs in rich-fate samples such as oils, Seo et al., 2016, recommended using a pre-column (DB-5 ms,5m

× 0.53mm i.d. × 0.25μm) connected to the analytical column DB-Wax (60m × 0.25mm i.d. × 0.5μm) in order to prevent the damage of this column. A packed column, 10% carbowax 20M with 2% KOH (to improve peak shape) on chromosorb WAW, 80/100 mesh, (1.8m × 2mm i.d.) was used in some cases with TEA detector (codes 10, 18). Moreover, methods based on comprehensive two dimensional GC (GC × GC) were developed in order to improve the selectivity of the separation (Ozel et al., 2010; Kocak et al., 2012). In these cases, the first GC-column, was a 5% phenyl stationary phase, BPX-5 or DB-5, (30m × 0.32 mm i.d. × 0.25 μm) while the second one was a 100% methyl-polysiloxane (RXI-17, 1.5m × 0.1 mm i.d. × 0.1μm) in the study of Ozel et al., 2010 and a 50% phenyl polysilphenylenesiloxane (BPX-50, 1.5m × 0.1 mm i.d. × 0.1μm) in the study of Kocak et al., 2012. Splitless is the main injection mode used in the methods listed in Table 2.4 although some authors used split injection with a split ratio up to 1:50 as the injected extract was 3 μl (Ramezani et al., 2015). To improve sensitivity and repeatability when small sample volumes are injected, from 1 to 3 μl, some authors used pulsed-splitless injection since in this way the sample is introduced more rapidly into the column (methods code 6, 11, 13). When larger volumes are injected (up to 10 μl), the use of a programmed temperature vaporizing (PTV) injector is recommended (method codes 15-16).

With regard to the detection system and as can be seen in Table 2.4, TEA detector is still used for the determination of VNAs (30 % of the GC methods) due to its high selectivity and sensitivity but at the same time MS is taking the first position (65% of the GC methods). Most of the GC-MS methods of Table 2.4 employed single quadrupole mass spectrometers operating in selective ion monitoring (SIM) or occasionally in full-scan and using electron ionization (EI) (codes 5, 8, 11, 14, 19,-20, 22, 23, 26). However, in this ionisation mode, the VNAs show extensive fragmentation with non-characteristic fragment ions and low intensity that affect negatively the sensitivity of the analytical method. Chemical ionization (CI) has proven to be more sensitive than EI for the analysis of VNAs because the softer ionization results in less molecular fragmentation giving as a result spectra with one or two ions with a relatively high abundance which can be used for quantitation. Ammonia (1 ml min$^{-1}$) positive

chemical ionisation (PCI) is proposed by several authors to analyse VNAs in meat products (codes 6, 15, 25). At this conditions, the base peak of the spectra is $[M+NH_4]^+$ for all VNAs although the protonated molecular ion $[M+H]^+$ with relative abundances between 16% and 33%, and some adducts such as $[M+N_2H_7]^+$ with relative abundances lower than 1.6%, were also obtained. In order to eliminate the use of ammonia, a toxic gas, and also to increase the sensitivity of the method by reducing the number of peaks in the spectra Huang et al., 2013 and more recently Scheeren et al., 2015, used methanol as a chemical ionization reagent for VNAs determination in food. At these conditions, the most abundant mass spectra peaks for all VNAs were the protonated molecular ions $[M+H]^+$ and no adducts were formed (codes 16, 21). Nevertheless, this authors employed SIM scanning mode and information about the potential advantage of tandem MS using an ion-trap and CI in terms of sensitivity and selectivity for the analysis of VNAs in food has not been found in the literature.

Sample preparation is still one of the most challenging task in the analysis of VNAs in food as food matrices are very complex and the level of VNAs in food is very low (low ng $g^{-1}$). As can be seen in Table 2.4, methods based on direct LLE using dichloromethane (DCM) (codes 1, 21, 24) or acetonitrile (Hermman et al., 2014) as extraction solvents or steam distillation (codes 2, 3, 14) and vacuum distillation (codes 10, 18) both, followed by liquid-liquid extraction (LLE), as the official method (AOAC, 2004), using DCM, are currently used for the extraction and purification of VNAs before the chromatographic analysis. Although these procedures provide good recoveries for the extraction of VNAs from food samples, they are time consuming, labour intensive and require large amounts of organic solvents (c.a. 200 ml for each sample). Therefore, solid phase extraction (SPE) is preferred. Some authors (codes 6, 9, 13, 15 and 25, Table 2. 4), used two-step SPE for the extraction and clean-up of VNAs from food samples obtaining recoveries between 70 and 100% for all VNAs. In these methods, Extrelut (diatomaceous earth) is used in the first SPE step and the compounds are eluted with hexane-dichloromethane while Florisil is the sorbent mainly selected for the clean-up using dichloromethane-methanol for elution. In order to completely eliminate fat, an additional LLE using methanol has been implemented by some authors

(codes 9, 13). To increase sample throughput, Sannino and Bolzoni, 2013 used diatomaceous earth ChemElut cartridges (Agilent) to adsorb the compounds previously extracted from the samples with NaOH/methanol. In this procedure DCM is proposed to elute VNAs from the cartridges and an additional clean-up step with Florisil was also used. Jurado-Sànchez et al., 2007, developed an automated SPE method using LiChrolut-EN as sorbent and only 150 µl of ethyl acetate- acetonitrile for sample elution obtaining recoveries between 95 and 102 % for the determination of VNAs in beverages although for dairy products, the previous precipitation of proteins with acetonitrile was needed (Jurado-Sànchez et al., 2011). In order to accelerate the extraction and also to reduce the consumption of organic solvents, Huang et al., 2013, developed a method for VNAs from meat samples that uses microwave-assisted extraction (30-ml of NaOH 0.01M, 100 ºC, 10 min) followed by a dispersive micro-solid phase (D-µ-SPE) extraction with Carboxen™ 1000 as adsorbing material and only 200 µl of DCM as elution solvent with acceptable recoveries (60 to 103 %). Superheated water has also been used to extract VNAs from meet samples (frankfurter sausages) by PLE previous to a clean-up with Florisil using diethyl ether-dichloromethane (60:40) as a elution solvent (Chienthavorn et al., 2014) with recoveries from 81 to 100%. More recently, Zeng et al., 2016, developed a method based on LLE using ethyl acetate followed by a QuEChERS (quick, easy, cheap, effective, rugged, and safe) method (PSA, C18 and GCB) to obtain a purified extract from soy sauce. Again, these methods are time consuming, labour intensive and require a considerable amount of organic solvents. In this context, microextraction techniques such as HS-SPME and more recently dispersive liquid-liquid microextraction (DLLME) have also been used for the analysis of VNAs in food. In Table 2.4, methods with codes 11, 20 and 26 used microwave-assisted extraction with alkaline solution (NaOH or KOH/NaOH) followed by dispersive liquid–liquid microextraction (DLLME) for the analysis of VNAs in meat samples. The DLLME procedure is very simple and was first reported by Rezaee et al., 2006. In brief, a solvent mixture including water immiscible extracting phase (µl range) and a disperser solvent are introduced in an aqueous sample. Partitioning of analytes between the extraction phase (fine droples of the solvents) and the sample takes place in a very short

time, typically less than two minutes, due to significantly large interface area between the two phases and fast mass-transfer. After extraction, the analytes-enriched solvent is separated from the sample by centrifugation and is analysed with or without further work-up, by chromatography. Although DLLME is low cost and quite efficient method with LOQs from 0.4 to 1.5 ng g$^{-1}$ (11, 20 and 26 Table 2-4), it has some drawbacks such as the non-selectivity of the extraction and the difficulty for automation (Sarafraz-Yazdi and Amiri, 2010).

With regard to SPME, Andrade et al., 2005, described a simple method using HS-SPME sampling and GC–TEA for the determination of NDMA, NDEA, NPIP, and NPYR in sausages. Only two commercialized fibres, polyacrylate (PA, 85 μm) and polydimethylsiloxane/divinylbenzene (PDMS/DVB, 65 μm), were evaluated, and the experimental conditions proposed were: equilibrium time (5- 30 min), salt addition (0-36%, w/w), extraction time (4- 46 min) and temperature (24- 66 ºC). In this study, NDMA and NDEA were more efficiently extracted using a PDMS/DVB, 65 μm fibre than NPIP and NPYR while for PA 85 μm fibre, the extraction efficiencies for these VNAs was reversed. Considering that in sausages NDMA and NDEA are the VNAs must frequently found, the author select the PDMS/DVB, 65 μm fibre for all VNAs extraction under the optimised extraction conditions (equilibrium time for 10 min, extraction time 25 min and temperature at 45 ºC) obtaining a LOQ of 10 ng g$^{-1}$. Ventanas and Ruiz (2006) employed SPME coupled to a direct extraction device for the analysis of VNAs in meat patties. This device enables the introduction of the PDMS/DVB SPME-fibre in the core of the semi-solid matrices and allows the headspace extraction resulting from the diffusion of VNAS from the sample to the internal body of the device through several holes on its surface. However, the method presented low reproducibility and poor linearity and the authors concluded that quantitation of VNAs in solid meat samples using SPME with this device was not reliable. Two more studies based on HS-SPME coupled to GC-MS have been reported for the analysis of NDMA in beer (Perez et al., 2008) and NDMA, NDEA, NDBA and NDPIP in red wine samples (Lona-Ramirez et al., 2016). In both studies, a PDMS/DVB, 65 μm SPME-fibre was used for VNAs extraction in accordance with the

previous work published by Andrade et al., 2005, and the HS-SPME conditions were also similar (Table 2-4) except that the equilibrium times were very high, 200 h and 4 h in the studies of Perez et al., 2008 and Lona-Ramirez et al., 2016, respectively. Although a little improvement in LOQ (1- 3 ng ml$^{-1}$) was achieved, these HS-SPME conditions (long time) yield to a high limitation for its application. To date any thorough optimization of HS-SPME for the analysis of VNAs in food have been reported in the literature.

## 2.3. RATIONALE OF THE STUDY

Furan and volatile N-nitrosamines are toxicants formed during the manufacturing process, especially heat treatment, in a wide range of foodstuffs. At present, food processing technologies able to prevent their formation are not available, although several modifications in food processing and regulations that limited the use of some additives, mainly for VNAs, have been established to reduce the generation and occurrence of these contaminants in food. It is known that there is a potential carcinogenic risk to humans even at low levels, but as commented in chapter 1, concentration limits in food have only been set for VNAs and for a few food commodities and countries. These limits, low ng g$^{-1}$ levels, show the importance of having available robust and highly sensitive analytical methods to control the presence of furan and VNAs in food.

As commented in the previous section, the headspace-gas chromatography–mass spectrometry (HS-GC–MS) method developed by the US Food and Drug Administration is the most commonly used for the analysis of furan in food. However, this method presents the drawback of using standard addition (furan-d$_4$ as an internal standard) for quantification which increases the analysis time and reduces the applicability of the method in high-throughput routine laboratories. In addition, the method has little selectivity and since no pre-concentration of furan is performed, it is difficult to improve limits of quantitation. Increasing extraction temperature can be an effective way to enhance the concentration of furan in the headspace and achieve lower

limits of quantification. However, the generation of furan during the analysis increases at high temperatures affecting the trueness of the method. For instance, Şenyuva and Gökmen (2005) demonstrated furan formation in green coffee and in tomato and orange juices at an incubation temperature of 40 ºC for 30 minutes. In addition, the use of GC split injection instead of splitless is recommended for HS methods as a large volume is injected (commonly 1 ml) which once again reduce the sensitivity of the method.

Regarding the analysis of VNAs in food, the extraction and clean-up methods currently used involve many preliminary steps that require large amounts of solvents, time-consuming and are difficult to be automated. In this context, we considered solvent-free headspace solid-phase microextraction (HS-SPME) the technique of choice. When we started our research the application of HS-SPME for the analysis of VNAs in food was very limited. Therefore, our aim was to evaluate this sample preparation technique coupled to gas chromatography in order to develop a method applicable for the routine analysis of trace amounts of VNAs in food samples. With the objective of improving sensitivity and achieve detection limits at the low ppb levels needed in food analysis, GC-positive chemical ionization MS/MS that till now has only been used for the analysis of VNAs in environmental samples, was employed.

## 2.4. DEVELOPMENT OF ANALYTICAL METHODS FOR THE ANALYSIS OF FURAN AND VOLATILE N-NITROSAMINES IN FOOD

The results of the studies performed in this thesis related to the establishment of analytical methodology for the analysis of furan in food by HS-SPME coupled to GC-MS are included in Paper I and Paper II. Paper I is devoted to the development of a manual HS-SPME-GC-MS method and included the optimization of HS-SPME parameters for furan extraction, the establishment of the chromatographic and mass spectrometry conditions for the separation and analysis of this compound and the obtained quality parameters. Paper II was focused to the automation of the previous developed HS-SPME-GC-MS method with the objective of having available a rapid, automated and reliable method to be applied in furan surveys. In addition, in order to

validate the proposed automated HS-SPME-GC-MS method, the results obtained in the analysis of foods containing a broad range of furan levels were compared with those found using the static direct headspace method (HS-GC-MS) proposed by US FDA that was taken as reference.

For volatile N-nitrosamines, the results obtained in the development of an analytical method based on automated HS-SPME coupled to GC-MS are presented in Paper III. The thorough optimization of HS-SPME parameters is described and MS/MS conditions in chemical ionization mode using methanol as a reagent are established. Moreover, in order to propose a valid method for routine control of VNAs in food, a validation parameters including linearity, specificity, precision, trueness, limit of quantification, decision limit (CC$\alpha$) and detection limit (CC$\beta$) were established.

**2.4.1. PAPER I**

**ANALYSIS OF FURAN IN FOODS BY HEADSPACE SOLID-PHASE MICROEXTRACTION–GAS CHROMATOGRAPHY–ION TRAP MASS SPECTROMETRY**

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# Analysis of furan in foods by headspace solid-phase microextraction–gas chromatography–ion trap mass spectrometry[☆]

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## Abstract

A headspace-solid-phase microextraction (HS-SPME) coupled to gas chromatography–ion trap mass spectrometry (GC–IT-MS) method is proposed for the analysis of furan in different heat-treated carbohydrate-rich food samples. The extraction efficiency of six commercially available fibres was evaluated and it was found that a 75 $\mu$m carboxen/polydimethylsiloxane coating was the most suitable for the extraction of the furan. Parameters affecting the efficiency of HS-SPME procedure such as extraction temperature and time, ionic strength, headspace and aqueous volume ratio ($V_h/V_w$), desorption temperature and time, were optimized. Quality parameters were established using spiked water and food samples. Linearity ranged between 0.02 and 0.5 ng g$^{-1}$ and run-to-run and day-to-day precisions for food samples were lower than 6% and 10%, respectively. The limit of detection (LOD) of the method is sample dependent and ranged from 8 to 70 pg g$^{-1}$, while the limit of quantification is from 30 to 250 pg g$^{-1}$. Isotope dilution using furan-d$_4$ is proposed for furan determination providing similar results to those obtained by standard addition with internal standard (US Food and Drug Administration method). The developed HS-SPME–GC–IT-MS method was applied to the analysis of furan in different Spanish food samples from a local market, and concentrations ranging from 0.17 to 2279 ng g$^{-1}$ were found.
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*Keywords:* Food analysis; Furan; GC–MS; Headspace solid-phase microextraction

## 1. Introduction

Furan ($C_4H_4O$) is a volatile synthetic heterocyclic compound, which is used in the synthesis of many industrial, agricultural and pharmaceutical products [1], and it is also occurring at low concentration levels (<200 ng g$^{-1}$) in many heat-processed foods and drinks. Its presence in food has been known since the 1960s as one of the Maillard reaction products formed in the thermal processes of foods such as birch syrup, caramel, coffee, bread and cooked meat [2–11]. Nowadays, the formation of furan remains unclear, although it is known that its generation is related to the thermal degradation of carbohydrates, thermal oxidation of polyunsaturated fatty acids and/or thermal decomposition of ascorbic acid or its derivatives [12–15]. In recent years, the study of furan in foods has received special atten-

tion by several international food organizations such as the US Food and Drugs Administration (FDA) and the European Food Safety Authority (EFSA) [16,17], because this compound has been found to exhibit carcinogenic and cytotoxic activity on animals and causes harmful effects on human health [18,19]. Due to its toxicity and carcinogenicity, furan has been included by the International Agency for Research on Cancer in the Group 2B as possibly carcinogenic to humans [20] and has been classified by the US Department of Health and Human Services as a human pathogen [21]. Therefore, there is a need to dispose of reliable methods for its determination in foods in order to obtain information about its occurrence as well as the source of exposure, mechanisms of formation and toxicology.

The analysis of furan in food is not easy due to its high volatility and low molecular weight and, moreover, it is present in foods at low concentration levels (ng g$^{-1}$ or pg g$^{-1}$). Gas chromatography–mass spectrometry combined with headspace (HS-GC–MS) is the method currently applied for the analysis of furan in foods, and this was proposed by the FDA as reference method [22–24]. This methodology has also been applied by the Swiss Federal Office of Public Health (SFOPH) to the analysis

of furan in Swiss market food products [25]. Nevertheless, the HS-GC–MS method provides relatively high limits of detection (2–4 $ng\,g^{-1}$) and no data about precision were reported. In addition, standard addition is recommended for quantification in order to avoid matrix interferences. Recently, the Health Canada modified and simplified the FDA method in order to reduce the analysis time by using isotope dilution achieving limits of detection of 0.1 $ng\,g^{-1}$ [26].

Headspace-solid-phase microextraction (HS-SPME) is a simple and a solvent-free alternative method to traditional headspace for food analysis [27]. Since furan occurs at a trace level in food, HS-SPME can provide enough selectivity and sensitivity for the analysis of furan with low limits of detection and minimum interferences of matrix compounds. To date, only three papers that propose the use of HS-SPME for the analysis of furan in foods are reported in the literature [28–30]. However, a thorough optimization of the HS-SPME method has not been performed. In fact, only Bianchi et al. [30] studied the effect of time and temperature on the extraction efficiency. Both split [28] and splitless injection modes have been used, but the split mode provides relatively high detection limits (0.3 $ng\,g^{-1}$). In order to improve peak shape with the splitless mode, Goldmann et al. [29] used a cryofocuser, although poor precision in the retention time was obtained (RSD > 0.5%) with values higher than those recommended by the Commission Decision 2002/657/EC [31].

In the present paper, a HS-SPME–GC–MS method for the analysis of furan in food commodities is optimized. For this purpose, in order to obtain maximum sensitivity and selectivity, we carried out a systematic study of the HS-SPME parameters that affect the extraction and desorption process. In addition, the applicability of isotope dilution method for the quantification of furan in solid and liquid food samples was investigated and the proposed method was applied to the analysis of furan in several food samples.

## 2. Experimental

### 2.1. Reagents and standards

Furan and [$^{2}H_4$] furan (furan-$d_4$) were obtained from Sigma–Aldrich (Munich, Germany) at a purity higher than 99%. Individual stock standard solutions of these compounds at a concentration of 12 $mg\,g^{-1}$ were prepared in methanol. For the preparation of these solutions, 20 μl of each pure analyte were transferred using a micro-syringe to a 2 ml amber sealed vial previously filled with methanol. Intermediate standard solutions of furan and furan-$d_4$ were prepared at a concentration of 120 $\mu g\,g^{-1}$ from the stock standard solutions by dilution with methanol. All these standard solutions were stored at 0 °C and were prepared weekly. Water working standard solutions at a concentration of 60 $ng\,g^{-1}$ were individually prepared daily by spiking 40 ml of water with appropriate volumes of the intermediate standard solutions. For furan determination by the isotope dilution method, six-calibration standard solutions were prepared by adding in weight, through the septum, an appropriate amount of the furan water working standard solution (60 $ng\,g^{-1}$) into a 40 ml screw-cap glass vial fitted with silicone-PTFE septa

containing a 10 mm × 5 mm PTFE-coated stir bar, 4 g of sodium chloride and an appropriate volume (15 ml) of water. In addition, 25 μl of the furan-$d_4$ water working standard solution (60 $ng\,g^{-1}$) were added to each calibration solution. The furan concentration of these calibration solutions ranged from 0.02 to 0.5 $ng\,g^{-1}$ while for furan-$d_4$ was 0.08 $ng\,g^{-1}$. For the quantification of the food samples by standard addition method, replicate analysis ($n = 3$) of each food sample were carried out spiking the sample with an adequate amount of water standard solution of furan at 0% ($n = 3$), 25% ($n = 2$), 50% ($n = 2$), 100% ($n = 1$) and 150% ($n = 1$) of the estimated concentration of furan in each food sample using furan-$d_4$ as internal standard at a concentration of 0.1 $ng\,g^{-1}$. Methanol of gas chromatography grade, water of organic trace grade and sodium chloride were purchased from Merck (Darmstadt, Germany).

### 2.2. Sample preparation

Several canned and/or jarred food samples such as apple juice (concentrated juice base), multi-flower honey, spinach and cheese soup, instant and ground coffee and baby foods (rice/potato/chicken, six-fruit and vegetables/sole) were purchased from a local supermarket in Barcelona (Spain). Food samples such as apple juice, honey, and baby foods were homogenised for 1 min at 4 °C immersing the sample in an ice/water bath and using a mixer and a Ultra-Turrax T25 basic disperser (IKA-Werke, Staufen, Germany). The soup sample was prepared according to the directions of the producer by adding the content of the soup envelope (35 g) to 250 ml of boiling water. To assure the complete preparation of the soup, it was magnetically stirred for 10 min in a closed glass vessel and stored at 4 °C before analysis. For ground coffee, 9 g of solid were used to prepare 50 ml of brewed coffee using a professional coffee-machine. After preparation, the brewed coffee was placed in a 40 ml screw-cap glass vial, which was stored at 4 °C before analysis. For instant coffee, furan was determined in the brewed coffee and in solid material. The brewed instant coffee was prepared according to the recommendations of the manufacturer. Briefly, 2 g of powder were mixed with 65 ml of boiling water, and the brewed coffee obtained was placed in a closed vial and stored at 4 °C before analysis. To determine the content of furan in the powder of instant coffee, 0.5 g of solid were mixed with 40 g of cold water (4 °C) in a 40 ml screw-cap glass vial. Due to the different concentrations of furan in the selected foods, the sample size was optimized for each sample (see Table 2).

### 2.3. GC–MS conditions

The determination of furan by GC–MS was carried out using a Trace GC 2000 gas chromatograph coupled to a GCQ/Polaris ion-trap mass spectrometer (ThermoFinnigan, Austin, TX, USA). For furan separation, several GC fused-silica capillary columns were tested: DB-5 ms (5% phenyl-, 95% methylpolysiloxane), 30 m × 0.25 mm I.D., 0.25 μm film thickness (J&W Scientific, Folsom, CA, USA), DB-17 (50% phenyl-, 50% methylpolysiloxane), 30 m × 0.25 mm I.D., 0.25 μm film thickness, and BPX-volatiles (cyanopropylphenyl

*Analysis of process-induced food carcinogens*

*M.S. Altaki et al. / J. Chromatogr. A 1146 (2007) 103–109* 105

polysilphenylene-siloxane), 60 m × 0.25 mm I.D., 1.4 μm film thickness (SGE Europe, Villebon, France). The best results were obtained using the BPX-volatiles capillary column. The carrier gas was helium at a constant flow-rate of 1.7 ml min$^{-1}$ held by electronic pressure control. The oven temperature programme was 35 °C (held for 2 min) to 230 °C at 20 °C min$^{-1}$ (held for 5 min). A SPME glass inlet liner (I.D., 0.75 mm, SGE) was used. The injector temperature was 275 °C, and the splitless injection mode (3 min) was used. The MS operating conditions were the following: positive electron ionization mode (EI+) using automatic gain control (AGC) with 70 eV of electron energy and 250 μA of emission current. The instrument was tuned using perfluorotributylamine (FC-43) according to the manufacturer's recommendations in order to achieve the best sensitivity. The ion source and transfer line temperatures were 200 °C and 280 °C, respectively. Electron multiplier voltage was set to 1350 V (10$^5$ gain) by automatic tuning. EI full-scan data acquisition was registered over the range *m/z* 35–100 at 0.73 s per scan (5 μscan per scan). Xcalibur version 1.2 software was used for control, general operation and data acquisition of the results. For furan determination, *m/z* 68 [M]$^{+\bullet}$ and *m/z* 39 [M-CHO]$^+$ were monitored for quantification and confirmation of furan, respectively. In the same way, *m/z* 72 [M]$^{+\bullet}$ and *m/z* 42 [M-C$^2$HO]$^+$ were monitored and used for furan-d$_4$.

### 2.4. Headspace-SPME procedure

SPME experiments were performed using a manual fiber holder supplied from Supelco (Bellefonte, PA, USA). Six commercially available fibres, polydimethylsiloxane (PDMS, 100 μm), Carboxen/polydimethylsiloxane (CAR/PDMS, 75 μm), polyacrylate (PA, 85 μm), polydimethylsiloxane/divinylbenzene (PDMS/DVB, 65 μm), Carbowax/divinylbenzene (CW/DVB, 65 μm) and a Stable-Flex divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm) were purchased from Supelco. Before use, each fibre was conditioned in the GC injection port under helium flow in accordance with the temperature and time recommended by the manufacturer. Fibre blanks were run periodically to ensure the absence of contaminants or carryover.

The SPME procedure was as follows: an optimal amount of the sample solution (see Section 2.2) was placed in a 40 ml screw-cap glass vial fitted with silicone-PTFE septa containing a 10 mm × 5 mm PTFE-coated stir bar, 4 g of sodium chloride and an appropriate volume (15 ml) of water. During the addition of the sample, the sample vial was immersed in an ice/water bath (4 °C) in order to prevent possible losses of the analyte. Then, 25 μl of the furan-d$_4$ in water working standard solution (60 ng g$^{-1}$) were added by weight through the septum of the sample vial. Before HS-SPME analysis, the sample vial was vortex mixed for 3 min, and conditioned for 15 min in a thermostatic water bath at a temperature of 25 °C. Then the sample and the calibration solutions were extracted using Carboxen/polydimethylsiloxane (CAR/PDMS 75 μm) fibre at 25 °C for 30 min using a constant magnetic agitation rate of 1200 rpm. Finally, thermal desorption of the analyte was carried out by exposing the fibre in the GC injector port at 275 °C for 2 min. The fibre was kept in the injector port for an additional time of 3 min, with the injector in split mode (purge on) in order to prevent possible carryover.

## 3. Results and discussion

### 3.1. HS-SPME optimization

Initially, different GC capillary columns such as DB-5 ms and DB-17 both of 30 m length and 0.25 μm film thickness, and BPX-volatiles of 60 m length and 1.4 μm film thickness were evaluated in order to optimize the chromatographic elution of furan. Due to the relatively higher polarity and film thickness of BPX-volatiles column, furan was eluted from the column with an appropriate retention time (5.3 min), while for the other columns the retention times were similar to the dead time of the column. Therefore, BPX-volatiles was chosen for furan determination. In addition, the use of conventional and SPME glass liners with the splitless injection mode as well as the effect of the carrier gas and programme oven temperature on the peak shape were evaluated. A symmetrical chromatographic peak was obtained using a relatively high constant flow of carrier gas (1.7 ml min$^{-1}$) using a initial oven temperature of 35 °C and increasing up to 230 °C at 20 °C min$^{-1}$. The use of a SPME glass liner (0.75 mm I.D.) instead of the conventional glass liner (3 mm I.D.) in the injector port made it possible to obtain higher sensitivity with a small peak width.

The first step in the optimization of SPME procedure was the selection of the appropriate fibre. Six fibres: 100 μm PDMS, 75 μm CAR/PDMS, 85 μm PA, 65 μm PDMS/DVB, 65 μm CW/DVB, and 50/30 μm DVB/CAR/PDMS, were evaluated in order to obtain the best sensitivity and selectivity for furan determination. For this purpose, a long extraction time (60 min) was applied to ensure that the maximum amount of furan was extracted. In addition, extraction temperature was fixed to 35 °C while desorption time in the GC injector port was 5 min. The desorption temperatures were within the recommended operating range for each fibre: 250 °C for PDMS, 290 °C for PA, 260 °C for PDMS/DVB, 220 °C for CW/DVB, and 280 °C for CAR/PDMS and 270 °C for DVB/CAR/PDMS. No carryover on second desorption was found for any of the fibres, indicating complete removal of analyte at these temperatures. The 75 μm CAR/PDMS provided an extraction yield higher than the other fibres, from 8-fold for DVB/CAR/PDMS to 650-fold for PA. This may be due to the strong retention of the analyte into the small pores of Carboxen layer. The dual-coated DVB/CAR/PDMS fibre showed a lower capacity to retain furan than CAR/PDMS probably due to the higher mean micropore diameter (17 Å) than that of Carboxen (10 Å), that seems to be ideal for the SPME analysis of small molecules in the C$_2$–C$_6$ range, such as furan. Therefore, a CAR/PDMS coating was selected for all subsequent experiments.

The effect of sampling temperature on the furan extraction yield was also examined from 25 °C to 40 °C using CAR/PDMS fiber and maintaining constant the other extraction parameters. As can be seen in Fig. 1A, by increasing the extraction temperature, a decrease in the relative response is produced. This is due
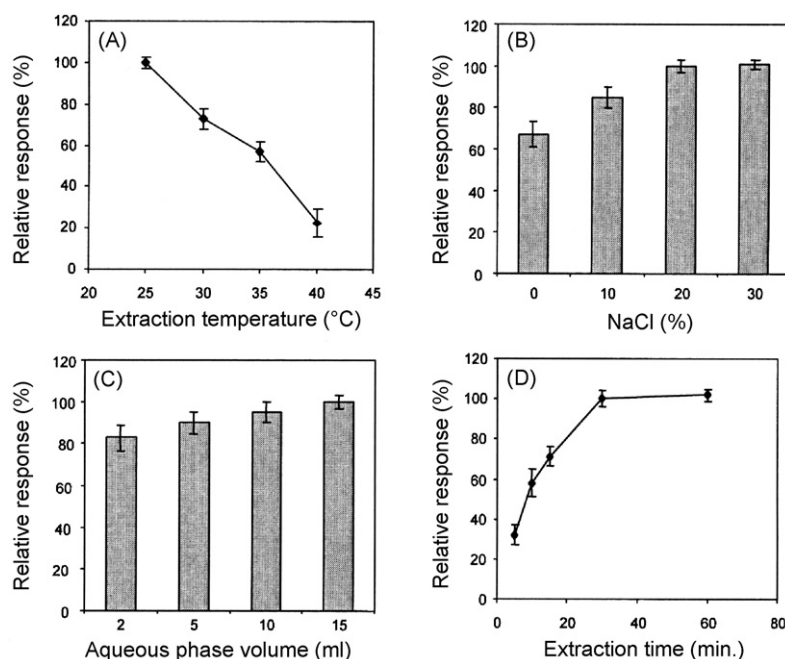
Fig. 1. Effect of (A) extraction temperature, (B) the addition of sodium chloride, (C) the aqueous phase volume, and (D) extraction time on the extraction efficiency of furan by HS-SPME. Conditions: 75 μm CAR/PDMS fibre; extraction time 60 min; desorption time 5 min.

to a decrease in the distribution constant of the furan between the headspace and the fibre coating, because the extraction process is exothermic. An extraction temperature of 25 °C was chosen since at this temperature the best furan response was obtained. This value is lower than that proposed by Goldmann et al. [29], but at the temperature they propose (50 °C) the sensitivity of the fibre is clearly lower than at 25 °C. Another important parameter that affects the efficiency of HS-SPME extraction procedure is the ionic strength. For many organic analytes, aqueous solubility decreases with increasing ionic strength, and thus the partitioning from the aqueous solution to the headspace is improved. Sodium chloride was used in order to determine the effect of the ionic strength in the extraction yield. Different amounts of NaCl in the range of 0–30% (w/w) were added to the sample vial in order to enhance the amount of furan in the vapour phase. As expected, the amount of furan absorbed onto the fibre greatly increased when the percentage of salt added achieved a concentration of 20% (w/w). At higher amounts of salt, a constant response was obtained (see Fig. 1B). So, a salt percentage at 20% (w/w) was selected as the optimal condition. In addition, the effect of the headspace and aqueous solution volume ratio ($V_h/V_w$) on the furan extraction yield was studied. This parameter was evaluated by varying the volume of the aqueous solution from 2 to 15 ml using a 40 ml glass vial and maintaining the concentration of furan constant. Volumes of aqueous phase higher than 15 ml (25 ml of headspace) were not studied because a volume of at least 25 ml of headspace is required for the complete spreading of the fibre. Fig. 1C shows the relative response of furan obtained at different aqueous volume. As can be seen, a slight increase in the furan response was observed when the aqueous solution volume increased. An aqueous volume of 15 ml (25 ml of headspace, $V_h/V_w = 1.67$) was chosen to obtain maximum extraction efficiency of furan. As a final step

towards the optimization of the extraction process, the extraction time required to reach equilibrium between the fibre coating and the headspace were determined. The extraction time profiles of furan were studied from 5 to 60 min using the optimal conditions previously established. Fig. 1D shows the absorption time profile of furan where it can be deduced that the time required to reach the equilibrium was 30 min, and this was considered as the optimal value.

Several desorption temperatures 230 °C, 275 °C and 290 °C, were also studied, maintaining constant the desorption time at 5 min. The results showed that the response increases when the temperature was changed from 230 °C to 275 °C, while no differences in the response were obtained at higher temperatures. So, 275 °C was selected as the optimal desoption temperature. Finally, the desorption time of furan was evaluated and 2 min were enough for the complete desorption.

In summary, the HS-SPME optimal conditions for the analysis of furan using a 75 μm CAR/PDMS fibre were: extraction temperature 25 °C, sampling time 30 min, stirring speed 1200 rpm, NaCl concentration 20% (w/w), headspace/aqueous volume ratio 25 ml/15 ml in a 40 ml glass vial, desorption temperature 275 °C and desorption time 2 min.

### 3.2. Quality parameters

To examine the performance of the proposed HS-SPME–GC–MS method, quality parameters were established. In order to study the linearity, water samples were spiked with appropriate amounts of furan over the range 0.02–0.5 ng g$^{-1}$, and were extracted using the established HS-SPME–GC–MS method. Good linearity with a correlation coefficient ($r^2$) higher than 0.999 was obtained. Limit of detection (LOD) and quantification (LOQ) based on a signal-to-noise ratio (S/N) of 3:1

*Analysis of process-induced food carcinogens*

*M.S. Altaki et al. / J. Chromatogr. A 1146 (2007) 103–109* 107

Table 1
Quality parameters of the HS-SPME–GC–IT-MS method

| Sample | Precision (RSD, %) | | LOD (pg g$^{-1}$) | LOQ (pg g$^{-1}$) |
|---|---|---|---|---|
| | Run-to-run[a] | Day-to-day[b] | | |
| *Spiked water standard solution* | | | 1 | 15 |
| Low level (0.03 ng g$^{-1}$) | 3 | 5 | | |
| High level (0.3 ng g$^{-1}$) | 1 | 3 | | |
| *Food samples* | | | | |
| Apple juice | 5 | 8 | 8 | 30 |
| Multi-flower honey | 4 | 7 | 15 | 55 |
| Spinach with cheese soup | 6 | 9 | 25 | 90 |
| Brewed instant coffee | 3 | 5 | 70 | 250 |
| Rice, potato and chicken baby food | 5 | 10 | 45 | 160 |

[a] $n = 3$.
[b] $n = 3$ replicates × 3 days.

and 10:1, respectively, were determined using both food samples and water standard solutions. For water standard solutions, water of organic trace grade spiked with furan at low concentration levels was used, and a LOD of 1 pg g$^{-1}$ and a LOQ of 15 pg g$^{-1}$ were obtained. For food samples, as no blank sample was found, LODs and LOQs were established using food samples with very low concentration levels. Alternatively, these quality parameters were also determined, spiking the food samples with furan-d$_4$ at low concentration levels. LOD and LOQ values obtained using both methods were in agreement and were sample-dependent. LODs ranged from 8 pg g$^{-1}$ in apple juice to 70 pg g$^{-1}$ for instant coffee were determined (Table 1), while LOQs between 30 and 250 pg g$^{-1}$ were obtained.

For run-to-run and day-to-day precision, three replicate analyses of water standard solutions spiked at two different levels (low level: 30 pg g$^{-1}$ and high level: 300 pg g$^{-1}$) were analyzed in 1 day and on 3 different days, respectively. Good precision was achieved with a relative standard deviation (RSD) of 1–3% for run-to-run and 3–5% for day-to-day (Table 1). In addition, the precision of the method was evaluated using food samples: an apple juice, a honey, a soup, a brewed instant coffee and a baby food (rice, potato and chicken). For run-to-run and day-to-day

precision, three replicate determinations for each sample were consecutively carried out in 1 day and on 3 non-consecutive days (3 replicates × 3 days), respectively. The precision achieved was good enough with values of relative standard deviations lower than 6% for run-to-run and 10% for day-to-day (Table 1).

### 3.3. Analysis of furan in food commodities

In order to evaluate the applicability of the optimized HS-SPME–GC–IT-MS method, several selected food samples were studied. All samples were analyzed in triplicate by the optimized method following the procedure described in Section 2. The quantification was performed by isotope dilution using as calibration solutions six water spiked with furan at concentrations within the linear range of the method and containing a constant amount of furan-d$_4$. The calibration solutions and food samples were analysed following the optimized HS-SPME–GC–IT-MS method.

The results obtained on the analysis of several food samples using the developed method are summarised in Table 2. Furan was detected in all samples at concentration levels ranging from 0.17 ng g$^{-1}$ for soup to 2279 ng g$^{-1}$ for powder instant

Table 2
Analysis of furan in food by HS-SPME–GC–IT-MS

| Food sample | Description | Sample size (g) | Concentration (ng g$^{-1}$) | | | |
|---|---|---|---|---|---|---|
| | | | HS-SPME and Isotopic dilution[a] | | HS-SPME and standard addition[b] | |
| | | | Mean | SD | Mean | SD |
| Apple juice | Concentrated juice based | 1 | 1.2 | 0.1 | 1.3 | 0.2 |
| Honey | Multi-floral | 0.5 | 5.8 | 0.2 | 6.0 | 0.3 |
| Soup | Spinach with cheese | 5 | 0.17 | 0.01 | – | – |
| Coffee | Powder instant coffee | 100 μl[c] | 2279 | 53 | 2353 | 176 |
| | Brewed instant coffee | 0.2 | 12.6 | 0.8 | – | – |
| | Brewed ground coffee (prof. machine) | 0.05 | 58 | 3.5 | – | – |
| Baby foods | Rice, potato (4%) and chicken (12%) | 0.5 | 16.6 | 1.0 | 14.3 | 1.8 |
| | Six fruits (73%) | 1 | 0.70 | 0.05 | – | – |
| | Vegetables with sole (8.2%) | 0.1 | 67 | 2 | – | – |

[a] $n = 3$ replicates.
[b] $n = 3$ standard addition curves.
[c] 100 μl of an instant coffee solution (0.5 g of powder coffee in 40 ml cold water).
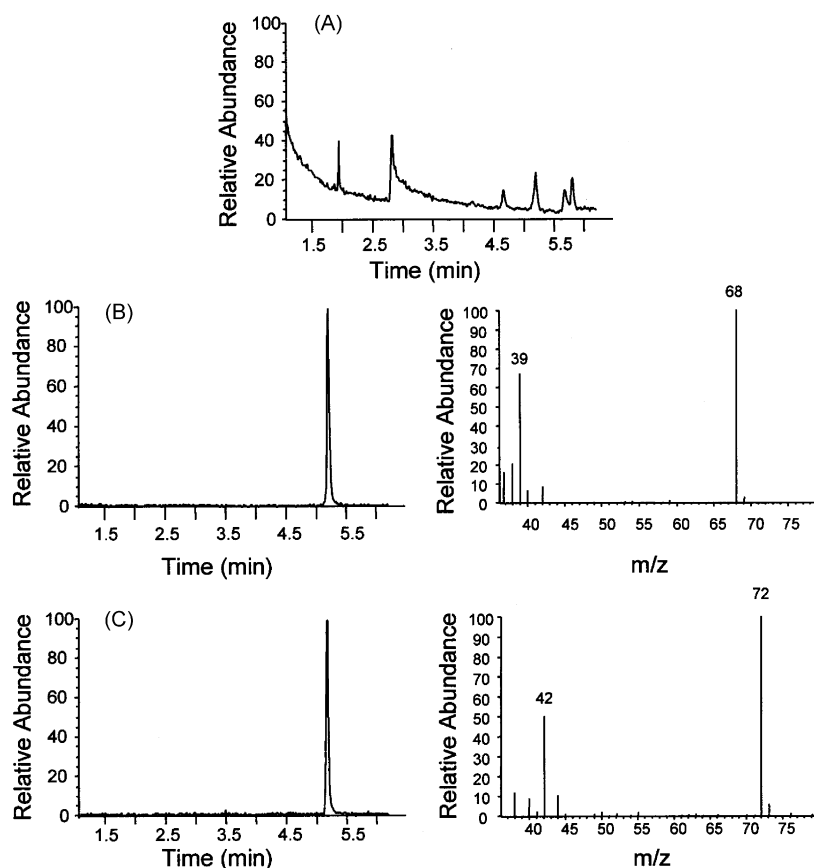
Fig. 2. HS-SPME–GC–MS chromatograms of (A) total-ion current (TIC), (B) reconstructed ion current $m/z$ 68 for furan, and (C) reconstructed ion current of $m/z$ 72 for furan-$d_4$, and the corresponding spectra of a honey sample obtained by the proposed method.

coffee, the precision being achieved between 3 and 8%. These concentrations agree with those reported by the FDA, the Swiss Federal Office of Public Health (SFOPH) and the EFSA [21]. For instance, the FDA reported the presence of furan in baby foods at concentration levels from not detected to 108 ng g$^{-1}$, while in the brewed ground coffee samples the concentration ranged from 5 to 85 ng g$^{-1}$. In addition, the SFOPH reported concentrations of furan in powder instant coffee ranging between 959 and 2740 ng g$^{-1}$ which are in agreement with that determined using the proposed method (2279 ng g$^{-1}$). For soup and six fruits-based baby foods, furan was found at lower concentration levels than those reported in the literature [17].

Fig. 2 shows as an example the GC–MS total ion current chromatogram of a honey sample and the single ion chromatograms of furan ($m/z$ 68) and furan-$d_4$ ($m/z$ 72) and their corresponding mass spectra. As can be seen, the proposed method provides high selectivity for furan determination and no interferences from other matrix compounds were detected. In order to assure the reliability of results obtained by isotope dilution, the standard addition method with internal standard proposed by the FDA for furan quantification [22–24] was also applied for the analysis of selected food samples such as a honey, an apple juice, a powder instant coffee and a baby food (rice, potato with chicken). For this purpose, replicate analyses ($n=3$) of each food sample were also carried out by standard addition method (Section 2.1), analysing the standard addition solutions using the opti-

mized HS-SPME–GC–MS procedure (Sections 2.3 and 2.4). In Table 2, the results obtained in the analysis of furan in the four food samples by standard addition method are included. As can be seen, good agreement between the two quantification methods was obtained and no significant differences were observed, demonstrating the validity of the isotope dilution method. Due to this good agreement, isotope dilution was proposed instead of standard addition, as recommended the FDA for quantification of furan in food, since isotope dilution is a rapid, simple and accurate method and also allows a high sample throughput in routine analysis.

## 4. Conclusions

The feasibility of headspace-SPME combined with GC–MS for the analysis of furan in food samples has been demonstrated. The CAR/PDMS fibre was found to be the most effective coating for the extraction of furan, with sensitivity higher than that for other commercially available fibres. Maximum responses were obtained using extraction conditions of 25 °C for 30 min with 20% (w/w) NaCl and a headspace/aqueous phase volume ratio of 25 ml/15 ml. The isotope dilution method provided similar results as the standard addition method recommended the FDA for quantification of furan in food The combination of HS-SPME and GC–MS showed good linearity, and the run-to-run and day-to-day precisions for foods were RSD <6% and <10%,

*Analysis of process-induced food carcinogens*

*M.S. Altaki et al. / J. Chromatogr. A 1146 (2007) 103–109* 109

respectively. The limits of detection were in the low pg g$^{-1}$ levels. The HS-SPME–GC–MS method was successfully applied to the analysis of furan in selected food products and can be proposed as a simple and accurate alternative to the FDA method for the analysis of furan in foods.

## Acknowledgments

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**2.4.2. PAPER II**

**AUTOMATED HEADSPACE SOLID-PHASE MICROEXTRACTION VERSUS HEADSPACE FOR THE ANALYSIS OF FURAN IN FOODS BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY**

# Automated headspace solid-phase microextraction versus headspace for the analysis of furan in foods by gas chromatography–mass spectrometry

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## ARTICLE INFO

## ABSTRACT

A simple, fast and fully automated method based on headspace solid-phase microextraction coupled on-line with gas chromatography–ion trap mass spectrometry (HS-SPME–GC–ITMS) is proposed for furan determination in foods. The performance of the proposed method was compared to the automated headspace-GC–MS method, proposed by the US Food and Drugs Administration (US FDA), in terms of repeatability, limits of the detection and quantification. Both methods gave similar results for furan determination in selected food samples, although slightly worse precision (RSD%, 9–12%) and higher limits of detection (from 5 to 20 times higher) were obtained by the headspace method. In addition, higher sample throughput in routine furan analysis was obtained using the proposed HS-SPME–GC–ITMS method with isotope dilution than using the US FDA method, which recommends standard addition for quantification. The proposed method provides good precision (RSD% <10%) and low limits of detection, ranging from 0.02 to 0.12 ng g$^{-1}$ depending on the sample. The developed HS-SPME–GC–MS method was used to analyse furan in several Spanish food commodities and concentrations ranging from 0.1 ng g$^{-1}$ to 1.1 μg g$^{-1}$ were found.

## 1. Introduction

Furan ($C_4H_4O$) is a volatile heterocyclic compound which is formed during the heat treatment of foods and drinks as one of the Maillard reaction products [1]. Furan occurs in a wide variety of foods, such as coffee, canned and jarred foods containing meat, and various vegetables [2,3], at concentration levels up to 174 ng g$^{-1}$. The common presence of furan suggests that there are probably multiple routes of formation rather than a single mechanism [4]. Nowadays, it is accepted that its generation is mainly related to the thermal degradation of carbohydrates, the oxidation of polyunsaturated fatty acids and the decomposition of ascorbic acid or its derivatives [5–11]. The occurrence of furan in food and drink is a cause for concern because it is both carcinogenic and cytotoxic in rats and mice [12–14]. It has been classified as possibly carcinogenic to humans (Group 2B) by the International Agency for Research on Cancer (IARC) [15], and has been included by the US Department of Health and Human Service in the human pathogen list [16]. Since furan has become a potential food safety issue, several international food organizations such as the US Food and Drugs Administration (FDA) and the EFSA have launched monitoring programs to survey the furan content of selected foods and beverages and to collect more information about furan formation, human exposure and toxicity [4,17]. Recently, the EFSA issued a call for more information on the occurrence of furan in foods [18]. Therefore, there is great interest in finding rapid, selective and sensitive analytical methods for obtaining reliable data to assess the risk to human health [19,20].

Due to its high volatility (B.P. 31.4 °C), furan is currently being analysed by headspace (HS) combined with gas chromatography–mass spectrometry (HS-GC–MS) [21,22]. This method, first proposed by the FDA in 2004, is relatively simple and well-established: a food sample in liquid or slurry form is heated at 80 °C for 30 min and the headspace is sampled and analysed by GC–MS. Furan is quantified by standard addition using furan-d$_4$ as internal standard. A similar method was used by the Swiss Federal Office of Public Health (SFOPH) for collecting data from a great number of foods likely to contain furan [3,23]. Since furan is formed during the analysis by sample heating [10,24–26], the FDA method was updated in 2006 by decreasing the headspace temperature from 80 to 60 °C [27]. This new FDA method provides limits of detection of 2–5 ng g$^{-1}$ for most food matrices. To date, the method has only been validated in-house [24] and a limited number of proficiency tests have been performed [21]. Recently, the Health Canada modified this headspace method [28], achieving limits of detection in foods between 0.8 and 4.85 ng g$^{-1}$. Nevertheless, the use of standard addition for quantification increases the analysis time and reduces the applicability of the method in a high-throughput routine laboratory operation.

Headspace solid-phase microextraction (HS-SPME) has also been used for furan determination. HS-SPME coupled with GC–MS has proved to be an excellent alternative to headspace for the analysis of volatile compounds at low concentration levels in food samples [29,30], since it provides enough sensitivity with minimum interferences of matrix compounds. HS-SPME has been used for the analysis of furan in coffee [31], orange juice [9] and foods in general [32–34]. Most of these methods are based on manual HS-SPME providing limits of detection in the low pg g$^{-1}$ range. In a previous paper, we proposed a manual HS-SPME method combined with isotope dilution and GC–IT–MS for the analysis of furan in foods [34]. This HS-SPME approach provided high selectivity and limits of detection between 8 and 70 pg g$^{-1}$. Although this method is rapid and effective for furan analysis, it is labour-intensive when a lot of samples are analysed. Therefore, full automation of HS-SPME could shorten total analysis time, thus improving productivity.

The aim of the present paper was to develop a fully automated HS-SPME method coupled on-line with GC–MS for routine analysis of furan in food commodities. The method was evaluated by comparing its performance to that of the US FDA method based on headspace, the usual reference procedure for furan analysis. Quality parameters of the two methods were established and compared using several food samples. Finally, the fully automated HS-SPME–GC–MS method was applied to the determination of furan in several food commodities.

## 2. Experimental

### 2.1. Chemicals and standards

Furan and [$^2$H$_4$] furan (furan-$d_4$) were purchased from Sigma–Aldrich (Munich, Germany) at purity higher than 99%. Individual stock standard solutions of furan and furan-$d_4$ at a concentration of 9 mg g$^{-1}$ in methanol were prepared by transferring 15 μl of each pure compound to a 2 ml amber sealed vial previously filled with methanol. Intermediate individual standard solutions of furan and furan-$d_4$ were prepared at a concentration of 45 μg g$^{-1}$ from the stock standard solutions by appropriate dilution with methanol. All these solutions were stored at 0 °C and prepared weekly. For HS-SPME analysis, individual water working standard solutions of furan and furan-$d_4$ at a concentration of 30 ng g$^{-1}$ were prepared daily by spiking 20 ml of water with appropriate volumes of the intermediate standard solutions. For furan determination by the isotope dilution method, eight-calibration standard solutions at concentrations ranging from 0.01 to 10 ng g$^{-1}$ were prepared by adding in weight, through the septum, an appropriate amount of the furan water working standard solution (30 ng g$^{-1}$) into a 20 ml sealed vial containing a 10 mm × 5 mm PTFE-coated stir bar, 2 g of sodium chloride and water (up to 8 ml). In addition, 70 μl of the furan-$d_4$ water working solution (30 ng g$^{-1}$) was added to each calibration solution, to achieve a concentration of 0.2 ng g$^{-1}$. For headspace analysis, individual water working standard solutions of furan and furan-$d_4$ at a concentration of 500 ng g$^{-1}$ were prepared daily from intermediate standard solutions and were used for the furan quantification by standard addition method. Water of organic trace grade, methanol of gas chromatography grade and sodium chloride of analytical grade were all obtained from Merck (Darmstadt, Germany).

### 2.2. Food sample preparation

A total of twenty-four food samples purchased from a local supermarket in Barcelona (Spain) were analysed for furan. Packaged food samples were stored at 4 °C to prevent possible losses of furan. Liquid samples (juices, honeys and broths) were homogenized in their own container for 1 min by manual shaking, while semi-solid samples (baby foods and sauces) were homogenized for 1 min at 4 °C by immersing the pot in an ice/water bath (15 min) and using a mixer and an Ultra-Turrax T25 basic disperser (IKA-Werke, Staufen, Germany). Cooked pulse samples (lentils, white kidney beans and chickpeas) were prepared in the same way as semi-solid samples by adding an appropriate amount of cold water (1:1, w/w) to facilitate the homogenization processes. For ground coffee, nine grams of solid coffee were used to obtain approximately 60 ml of brewed coffee, using both an automatic espresso coffee machine and a home coffee pot-brewer. After preparation, the brewed coffee was placed in a 40 ml screw-cap glass vial, which was stored at 4 °C before analysis. Furan in instant coffee was determined in both brewed coffee and powdered material. The brewed instant coffee was prepared according to the recommendations of the manufacturer, mixing 2 g of powder with 60 g of boiling water. To determine the content of furan in powdered instant coffee, 0.5 g of powder was mixed with 40 ml of cold water (4 °C) in a 40 ml closed glass vial. Soup samples (dehydrated material) were prepared in line with the manufacturer's indications. Approximately 50 g of solid soup was mixed first with 100 g of warm water until complete dissolution and then with 200 g of water. The mixture was heated for 15 min with manual shaking. After preparation and the homogenization process, all samples were immediately kept in a closed vial without headspace to minimize the possible loss of furan.

### 2.3. GC–MS conditions

All GC–MS analyses were performed on a CP-3800 gas chromatograph coupled with a Saturn-2200 ion trap mass spectrometer (Varian, Mississauga, Canada). A BPX-volatile (cyanopropylphenyl polysilphenylene-siloxane), 60 m × 0.25 mm I.D., fused-silica capillary column (SGE Europe, Villebon, France) of 1.4 μm film thickness was used for chromatographic separation. The oven temperature program was 35 °C (held for 2 min) to 230 °C at 20 °C min$^{-1}$ (held for 5 min). Helium was used as carrier gas at a constant flow-rate of 1.7 ml min$^{-1}$ held by electronic flow control (EFC). The injector temperature was maintained at 275 °C and the splitless injection mode (3 min) was used for HS-SPME experiments, while for headspace (US FDA method) the injector was operated in split injection mode at 2:1 split ratio. An SPME glass inlet liner (I.D., 0.75 mm, SGE Europe) and a 23-gauge Merlin Micro-seal septum (Supelco, Bellefonte, PA, USA) were used for SPME analysis, while for headspace a split inlet liner (I.D., 3 mm, SGE Europe) was used. The ion trap MS was operated in electron ionization (EI) mode with 70 eV of electron energy and 30 μA of emission current, using automatic gain control (AGC). The instrument was tuned using perfluorotributylamine (FC-43) according to the manufacturer's recommendations to achieve the best sensitivity. Manifold, ion source trap and transfer line temperatures were set at 80, 200 and 280 °C, respectively. The electron multiplier voltage and the axial modulation amplitude were set to 1350 V ($10^5$ gain) and 4.0 V, respectively. In addition, a maximum ionization time of 25,000 μs, a pre-scan ionization time of 100 μs with a background mass of 45 $m/z$ and an RF dump value of 650 $m/z$, were set for all experiments. For data acquisition, EI full-scan mode was used over the mass range $m/z$ 35–100 at 0.75 s/scan (7 μscan per scan). Varian MS Workstation software (version 6.42) was used for control, general operating and data acquisition. For quantification and confirmation, $m/z$ 68 [M]$^{+\cdot}$ and $m/z$ 39 [M-CHO]$^+$ for furan and $m/z$ 72 and $m/z$ 42 [M-C$^2$HO]$^+$ for furan-$d_4$ were monitored.

### 2.4. Automatic HS-SPME method

The HS-SPME experiments were carried out with a 75-μm Carboxen-polydimethylsiloxane fibre (CAR/PDMS) (Supelco, Bellefonte, PA, USA) on a CTC Combi-Pal autosampler (CTC Analytics

AG, Zwingen, Switzerland), which was equipped with a sample tray for 32 vials of 20 ml, an SPME fibre conditioning station, and a temperature-controlled single magnet mixer tray (SMM tray) (Chromtech, Idstein, Germany). CTC Combi-Pal autosampler was controlled and programmed with the Cycle composer software version 1.5.3. Before use, the CAR/PDMS fibre was conditioned at 300 °C under helium flow for 1 h in accordance with the manufacturer's recommendations. Fibre blanks were run daily to ensure the absence of contaminants or carry-over.

After optimization, the automatic HS-SPME procedure used for furan determination in the selected food samples was as follows: 3 g of the prepared and homogenized sample (for baby food, brewed coffee, soup and broth, 1–2 g were used) was quickly transferred to a 20 ml headspace vial containing a PTFE-coated stir bar, 2 g of sodium chloride and an adequate amount of water (up to 8 ml). During preparation, the sample vial was immersed in an ice/water bath (4 °C) to prevent possible losses of the analyte. The vial was then spiked with 70 μl of furan-$d_4$ water working solution (30 ng g$^{-1}$) by weight through the septum of the vial and was vortexed for 3 min before analysis. Then the sample and calibration solutions were placed in the sample tray of the autosampler and were analysed by means of the automatic HS-SPME procedure. Before HS-SPME analysis, the vials were conditioned in the SMM tray for 5 min at a temperature of 30 °C and under a magnetic agitation rate of 750 rpm. At the end of this time period, the CAR/PDMS fibre was cleaned for 1 min at 275 °C using the SPME fibre conditioning station, and the sample was extracted from the headspace at 30 °C for 20 min with a constant magnetic agitation rate of 750 rpm. Thermal desorption of the analyte was accomplished by exposing the fibre in the GC injector port at 275 °C for 2 min. To reduce the analysis time and to obtain a high sample throughput, a new HS-SPME experiment was performed while the GC was being run.

### 2.5. Headspace method

Headspace analysis of furan in food was performed following the proposed US FDA method [27] but using a BPX-volatiles as GC column and a CTC Combi-Pal autosampler for headspace analysis. This autosampler was equipped with a 1 ml gas-tight headspace syringe (1001N CTC, Hamilton Company, Bonaduz, Switzerland), a syringe heater and plunger holder (CTC Analytics), was used. For conditioning and cleaning the headspace syringe, the CTC syringe heater was set to 150 °C for 2 min with helium flushing. Furan was determined by standard addition method with internal standard, as proposed in the FDA method. For this purpose, replicate analyses ($n = 3$) of the food sample were carried out by transferring an adequate amount of the homogenized sample (5 g for solid and semi-solid samples and 10 g for liquid samples) to a 20 ml headspace vial containing a PTFE-coated stir bar and an appropriate amount of water saturated with NaCl (up to 10 ml). Then the sample was spiked with adequate amounts of a furan aqueous working standard solution (500 ng g$^{-1}$) at 0% ($n = 3$), 25% ($n = 2$), 50% ($n = 2$), 100% ($n = 1$), 150% ($n = 1$) and 200% ($n = 1$) of the estimated concentration of furan in sample. Finally, furan-$d_4$ was added to each sample vial to obtain a concentration of 200% of the estimated furan content. Automated headspace sampling conditions were as follows: the sample vial was conditioned for 30 min at an incubation temperature of 60 °C stirring at 750 rpm using the temperature-controlled single magnet mixer tray (Chromtech, Idstein, Germany). The temperature of the syringe heater was set to 100 °C and the syringe was flushed with helium before and after each extraction. Headspace (1 ml) was sampled at 100 μl s$^{-1}$ and injected into the GC port at 275 °C and with an injection speed of 250 μl s$^{-1}$. GC–MS conditions for furan determination using the headspace method were the same as those used for SPME experiments (Section 2.3).

**Table 1**
Effect of the extraction temperature, extraction time, headspace/aqueous volume ratio and stirring rate on the response of furan using automated HS-SPME (optimum conditions are indicated in bold).

| Temperature | | Time | | Volume ratio | | Stirring rate | |
|---|---|---|---|---|---|---|---|
| °C | RR[*] | min | RR | $V_h/V_w$ | RR | rpm | RR |
| **30** | **100%** | 5 | 42% | 5.7 | 75% | 0 | 62% |
| 35 | 78% | 10 | 65% | 3 | 87% | 250 | 80% |
| 40 | 43% | **20** | **100%** | **1.5** | **100%** | 500 | 91% |
| | | 30 | 100% | | | **750** | **100%** |

[*] Relative response ($n = 3$).

## 3. Results and discussion

### 3.1. Automated HS-SPME method

The optimum key parameters affecting HS-SPME efficiency for furan determination in food were previously reported for manual HS-SPME [34]. Nevertheless, to automate this method some SPME conditions such as extraction time and temperature, sampling stirring speed and headspace/aqueous volume ration were optimized because of certain characteristics of the Combi-Pal autosampler (vial size up to 20 ml, stirring speed ≤750 rpm and extraction temperature ≥30 °C) may affect SPME efficiency. Initially, the effect of sampling temperature on the furan extraction yield was examined from 30 to 40 °C using CAR/PDMS fiber and maintaining constant the other extraction (extraction time 30 min, stirring speed 750 rpm and 20% (w/w) of NaCl) and desorption conditions (275 °C for 2 min). Table 1 shows the relative response of furan obtained at the different conditions studied using an aqueous standard solution of furan at 0.08 ng g$^{-1}$. As can be seen, the response of furan decreased when temperature increased. So, 30 °C was chosen for subsequent experiments because the temperature-controlled mixer stay (SMM tray) does not permit work at lower temperatures. In addition, the highest extraction efficiency of furan was obtained at the maximum stirring speed allowed by the autosampler (750 rpm) and it was chosen as optimal value. The effect of the headspace/aqueous volume ratio ($V_h/V_w$) on furan extraction was also studied using 20 ml glass vials and maintaining constant the other parameters. The best results were obtained using an aqueous volume of 8 ml (12 ml of headspace). Aqueous volumes higher than 8 ml ($V_h < 12$ ml) were not studied because a minimum volume of 12 ml of headspace is required for the complete spreading of fibre. Finally, extraction time, from 5 to 30 min, was evaluated and 20 min was enough to reach equilibrium. Other HS-SPME parameters, such as ionic strength and desorption temperature and time, were set according to those previously optimized by manual SPME [34].

Quality parameters of the HS-SPME method such as linearity, instrumental limit of detection and quantification and repeatability were established. Good linearity, between 0.01 and 10 ng g$^{-1}$, with correlation coefficients ($r^2$) higher than 0.999 was obtained. Instrumental limit of detection (iLOD) and quantification (iLOQ) based on a signal-to-noise ratio (S/N) of 3:1 and 10:1, were determined using water standard solutions and were 1.4 and 5 pg ml$^{-1}$, respectively. The precision of the automated HS-SPME method was determined analysing five water standard solutions spiked at two concentration levels, 0.05 and 1 ng ml$^{-1}$. Relative standard deviations (RSD %) lower than 3% were obtained for the two levels.

In addition, the variability of the method associated with the SPME manufacturing process, when different SPME fibres were used, was also examined. For this, fifteen aqueous standard solutions spiked with furan at 0.1 ng g$^{-1}$ were analysed under the optimal HS-SPME conditions, using three previously conditioned 75 μm CAR/PDMS fibres obtained from different lots. After five

**Table 2**
Analysis of furan in selected foods by automated HS-SPME and HS methods.

| Food sample | Description | Concentration (ng g$^{-1}$)[a] | | | | | | | | Significance level (P-value)[b] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Automated HS-SPME | | | | HS (US FDA method) | | | | |
| | | Mean ± S.D. | RSD (%) | LOD (ng g$^{-1}$) | LOQ (ng g$^{-1}$) | Mean ± S.D. | RSD (%) | LOD (ng g$^{-1}$) | LOQ (ng g$^{-1}$) | |
| Apple juice | Concentrated base | 1.10 ± 0.08 | 7 | 0.02 | 0.05 | 1.25 ± 0.13 | 10 | 0.42 | 1.39 | 0.1716 |
| Honey | Multi-floral | 4.8 ± 0.2 | 5 | 0.03 | 0.10 | 5.2 ± 0.45 | 9 | 0.58 | 1.93 | 0.2122 |
| Baby food | Chicken with rice | 15.7 ± 1.3 | 8 | 0.06 | 0.20 | 17.1 ± 2.1 | 12 | 0.80 | 2.86 | 0.3766 |
| Coffee | Brewed instant coffee | 35.0 ± 2.0 | 6 | 0.12 | 0.40 | 31.1 ± 3.7 | 12 | 0.62 | 2.06 | 0.1802 |
| Pulses | Cooked chickpeas | 0.24 ± 0.02 | 8 | 0.05 | 0.17 | n.d. | – | 0.50 | 1.67 | – |

n.d.: not detected.

[a] $n = 3$.

[b] Significant differences between methods for P-value < 0.05 (at 95% confidence level).

independent analyses with each fibre, the results obtained showed no significant differences between the fibres, with relative standard deviations lower than 10%. The durability of the fibre was also examined for any changes in the sensitivity of the fibre during its lifetime. The durability of the 75 μm CAR/PDMS fibre was estimated through a control standard solution and no significant differences on the extraction yield of furan were found after 150 analyses and only a 15% decrease was observed after 200 analyses. The stability of furan in sample vial before analysis was also investigated using several food samples: an apple juice, a baby food and a brewed coffee. For this purpose, four sample vials of each food matrix were prepared and analysed after 0, 1, 6 and 15 h. During this time, the sample vials were placed in the sample tray of autosampler at laboratory temperature (21 ± 2 °C). For all food samples and standing times, good agreement (RSD < 12%) in the results was obtained demonstrating the stability of furan in sample vials.

### 3.2. Comparison of HS-SPME and HS methods for the analysis of furan

To date, no comparative study between headspace extraction, the most commonly used method proposed by the US FDA, and HS-SPME for the analysis of furan in foods has been performed. In this paper, several food samples, such as apple juice (liquid sample), honey and coffee (rich flavours sample), chicken pap baby food (semi-solid sample) and cooked chickpeas (solid sample), were analysed in triplicate by automated headspace (HS) and HS-SPME, both coupled to GC–MS under the optimal conditions described in Section 2. Quantification with automated HS-SPME was performed by isotope dilution using furan-$d_4$. For HS, the standard addition method with the internal standard proposed by the US FDA for furan quantification was employed [27]. The results obtained in the analysis of furan by the two methods in selected food samples are summarized in Table 2. As can be seen, furan was detected in all selected food samples by using automated HS-SPME at concentrations ranging from 0.24 ng g$^{-1}$ for cooked chickpeas to 35.0 ng g$^{-1}$ for brewed instant coffee. In order to assure that the matrix did not affect the reliability of the results obtained by isotope dilution, the standard addition method was also applied for the analysis of these samples using HS-SPME and no significant differences were observed between both quantification methods. In addition, these results were consistent with those obtained with the HS method. To compare the results of the two methods, a statistical treatment of the data was performed
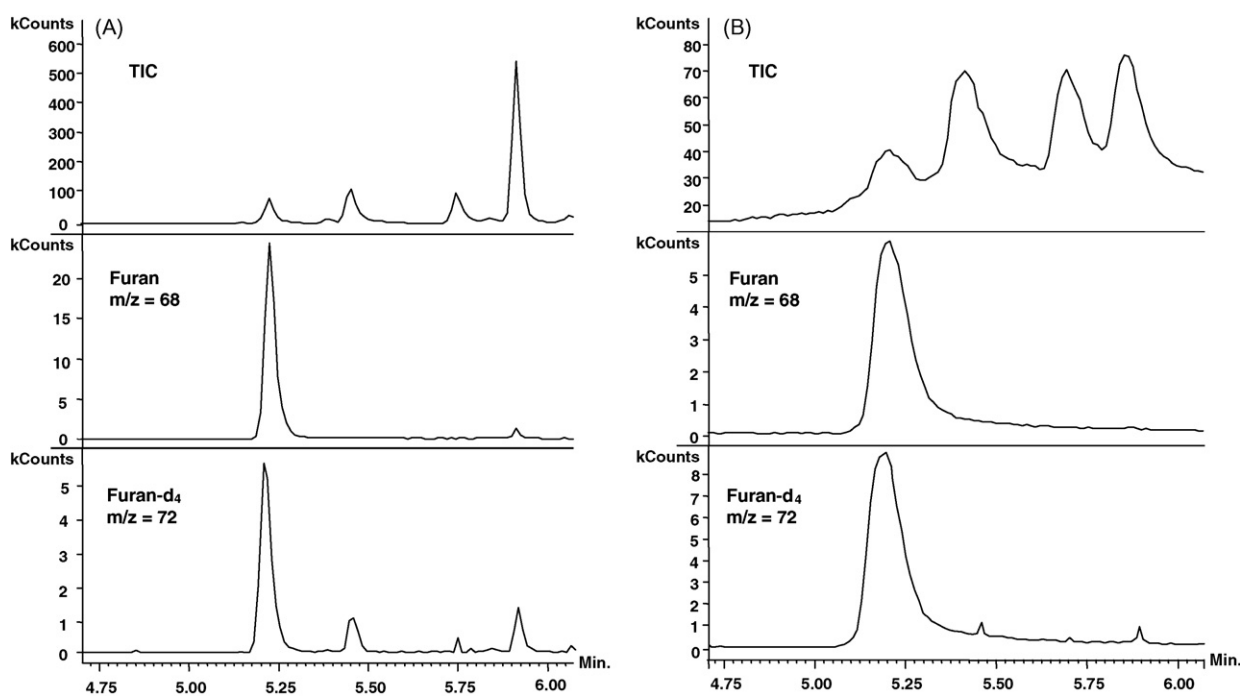


**Fig. 1.** GC–MS chromatograms of the total-ion-current (TIC) and the reconstructed ion current *m/z* 68 for furan and *m/z* 72 for furan-$d_4$, obtained by (A) automated HS-SPME method and (B) HS method proposed by the FDA.

**Table 3**
Analysis of furan in food by HS-SPME–GC–IT–MS.

| Food sample | Description | Concentration[a] (ng g$^{-1}$) | | Published data (ng g$^{-1}$) [ref.] |
|---|---|---|---|---|
| | | Mean | RSD (%) | |
| Juice | Apple | 1.7 | 3 | 1–4 [2,3] |
| | Orange | 0.7 | 4 | 2.5 [32] |
| | Multi-fruit | 2.0 | 3 | 6 [23] |
| | Tropicana | 2.3 | 3 | 6 [23] |
| Honey | Multi-floral (brand 1) | 3.6 | 7 | 3–10 [4] |
| | Multi-floral (brand 2) | 6.5 | 5 | 3–10 [4] |
| Soup | 15 vegetable soup | 0.5 | 10 | 19–49 [23,28] |
| and | Fish and shellfish with rice and pea soup | 39.0 | 3 | – |
| broth | Chicken with vermicelli soup | 5.0 | 5 | – |
| | Beef with vegetable broth | 2.0 | 5 | – |
| | Vegetable broth | 0.3 | 10 | – |
| Sauce | Fried tomato with olive oil (brand 1) | 0.6 | 8 | – |
| | Fried tomato with olive oil (brand 2) | 1.0 | 5 | – |
| | Ketchup with 6 vitamins | 0.9 | 7 | – |
| | Mayonnaise | 0.1 | 10 | – |
| | Cocktail sauce | 0.1 | 10 | – |
| Pulses | Cooked lentils | 1.0 | 8 | 3 [23] |
| | Cooked white kidney beans | 1.2 | 8 | – |
| Baby | Multi-fruit pop | 0.5 | 7 | 1–16 [23,28] |
| food | Mixed vegetable pop | 1.0 | 9 | 35–150 [2,23,28,33] |
| | Beef with spaghetti pop | 40 | 10 | 42–100 [23,28] |
| Coffee | Brewed natural coffee (automatic espresso machine) | 70 | 4 | 46–146 [21,23] |
| | Brewed natural filter coffee (filter-home pot brewer) | 20 | 7 | 9–40 [21,23] |
| | Brewed natural instant coffee | 35 | 5 | 36.9–51.3 [23] |
| | Brewed decaffeinated instant coffee | 28 | 5 | 8–31 [21] |
| | Powdered natural instant coffee | 1100 | 3 | 44–2200 [21,23] |
| | Powdered decaffeinated instant coffee | 820 | 5 | 309–2800 [21,23] |

[a] $n = 3$ replicates.

using the Student's *t*-test for equal or/and unequal variances and the results (*P*-value) are shown in Table 2. As can be seen, no significant differences were observed between the results with the two methods (*P* > 0.05), although the precision achieved by automated HS-SPME (RSD, 5–8%) was better than that found for the HS method (RSD, 9–12%). Limits of detection and quantification were also determined in food samples for the two methods. Since no blank food samples were found, samples spiked with furan-d$_4$ at very low concentration levels were used to estimate LODs and LOQs. The results given in Table 2 show that automated HS-SPME provided LOD values between 0.02 ng g$^{-1}$ (apple juice) and 0.12 ng g$^{-1}$ (brewed instant coffee), while for the HS method LODs were between 0.42 ng g$^{-1}$ (apple juice) and 0.80 ng g$^{-1}$ (chicken baby food). These results show that the automated HS-SPME method provided LODs from 5- to 20-fold lower than those obtained with the HS method, which is mainly because the HS-SPME technique has a higher preconcentration capacity than HS and because the HS-SPME method used the splitless injection mode instead of split injection. In addition, the HS method applied in this study provided LOD, LOQ and RSD values very close to those reported by FDA method [24], demonstrating that it can be used for comparative purposes. Fig. 1 shows as an example the GC–MS total ion current (TIC) chromatograms of a brewed instant coffee sample and the single ion chromatograms of *m/z* 68 (furan) and *m/z* 72 (furan-d$_4$) obtained by the two methods. As can be seen, higher sensitivity and better peak shape were achieved with the HS-SPME method mainly due to the use of a narrow SPME inlet liner (I.D., 0.75 mm). Moreover, no interfering compounds from matrix components were found in any of the analysed samples when comparing spectra of standards and samples. In addition, a higher sample throughput in routine furan analysis was obtained using HS-SPME–GC–MS with isotope dilution than HS with standard addition.

### 3.3. Analysis of furan in food commodities

To examine the feasibility of the automated HS-SPME method, several selected food commodities (27 different samples) were analysed. Furan was determined in triplicate by isotopic dilution, using the optimized method. The results obtained for the samples analysed are summarized in Table 3. Furan was detected and quantified in all samples at concentration levels ranging from 0.1 ng g$^{-1}$ for mayonnaise and cocktail sauces to 1.1 μg g$^{-1}$ for powdered natural instant coffee, with a precision better than 11%. These results are consistent with data published in the literature for similar food samples (Table 3). For instance, concentrations of furan in apple, orange and multi-fruit juices ranging from 1 to 6 ng g$^{-1}$ have been reported [2,3,23,32], which corroborates the figures determined using the proposed method for fruit juices (0.7–2.3 ng g$^{-1}$). In addition, furan levels found by EFSA in honey samples (3–10 ng g$^{-1}$) [4] were similar to those obtained in this study (3.6–6.5 ng g$^{-1}$). For soup and broth samples, a wide range of furan concentrations have been reported in the literature (3–125 ng g$^{-1}$) [2–4] due to the different ingredients and cooking procedure. In this case, furan was found at low concentration levels, with the lowest value (0.3 and 0.5 ng g$^{-1}$) being those found for vegetable soup and broth samples. This could be attributed to the low presence of furan in vegetables often below the detection limits [23]. Furan in sauces was also determined at low concentration levels, between 0.1 and 1 ng g$^{-1}$.

In baby food samples, furan concentrations ranging from 1 to 150 ng g$^{-1}$ have been reported depending on the food matrix. In this study, furan was found in multi-fruit and mixed vegetable samples at low concentration levels (0.5–1 ng g$^{-1}$), while for beef with spaghetti baby food a higher level of furan (40 ng g$^{-1}$) was found. Finally, the brewed natural coffee sample obtained from an automatic espresso machine gave a higher furan concentration level (70 ng.g$^{-1}$) than the filter-home pot brewer sample (20 ng.g$^{-1}$),

which confirmed previous data published by Zoller et al. [23], who indicate that the highest furan values are found in coffee prepared with an automatic espresso machine.

## 4. Conclusions

The automated headspace-SPME technique combined with GC–ion trap–MS has shown to be fast, sensitive and suitable for the analysis of furan in food commodities at low ng g$^{-1}$ levels using isotope dilution. The proposed method provided low limits of detection, between 0.02 ng g$^{-1}$ for apple juice and 0.12 ng g$^{-1}$ for brewed instant coffee, which were from 5- to 20-fold lower than those obtained with the HS-GC–MS method. In addition, both methods gave similar results for furan determination in selected food samples, although a relatively worse precision with HS method was obtained. In addition, HS-SPME–GC–MS is able to provide accurate and precise results (RSD% <11%) for the analysis of foods containing a broad range of furan levels with enough selectivity and sensitivity. Therefore, the HS-SPME–GC–MS method can be proposed as an alternative to the FDA method for routine analysis of furan in foods.

## Acknowledgments

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**2.4.3. PAPER III**

**AUTOMATED HEADSPACE SOLID-PHASE MICROEXTRACTION COMBINED WITH GAS CHROMATOGRAPHY - CHEMICAL IONIZATION - TANDEM MASS SPECTROMETRY FOR THE ANALYSIS OF VOLATILE N-NITROSAMINES IN FOOD MATRICES**

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## 1. Introduction

Volatile N-nitrosamines (VNAs) constitute a potentially carcinogenic and mutagenic class of compounds, which can be present in the environment and in a wide variety of food products [1]. VNAs are formed during food processing and storage by reaction of nitrosating agents, such as nitrites and/or nitrates [2], which are added as preservatives, and those substances present in the food matrix containing an amino group, such as amines and amides, proteins, peptides, and amino acids [3–6]. In 1978, the International Agency for Research on Cancer classified two VNAs, *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA), as probably carcinogens to humans (Group 2A), whereas *N*-nitrosodibutylamine (NDBA), *N*-nitrosopiperidine (NPIP) and *N*-nitrosopyrrolidine (NPYR) were included in the group of the possible carcinogens [7]. Despite the high toxicity of VNAs even at low-dose exposure [8], only few countries have regulations limiting their occurrence in food and moreover, important differences in the maximum permitted levels among the countries can be found. For instance, the maximum level of NDMA for beer in some European countries (Germany, Italy and Switzerland) has been set at 0.5 $\mu$g kg$^{-1}$, while in the United States of America (USA) the admissible value is ten times higher, 5 $\mu$g kg$^{-1}$ [3]. For meat and meat products, levels established in different countries such as, USA, Russia or China, range from 2 to 15 $\mu$g kg$^{-1}$ depending on the foodstuffs and the compounds legislated [3,5,9]. The determination of VNAs in food is currently performed by gas chromatography with thermal energy analyser detection (GC-TEA) [4,10–15] or coupled to mass spectrometry (GC-MS) [5,6,16–19], although the use of liquid chromatography with fluorescence [20] and UV detection [21] or coupled to tandem mass spectrometry (MS/MS) [15,22–25] has also been reported. TEA offers the

sensitivity required for VNA determination [7] but this detector is not usually found in routine laboratories and therefore GC-MS operating in electron ionisation (EI) is the technique most frequently used [26–31]. However, lately the use of positive chemical ionization (CI) has increased since it produces less fragmentation than EI thus improving sensitivity. Both, methanol [16,17] and ammonia [6,18,19,32] have been employed as CI reagent gases but methanol has the advantage of being less expensive and safer than ammonia and in addition, it offers less fragmentation and reduce the formation of adducts with VNAs, improving the sensitivity and specificity for VNA determination [17]. Both, quadrupoles and ion trap analysers have been used with GC-CI-MS but when the reagent gas is methanol, ion traps are used since they are able to work at the low pressures generated by liquid reagents.

Since VNAs in food are present at very low concentration levels ($\mu$g kg$^{-1}$), extraction methods that allow the enrichment and purification of the extracts are required. Currently, the analysis of VNAs in liquid and solid foods is performed by solvent extraction [15,17,23,33], low-temperature vacuum distillation or steam distillation followed by liquid-liquid extraction [4,11,14,34], solid-supported liquid extraction combined with solid phase extraction [6,19,32,35–37], and microwave-assisted extraction combined with dispersive solid-phase or liquid-liquid extraction [16,20,31]. Most of these methods are labour-intensive and time consuming and require large amounts of organic solvents. Headspace solid-phase microextraction (HS-SPME) combined with GC-TAE [13,38] and GC-EI-MS [26–28,39], has been demonstrated to be applicable for the analysis of specific VNAs in beverages and meat products. Nevertheless, these couplings provide limits of detection in the range of 1-3 $\mu$g kg$^{-1}$ for meat products and from 0.12 to 0.35 $\mu$g L$^{-1}$ for beer and wine, which are slightly higher

than concentrations reported in the literature for VNAs in these samples [17,30,31]. With regard to HS-SPME combined with GC-CI-MS, up to date this technique has been only used for the analysis of VNAs in environmental samples [40–42] and it has not been evaluated for the analysis of foods.

The aim of this work was to develop an automated and solvent-free method for routine analysis of trace amounts of VNAs in food and beverages. To meet this goal, the proposed method uses the combination of HS-SPME and GC methanol CI-MS/MS. HS-SPME extraction parameters and CI-MS/MS instrumental conditions were optimized to achieve maximum sensitivity and selectivity. The proposed method was validated and applied to the analysis of VNAs in processed meat products and beers collected from local supermarkets from Barcelona (Spain).

## 2. Experimental

### 2.1. Reagents and standards

A stock standard solution containing a mixture of nine VNAs, including *N*-nitrosodimethylamine (NDMA), *N*-nitrosomethylethylamine (NMEA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosomorpholine (NMOR), *N*-nitrosopiperidine (NPIP), *N*-nitrosodipropylamine (NDPA), *N*-nitrosodibuthylamine (NDBA), and *N*-nitrosodiphenylamine NDPheA, at 2 mg mL$^{-1}$ in methanol was purchased from Supelco (Bellefonte, PA, USA). Individual pure standards of [$^2$H$_6$]-NDMA (NDMA-d$_6$), [$^2$H$_{10}$]-NDEA (NDEA-d$_{10}$), [$^2$H$_8$]-NPYR (NPYR-d$_8$), and [$^2$H$_{14}$]-NDPA (NDPA-d$_{14}$), used as surrogate internal standards for quantification, were obtained from C/D/N isotopes Inc. (Pointe-Claire, Quebec, Canada) at purity above 99%. A stock standard mixture of deuterated VNAs was prepared in

methanol at a concentration of 2 mg mL$^{-1}$ from the pure standards. All standard solutions were kept in amber vials without headspace and stored at -18 °C. Aqueous working standard solutions of native and deuterated VNAs at 125 ng mL$^{-1}$ and 25 ng mL$^{-1}$ were daily prepared from the respective stock standard solutions. For calibration, a set of seven aqueous solutions at concentrations ranging from 0.03 to 10 ng mL$^{-1}$ of each VNA and 3 ng mL$^{-1}$ of deuterated VNAs were prepared by adding appropriate amounts of the working solutions into a 20-mL HS vial containing a 10 mm × 5 mm PTFE-coated stir bar, 0.1 g of sodium hydroxide, 3 g of sodium sulphate and 10 mL of water to obtain a final volume of about 12 mL.

Anhydrous sodium sulphate, sodium hydroxide and sodium chloride of analytical grade were obtained from Merck (Darmstadt, Germany). Methanol of liquid chromatography grade and dichloromethane of gas chromatography grade were purchased from Sigma-Aldrich (Munich, Germany), and ultra-pure water was supplied by J.T. Baker (Phillipsburg, NJ, USA).

## 2.2. Sample preparation

A total of 18 processed meat samples, including dry-cured and cooked pork ham, Frankfurt sausage, cured sausage, Spanish chorizo sausage, bacon, mortadella and pork liver pâté, and 12 beer samples (6 beers with an ethanol content of about 5.4% and 6 non-alcoholic beers), selected from the most consumed brands in Spain, were collected randomly from local supermarkets in Barcelona (Spain). All foods and beers were stored in their original packaging at 4 °C for keeping the quality and safety conditions. For beer sample preparation, a portion of about 100 mL was transferred to a beaker and degassed by magnetic stirring at 1,200 rpm for 10 min. For meat-based food, 200 g of

solid sample were mixed with 400 mL of ultrapure water and homogenised for 5 min at 4 ºC using a mixer and an Ultra-Turrax T25 basic disperser (IKA-Werke, Staufen, Germany). After preparation, all samples were kept in a closed vial without headspace and stored at 4 ºC under analysis. For validation, a meat-based food (cooked ham) and a beer, without detectable amounts of VNAs, spiked at concentrations levels close to the method limit of quantification of each compound were used. Fortification was performed by adding adequate amounts of native and deuterated VNAs to the blank samples which were kept at 4 ºC in the dark for 24 hours for equilibration before analysis.

## 2.3. Automatic HS-SPME procedure

HS-SPME experiments were carried out on a CTC Combi-Pal autosampler (CTC Analytics AG, Zwingen, Switzerland), which was equipped with a sample tray for 32 vials of 20 ml, a SPME fibre conditioning station, and a temperature-controlled single magnet mixer tray (SMM tray) (Chromtech, Idstein, Germany). For SPME analysis, five commercially fibres were tested: 100 µm polydimethylsiloxane (100 µm-PDMS), 75 µm Carboxen/polydimethylsiloxane (75 µm-CAR/PDMS), 85 µm polyacrylate (85 µm-PA), 65 µm polydimethylsiloxane/divinylbenzene (65 µm-PDMS/DVB) and a 50/30 µm Stable-Flex divinylbenzene/Carboxen/polydimethylsiloxane (50/30 µm-DVB/CAR/PDMS) of 23 gauge needle size were purchased from Supelco (Bellefonte, PA, USA). Before use, each fibre was conditioned in a heated GC split/splitless injection port according to the manufacturer's recommendations. The HS-SPME established optimal conditions were: 10 g of the prepared meat slurry (equivalent to 3.3 g of food sample) or 10 g of beer samples were quickly placed in a 20 ml headspace vial

immersed in an ice/water bath (4ºC) containing a 10 mm × 5 mm PTFE-coated stir bar, 0.1 g of sodium hydroxide, and 3 g of sodium sulphate. After that, 300 µL of deuterated-VNAs aqueous working solution (125 ng mL$^{-1}$) were added and the sample was kept at 4ºC for 24 h to ensure equilibration. Before HS-SPME analysis, the sample vial was vortexed for 5 min and conditioned in a magnet mixer tray (SMM tray) for 15 min at 70 ºC using a stirring rate of 750 rpm. The fibre was cleaned for 1 min at 275ºC using the SPME fibre conditioning station, and the sample was extracted from the headspace at 70 ºC for 45 min at a constant stirring rate of 750 rpm. Thermal desorption of the analytes was accomplished by exposing the fibre in the GC injector port at 275ºC for 2 min. To reduce the analysis time and to obtain a high sample throughput, a new HS-SPME extraction was performed while the GC analysis was running. Further details about the optimisation of the HS-SPME procedure are given in section 3.2.

## 2.4. GC-MS analysis

GC-MS analysis was performed on a CP-3800 gas chromatograph coupled to a Saturn-2200 ion-trap mass spectrometer (Varian, Mississauga, Canada). Automatic injections were performed using a CTC Combi-Pal autosampler (CTC Analytics AG, Zwingen, Switzerland) working in splitless injection mode (2 min) at 275 ºC. For HS-SPME experiments, the injector port was fitted with an SPME inlet liner (I.D., 0.75 mm, SGE Europe) and a 23-gauge Merlin Micro-seal septumless injector cap (Supelco, Bellefonte, PA, USA), while for liquid sample injection (1 µl) a splitless inlet liner (I.D., 2 mm, SGE Europe) was used. Chromatographic separation of the target compounds was accomplished on an Rtx-5Sil MS (5% phenyl-, 95% dimethylpolysiloxane) fused-silica capillary column (Restek, Bellefonte, PA, USA), 30

m × 0.25 mm I.D. and 1 μm film thickness. Helium (99.999%, Abelló-Linde S.A., Barcelona, Spain) was used as carrier gas at a constant flow rate of 1.2 mL min$^{-1}$ held by electronic flow control. The oven temperature was programmed from 35 ºC (held for 4 min) to 85 ºC at 8 ºC/min and then to 130 ºC at 3 ºC/min and finally to 250 ºC at 20 ºC/min (held for 5 min). The GC-MS transfer-line, ion-trap manifold and ion-trap source temperatures were set at 250 ºC, 80 ºC, and 175 ºC, respectively. The axial modulation amplitude was adjusted at 4.0 V for all experiments. The multiplier offset was ±200 V and the electron multiplier voltage was fixed at 1,600 V ($10^5$ gain). Methanol was used as reagent gas for chemical ionization (CI) with a filament emission current of 80 μA. Optimal CI operating parameters were as follows: target total ion current of 2,000 counts, CI storage level of *m/z* 19, background mass of *m/z* 40, maximum pre-scan time of 200 μs, ionization time of 2,000 μs and reaction time of 128 ms. MS/MS measurements were performed by collision-induced dissociation (CID) working in resonance excitation mode with a precursor isolation window of ±1 *m/z*, applying during 2 ms and an excitation time of 10 ms. Other MS/MS operating conditions were: broadband ejection mode at high edge amplitude of 20 V, low and high edge offsets of 6 and 2 steps, a modulation range of 2 steps at a rate of 3,000 μs/step, and an excitation storage level of 35 *m/z*. Table 1 shows the optimal CID excitation voltages and the precursor ([M+H]$^+$) and product ions selected for quantification and confirmation by multi-reaction monitoring (MRM) mode. The MS/MS acquisition method was time-programmed in six segments for monitoring the transitions selected for each target compound. Quantification was performed by internal standard method using NDMA-d$_6$ for NDMA, NDEA-d$_{10}$ for NMEA and NDEA, NPYR-d$_8$ for NPYR and NMOR, and NDPA-d$_{14}$ for NDPA, NPIP and NDBA as internal standards. Saturn

GC-MS WorkStation version 6.4 software was used for instrument control and data processing. Cycle Composer software version 1.5.3 was employed for programming and controlling the CTC Combi-Pal autosampler.

## 2.5. Method validation

Validation parameters of the HS-SPME GC-CI-MS/MS method, including linearity, specificity, precision, trueness, limit of quantification, decision limit (CCα) and detection limit (CCβ), were evaluated. Linearity of the relative response (analyte/internal standard) was established by least-squares regression analysis of the data obtained by analysing aqueous calibration solutions (7-points) within the range of 0.03 to 10 ng mL$^{-1}$ of each compound and the deuterated VNAs at 3 ng mL$^{-1}$. The goodness-of-fit of the data were considered acceptable when determination coefficient (R$^2$) was greater than 0.99 and residuals are randomly distributed. Method specificity was studied by analysing the presence of any interfering compounds that could affect the identification and quantification of the target compounds in 20 blank samples per matrix (meat-based food and beer). Trueness was evaluated by means of recovery experiments, analysing six blank samples per matrix fortified with the target compounds at concentration levels of 1, 1.5 and 2 times the limits of quantification according to the Commission Decision 2002/657/EC [47]. The precision of the method was determined under repeatability and reproducibility conditions by replicate analysis of spiked meat and beer blank samples on the same day (n=6) (intra-day precision) and in three non-consecutive days (n= 6 replicates x 3 days) (inter-day precision). The decision limits (CCα) and the detection capabilities (CCβ) were estimated analysing 20 blank samples per matrix spiked at the LOQ and CCα levels, respectively. The CCα and CCβ of each

compound were calculated as the mean values of the found concentration plus 1.64 times of the corresponding standard deviation. Statistical analysis for $CC_{\alpha}$ and $CC_{\beta}$ was performed at the 95% confidential level.

For quality control procedural blanks were daily analysed to ensure the absence of interfering compounds and possible carryover between samples. GC separation, sensitivity of GC-CI-MS/MS system, and validity of the HS-SPME calibration were checked and the identification of VNAs was confirmed by: (a) retention times of analytes *vs* standards within ±2 s, (b) signal-to-noise ratio of the selected product ions greater than 3, and (c) isotope ratios between quantifier and qualifier ions within ±20 % of the theoretical value.

## 3. Results and discussion

### 3.1. GC-CI-MS/MS optimization

GC-(methanol) CI-MS/MS methods using large volume injection or sample preconcentration techniques with high enrichment factors, such as dispersive micro solid-phase extraction [16] and SPE [6,17–19] have been reported in the literature for the analysis of VNAs in food samples, but SPME, a  non-exhaustive extraction method, has not been used until now because not enough sensitivity was achieved for this application. So, experiments were conducted to improve sensitivity and to establish the optimal CI conditions taken as reference the CI-MS/MS method proposed by the U.S. EPA that uses methanol as reagent for the analysis of VNAs in water [44].  Then, the effect of ion-trap temperature (150 ºC, 175 ºC and 200 ºC), filament emission current (from 10 µA to 100 µA), and detector multiplier offset (from ±0 V to ±300 V), in the response was investigated using an electron energy of 70 eV. For this optimisation, a

standard solution of native and isotopically labelled VNAs at 1 µg mL$^{-1}$ was injected in the GC-MS system. Figure 1 (A and B) shows the signal-to-noise ratio of the protonated molecule ([M+H]$^+$) obtained at the different ion-trap temperatures and filament emission currents. The highest S/N ratios were achieved at 175 ºC for all the compounds, for instance, for NDMA they were two-fold higher than those attained at 150 ºC or 200 ºC. In addition, S/N ratios about 20% higher were obtained using a filament emission current of 80 µA instead of the value (50 µA) proposed by the U.S. EPA method. At these conditions, the optimal multiplier offset was found at ± 200 V for all the compounds. Other CI parameters, such as target total ion current (TIC) and maximum ionisation and reaction time were optimised. Figure 1C shows the relative S/N ratios obtained for all the compounds at TIC values of 1,000, 2,000 and 5,000 counts (pre-scan ionisation time of 200 ms). Generally, target TIC values about 5,000 counts (default value) are recommended for CI experiments to avoid space charging effects. Nevertheless, a significant decrease (between 30% for NPYR and 50% for NDMA), of the S/N ratios were obtained at the default value compared with those found at 2,000 counts (Figure 1C) and so, it was selected as optimum. Optimal values of CID excitation voltage were obtained between 0.44 and 0.66 V (Table 1) with a CID excitation time of 10 ms. Figure 2 shows as an example, the CI-MS/MS spectra of NDMA obtained at CID excitation voltages of 0, 0.4 and 0.62 V (optimum value). To improve the selectivity, the isolation window of the precursor ions was set at ±0.5 *m/z* because good S/N ratio was obtained. The effect of MS/MS parameters that affect isolation and dissociation of the precursor ions such as the high edge amplitude (used to eject masses above the precursor ions), and the isolation time (to accumulate the

precursor ions into the ion-trap), were evaluated. Optimum values for the high edge

amplitude and isolation time were 20 V and 2 ms respectively.
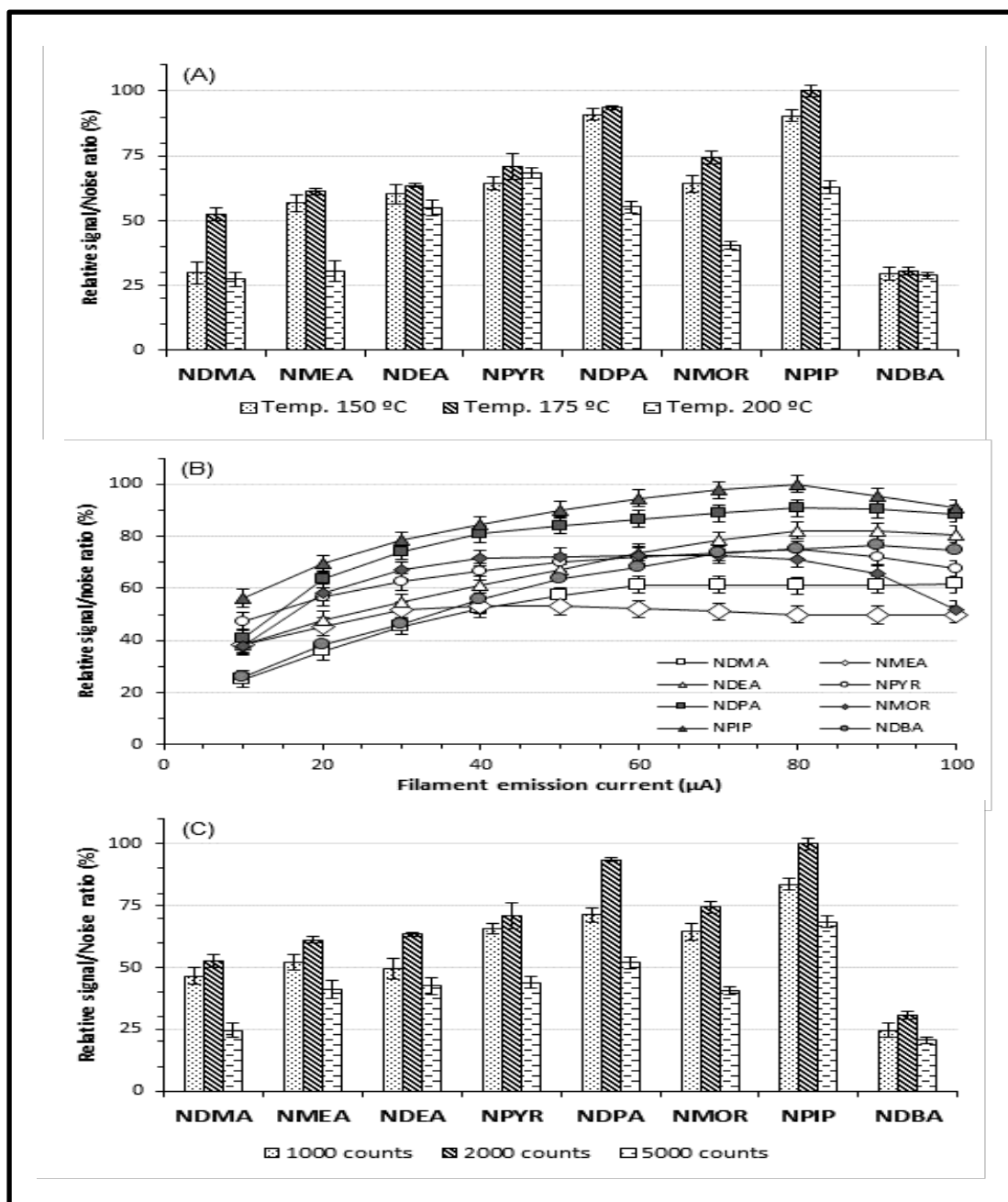


**Figure 1**: Effect of GC-CI-MS/MS parameters on the signal to noise (S/N) ratio:
     (A) ion-trap temperature, (B) filament emission current and
     (C) target of total ion current.

**Table 1.** GC-CI-MS/MS conditions for VNAs determination.

| Compound | Retention time ($t_R$, min) | Segment | | | MS-MS transition | | | Excitation |
| | | Time (min) | Scan range (m/z) | Scan rate (s/scan) | Precursor ion (m/z) | Products ion (m/z) | | CID voltage (V) |
|---|---|---|---|---|---|---|---|---|
| NDMA-$d_6$ | 5.66 | 5 - 7.5 | 40 - 85 | 0.55 | 81 | 46 | | 0.55 |
| NDMA | 5.74 | 5 - 7.5 | 40 - 85 | 0.55 | 75 | 47 (44) | | 0.66 |
| NMEA | 8.34 | 7.5 – 9.5 | 40 - 93 | 0.55 | 89 | 61 (47) | | 0.52 |
| NDEA-$d_{10}$ | 10.12 | 9.5 – 12.5 | 40 - 115 | 0.56 | 113 | 81 | | 0.50 |
| NDEA | 10.29 | 9.5 – 12.5 | 40 - 115 | 0.56 | 103 | 75 (47) | | 0.50 |
| NPYR-$d_8$ | 15.85 | 14.0 – 17.0 | 40 - 150 | 0.57 | 109 | 61 | | 0.52 |
| NPYR | 15.99 | 14.0 – 17.0 | 40 - 150 | 0.57 | 101 | 55 (47) | | 0.52 |
| NDPA-$d_{14}$ | 16.12 | 14.0 – 17.0 | 40 - 150 | 0.57 | 145 | 97 | | 0.46 |
| NDPA | 16.23 | 14.0 – 17.0 | 40 - 150 | 0.57 | 131 | 89 (47) | | 0.46 |
| NMOR | 16.25 | 14.0 – 17.0 | 40 - 150 | 0.57 | 117 | 86 (87) | | 0.48 |
| NPIP | 17.86 | 17.0 – 20.0 | 40 - 120 | 0.56 | 115 | 69 (47) | | 0.44 |
| NDBA | 24.54 | 20.0 – 27.0 | 40 - 165 | 0.56 | 159 | 57 (47) | | 0.46 |
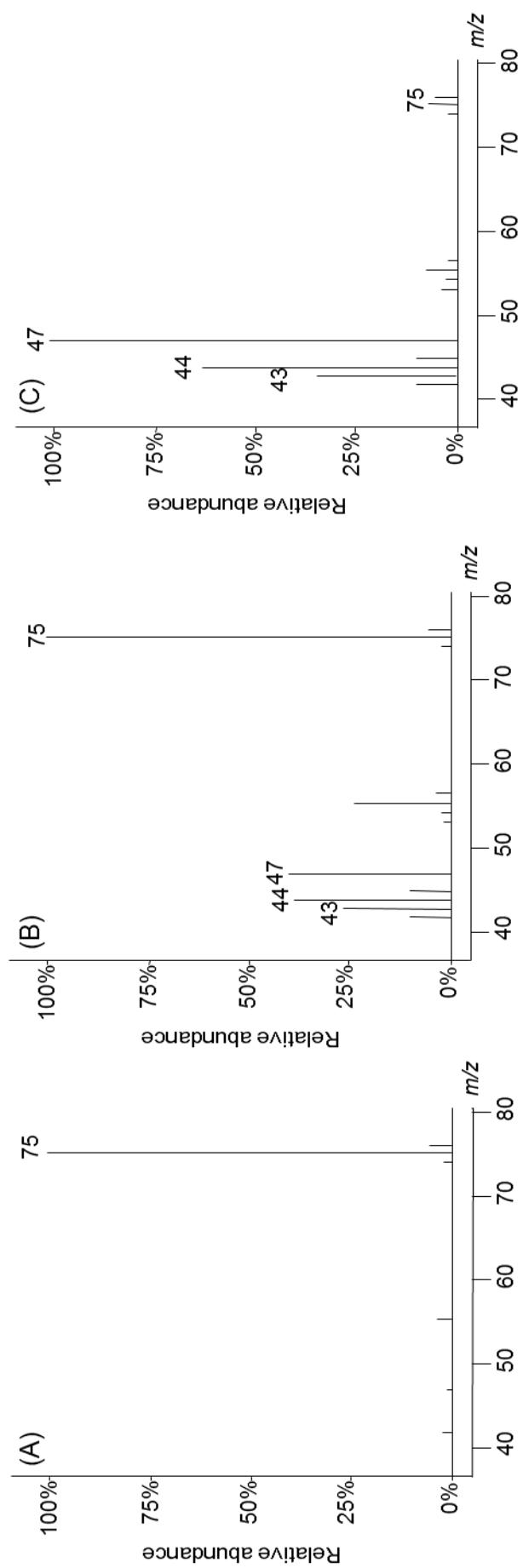
**Figure 2**: Methanol chemical ionization MS/MS spectra of NDMA at a CID excitation voltage of: (A) 0 V, (B) 0.4 V and (C) 0.62 V (optimum value).

As a result of this GC-CI-MS/MS optimisation instrumental limits of detection ranging from 0.21 pg injected for NDPA to 0.32 pg injected for NMOR were obtained which were from 15 to 70-fold lower than those of the US EPA method 521 [43–45].

## 3. 2. HS-SPME optimization

The first step in the SPME optimization was the selection of the appropriate fibre for the NA determination. Five commercially available SPME fibres: 100-μm PDMS, 75-μm CAR/PDMS, 85-μm PA, 65-μm PDMS/DVB, and 50/30-μm DVB/CAR/PDMS, were tested to obtain the best sensitivity and selectivity for VNAs determination. To this end, aqueous standard solutions of VNAs (5 ng mL$^{-1}$) were analysed using the five fibres using an extraction time of 30 min and a pre-equilibration time of 10 min to ensure that the maximum amounts of VNAs were extracted. In addition, the stirring rate, the extraction temperature and desorption time were set to 500 rpm, 50 ºC and 5 min, respectively. The desorption temperatures were within the recommended operating range for each fibre. No carryover on second desorption was found for any fibre, indicating complete removal of analytes at these conditions. Figure 3 (A) shows the relative peak area (%) obtained for VNAs using the studied fibres. The CAR/PDMS fibre provided the highest extraction yields for VNAs of low molecular weight, such as NDMA, NMEA and NDEA, while the PDMS/DVB fibre showed a greater capacity to retain the low volatile VNAs. This behaviour could be attributed to the higher surface area (720 m$^2$ g$^{-1}$) and the relatively high percentage of micro-porous ( 2-20 Å) of the CAR/PDMS fibre that favours the retention of small molecules [46]. Considering that NDMA, NMEA, NDEA and NPYR are the most commonly VNAs found in food samples, CAR/PDMS fibre was selected for all subsequent experiments.
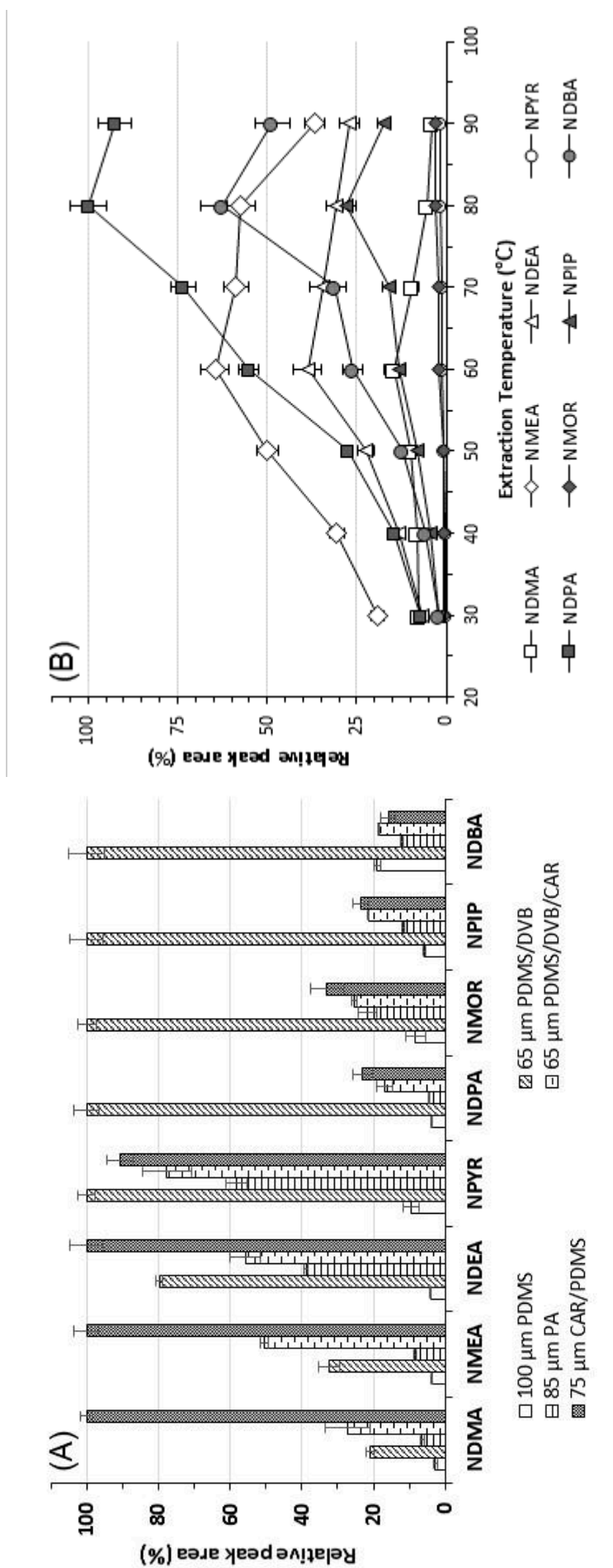
**Figure 3**: Optimization of HS-SPME parameters. Effect of: (A) fibre type and (B) extraction temperature.

After fibre selection, the effect of temperature on the NA extraction yield was examined from 30 ºC to 90 ºC, remaining constant the rest of HS-SPME parameters. As Figure 3(B) shows, maximum relative peak areas were obtained at temperatures of 60ºC for NDMA, NMEA and NDEA, while for NPYR, NDPA, NPIP and NDBA a higher temperature, of about 80 °C, was required. Therefore, an extraction temperature of 70 °C was selected as a compromise for all the compounds.

Blank beer and meat (cooked ham) samples spiked at 5 ng g$^{-1}$were used to confirm the suitability of the selected extraction temperature (70 ºC) for the analysis of food samples and to evaluate the effect of the addition of salts and pH on the extraction. Sodium chloride and sodium sulphate (30%, w/w) were used as salting out reagents and $Na_2SO_4$ was selected as salting out agent since the response of VNAs obtained with $Na_2SO_4$ was about 2-fold higher than that with NaCl. Moreover, the effect of the amount of $Na_2SO_4$ (0-30%, w/w) was also evaluated and as expected, the response increased with the percentage of salt. Regarding the pH, any significantly effect was observed for beer while for meat a slight improvement was found at high pH probably because at these conditions the extraction of analytes from the sample is favoured. In addition, the alkaline medium avoids the formation of VNAs during the sample extraction by blocking the nitrosation reactions [32]. Therefore, 0.1 g of NaOH to both calibration and sample solutions before HS-SPME extraction were added. Headspace/sample volume ($V_h/V_s$) ratio was also investigated using volumes of 2.5 mL, 6 mL and 12 mL ($V_h/V_s$: 7, 2.3 and 0.67, respectively) of the spiked blank sample solutions (beer and meat) in a 20 mL vial. Maximum responses were obtained using a low $V_h/V_s$ ratio (0.67) which corresponds to 8 mL headspace (12 mL of aqueous phase). Moreover, a sample stirring rate of 750 rpm provided the highest extraction yield. With

regard to the extraction time it was observed that equilibrium was not reached after 120 min, and 45 min was selected since more that 80% of the maximum extraction yield was obtained. Finally, the quantitative desorption of the analytes from the fibre coating into the injector port was achieved in 2 min using a temperature of 275 ºC.

## 3.3. Method validation

Validation parameters of the HS-SPME GC-CI-MS/MS method are given in Table 2. Linear calibration curves were obtained using internal standard method with coefficients of determination ($R^2$) ranging from 0.994 for NDBA to 0.999 for NDMA. Limits of quantification, based on a signal-to-noise ratio (S/N) of 10, were determined using blank beer and cooked ham samples spiked at low concentration levels (0.6 ng g$^-$$^1$). LOQs ranged from 0.10 to 0.38 ng g$^{-1}$ for both matrices and are similar to those reported in the literature using other sample treatments, such as liquid-liquid or liquid-solid extraction and SPE, combined with GC-CI-MS/MS [6,17,18,32]. These LOQs are lower than those established as maximum admissible levels in USA for cured meat products (10 ng g$^{-1}$) and beer and bacon (5 ng g$^{-1}$) [3,39], in some European countries for NDMA in beer (0.5 ng g$^{-1}$) and in Estonia and China for meat and meat products (3 ng g$^{-1}$) [3,5,9]. To study the specificity of the method, a representative number of blank beer and meat samples (n=20 per matrix) were analysed. Good specificity was achieved for both matrixes since the presence of interfering peaks at the retention times of VNAs were not observed. Trueness of the method was also assessed through recovery studies. Since no certificated reference materials of VNAs in food are available, the trueness was evaluated using blank food samples (beer and cooked ham) spiked with VNAs at concentration levels of 1, 1.5 and 2 times the LOQ (0.40, 0.60 and 0.80 ng g$^{-1}$).

Recoveries (Table 2) ranging from 90% to 106% were obtained for both matrices and these values were always within the recommended range 70% - 110% [47] for samples spiked at concentrations between 0.4 and 0.8 ng g$^{-1}$. Precision was also investigated by analysing the same spiked samples in the same day (inter-day precision) and in three non-consecutive days (intra-day precision). Inter-day precision, expressed as relative standard deviations (RSD%, n=6), ranged between 5% and 10% for meat and beer (Table 2), while for inter-day precision the RSD (%) values were always lower than 12%. Finally, decision limit (CCα) and detection capabilities (CCβ) were determined by analysing a representative number of blank samples (n=20) per matrix spiked at the LOQ and CCα concentration levels, respectively. Values obtained for CC$_\alpha$ and CC$_\beta$ that ranged from 0.40 to 0.44 ng g$^{-1}$, and between 0.44 and 0.50 ng g$^{-1}$ for all the VNAs and matrices, respectively (Table 2). These results reveal that the combination of HS-SPME and GC-CI-MS/MS is capable of achieving a high precision with good linearity and a proper sensitivity for the determination of VNAs in food products. Figure 4 shows as an example the GC-CI-MS/MS chromatograms of a cooked ham sample spiked at a concentration of 2 ng g$^{-1}$ of each VNA.

## 3.4. Analysis of food samples

The proposed method was applied to the determination of VNAs in beer and processed meat products selected from the most consumed brands commercialised in Spain. A total of 18 meat samples, including dry-cured and cooked ham, frankfurter sausage, cured sausage, and Spanish chorizo, and 12 beer samples, were analysed in triplicate and the results are given in Table 3. The target compounds were not found in any of the analysed beer samples at concentrations levels above LOQs.

**Table 2.** Validation parameters of the proposed HS-SPME GC-CI-MS/MS method for the VNAs determination in beer and cooked ham.

| Parameter | Matrix | Compound | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | NDMA | NMEA | NDEA | NPYR | NDPA | NMOR | NPIP | NDBA |
| Linearity ($R^2$) [a] | | 0.999 | 0.997 | 0.998 | 0.997 | 0.998 | 0.998 | 0.998 | 0.994 |
| Recovery (%) [b] | | | | | | | | | |
| Level 0.40 ng g⁻¹ | Beer | 93 | 103 | 95 | 94 | 103 | 90 | 107 | 95 |
| | Cooked ham | 95 | 106 | 98 | 106 | 101 | 103 | 99 | 104 |
| Level 0.60 ng g⁻¹ | Beer | 98 | 101 | 97 | 105 | 98 | 105 | 97 | 103 |
| | Cooked ham | 104 | 97 | 105 | 95 | 99 | 90 | 106 | 96 |
| Level 0.80 ng g⁻¹ | Beer | 99 | 97 | 104 | 97 | 101 | 97 | 105 | 96 |
| | Cooked ham | 97 | 103 | 101 | 103 | 104 | 96 | 95 | 102 |
| Intra-day precision (RSD %) [b] | | | | | | | | | |
| Level 0.40 ng g⁻¹ | Beer | 6 | 8 | 6 | 9 | 7 | 9 | 10 | 6 |
| | Cooked ham | 8 | 9 | 6 | 9 | 9 | 10 | 9 | 7 |
| Level: 0.60 ng g⁻¹ | Beer | 5 | 5 | 5 | 9 | 7 | 9 | 7 | 5 |
| | Cooked ham | 5 | 8 | 7 | 9 | 8 | 10 | 9 | 6 |
| Level: 0.80 ng g⁻¹ | Beer | 5 | 5 | 4 | 7 | 6 | 8 | 5 | 4 |
| | Cooked ham | 5 | 5 | 5 | 7 | 7 | 8 | 8 | 5 |
| Inter-day precision (RSD %) [c] | | | | | | | | | |
| Level 0.40 ng g⁻¹ | Beer | 9 | 9 | 8 | 9 | 8 | 11 | 11 | 8 |
| | Cooked ham | 8 | 9 | 6 | 10 | 9 | 12 | 11 | 8 |
| Level: 0.60 ng g⁻¹ | Beer | 7 | 6 | 7 | 10 | 8 | 9 | 10 | 6 |
| | Cooked ham | 7 | 8 | 8 | 9 | 8 | 11 | 11 | 6 |
| Level: 0.80 ng g⁻¹ | Beer | 7 | 5 | 5 | 8 | 6 | 9 | 9 | 5 |
| | Cooked ham | 6 | 9 | 8 | 8 | 7 | 9 | 8 | 8 |
| LOQ (ng g⁻¹) | Beer | 0.26 | 0.18 | 0.10 | 0.36 | 0.14 | 0.36 | 0.32 | 0.12 |
| | Cooked ham | 0.30 | 0.19 | 0.12 | 0.38 | 0.16 | 0.38 | 0.34 | 0.14 |
| CCα (ng g⁻¹) | Beer | 0.40 | 0.42 | 0.40 | 0.42 | 0.42 | 0.42 | 0.42 | 0.42 |
| | Cooked ham | 0.42 | 0.44 | 0.42 | 0.44 | 0.44 | 0.44 | 0.44 | 0.44 |
| CCβ (ng g⁻¹) | Beer | 0.44 | 0.46 | 0.44 | 0.48 | 0.44 | 0.48 | 0.48 | 0.44 |
| | Cooked ham | 0.48 | 0.48 | 0.46 | 0.50 | 0.46 | 0.50 | 0.50 | 0.46 |
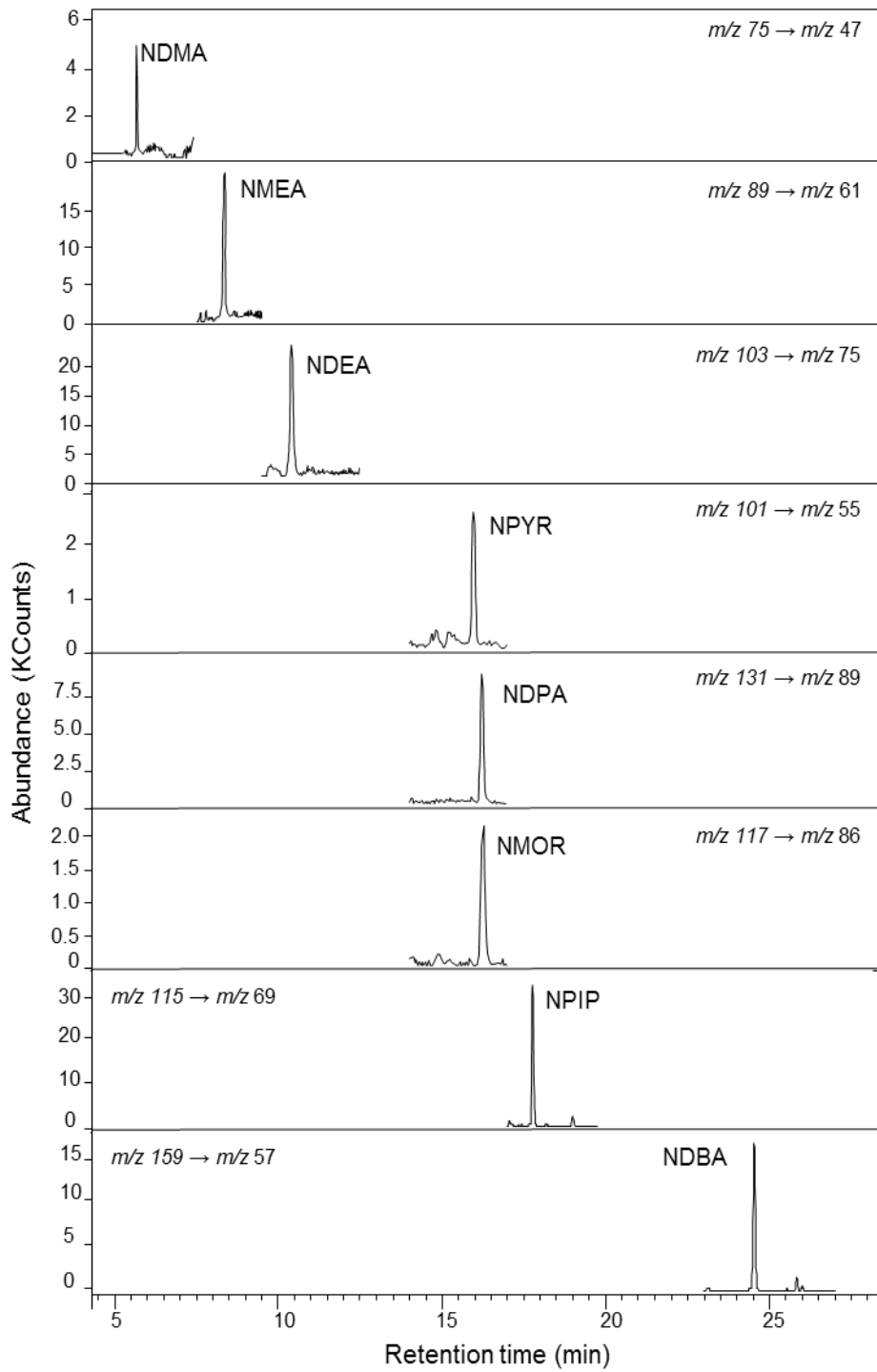
**Figure 4:** GC-CI-MS/MS chromatograms of a cooked ham sample spiked at a concentration of 2 ng g$^{-1}$ of each volatile *N*-nitrosamine.

These results confirm the decreasing trend of NA levels in beer as a consequence of the actions applied for reducing their presence, such as the decrease of the pH of the barely before kilning, the removal of the rootlets (in which amines are known to be concentrated) and the use of indirect-fired kilning of the malt. Regarding food samples, VNAs were only detected in 3 out 18 samples. NDMA and NPYR were found at concentrations from 2.4 to 3.7 ng g$^{-1}$ in one sample of cured sausage and in two Spanish chorizo samples, while the rest of VNAs they were either not detected or present at levels below their limits of quantification. The presence of NDMA and NPYR at significant levels in Spanish chorizo and salami-type sausages can be explained by the occurrence of precursors in the seasonings added to obtain their characteristic taste. For instance, the addition  of large quantity of sweet paprika, which is known to contain pyrrolidine, or of high amounts of spices, like black pepper in salami-type sausage during the production of Spanish chorizo, can be explain the NPYR formation in these samples [14]. Although the number of samples analysed in this work is limited, the results obtained indicated the effectiveness of the measures adopted by the manufactures to avoid the formation of VNAs by reducing the presence of sodium nitrate and/or the addition of ascorbic acid to ensure the conversion of nitrite to nitric oxide.

## 5. Conclusions

A headspace-solid phase microextracion method combined with GC-CI-MS/MS employing methanol as reagent gas was developed for N-nitrosamine determination in beer and processed meat products. CI-MS/MS parameters such as ion-trap temperature, filament emission current, CI reaction time and isolation time of precursor ions were

found to be critical to improve sensitivity. For HS-SPME the best results were found using a CAR/PDMS fibre working at non-equilibrium conditions, alkaline medium (NaOH) and high ionic strength. The method is simple, easy to automate, show an acceptable analysis time (45 min), and provides good selectivity and sensitivity, with limits of quantification (0.10-0.38 µg kg$^{-1}$) lower than the maximum levels established by international regulations. In addition, the developed method showed good recoveries (70-119%) and precision (RSD% < 12%) with detection capabilities (CC$\beta$) ranging from 0.44 to 0.50 µg kg$^{-1}$ for both meat and beer samples. This HS-SPME GC-CI-MS/MS method can be proposed for the routine analysis of VNAs in beer and processed meat products.

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## 2.5. DISCUSSION OF THE RESULTS

### 2.5.1. Headspace solid-phase microextraction optimisation

Since furan has a very high volatility, it was necessary, as a first step, to design a procedure able to prevent its loss during the preparation of the sample for HS-SPME. So, as described in Papers I and II, all samples were refrigerated before treatment and chilled laboratory equipment was always used for sample handling which in addition, was always carried out as quick as possible. For instance, the homogenization, for solid and semi-solid samples, was quickly performed using a chilled blender with the sample and sample vial immersed in an ice bath. This procedure was also used in sample preparation (beer and meat products) for the analysis of VNAs in food (Paper III) even though these compounds are less volatile than furan. The addition of analytes (target and/or IS) to prepare calibration standard solutions or samples was always performed using a chilled GC-syringe and through the septum of already prepared and sealed HS-vial.

To establish the optimum experimental conditions for HS-SPME methods for the analysis of furan and VNAs in food parameters affecting extraction and thermal desorption were studied. Direct immersion SPME (DI-SPME) was not evaluated since for the analysis of volatile compounds in complex matrices such as foods, is better do not immerse the fibre in the corresponding aqueous suspensions.

As the sensitivity of HS-SPME is determined by the distribution coefficients of the analyte between the fibre, the headspace and the sample the first studied parameter was the selection of the most appropriate fibre that provided the highest extraction efficiency of furan (Paper I) and VNAs (Paper III) from food samples. The tested commercial SPME fibres, their conditioning before the use as well as the HS-SPME and GC-MS conditions established for furan and VNAs analyses are described in the experimental section of Paper I and Paper III, respectively. The results showed that for furan, the best extraction efficiency was obtained using the carboxen/polydimethylsiloxane (75μm CAR/PDMS) fibre which was several times, up to 650-fold, more efficient than the rest of the examined fibres (Paper I). For VNAs extraction, and as can be seen in Figure 3-A of Paper III, effective extractions of the

small molecular size VNAs, namely NDMA, NMEA and NDEA were also obtained using the 75μm CAR/PDMS fibre. The high retention of both, furan and these small VNAs, can be attributed to the great surface specific area, small diameter size of pores and relative high amount of small pores (micropores) of this fibre (Table 2.5) that favours the strong retention of small molecules such as furan, NDMA, NMEA and NDEA.

Table 2.5: Physical properties of the sorbent used in SPME fibre (Shirey, 2009).

| Material | Surface area ($m^2 g^{-1}$) | Porosity (ml $g^{-1}$) | | | | Average Micropore Diameter (Å) |
|---|---|---|---|---|---|---|
| | | Macropore > 500 Å | Mesopore 20- 500 Å | Micropore 2- 20 Å | Total | |
| DVB | 750 | 0.58 | 0.85 | 0.11 | 1.54 | 16 |
| Carboxen | 950 | 0.23 | 0.26 | 0.29 | 0.78 | 12 |

The large molecular size VNAs, namely NDPA, NPIP, NMOR and NDBA, were more efficiently extracted with the divinylbenzene/polydimethylsiloxane fibre (65μm PDMS/DVB) probably because DVB particles have a porous structure with a larger average diameter size than CAR/PDMS fibre (Table 2.5). Since the CAR/PDMS fibre showed the best extraction efficiency for furan as well as for NDMA, NMEA and NDEA which are the VNAs most frequently found in food products, this fibre was select as optimum for the analyses of both, furan (Papers I and II) and VNAs (Paper III). The good performance of 75μm CAR/PDMS fibre for furan found in this thesis is in agreement with the results published by other authors, some of them before (Goldmann et al., 2005 and Bianchi et al., 2006) and others after (Table 2.3) our study. For VNAs, we were the first to report the use of 75μm CAR/PDMS fibre for VNAs extraction from food instead of the 65μm PDMS/DVB used in all previous published papers (SPME methods in Table 2.4), allowing to improve the extraction efficiency of the VNAs most commonly detected in food.

The effect of temperature on the extraction using the 75μm CAR/PDMS fibre was studied from 25 to 40 ºC for furan (Paper I) and from 30 to 90 ºC for VNAs (Paper III). A high extraction temperature in HS-SPME can reduce the extraction time making

the whole procedure faster and increase the extraction efficiency as more analyte is present in the HS-phase. However, the fibre coating-headspace distribution constant is reduced which negatively affects method sensitivity. Therefore, in general, temperature may be increased to a level, as far as the reduction in sensitivity can be tolerated. As can be seen in Figure 1-A, Paper I, a decrease in furan extraction efficiency was observed when the extraction temperature was increased indicating that the previously mentioned negative effect is more important than the positive one. Therefore an extraction temperature of 25 ºC was selected as optimum. This extraction temperature is c.a. 3-times lower than that used for furan analysis by direct HS methods (Table 2.2) which has the advantage of reduce the possible furan formation during the analysis and provides a high furan pre-concentration on the SPME-fibre thus increasing the sensitivity of the HS-SPME method. However, in the developed automated HS-SPME method (Paper II) a temperature of 30 ºC, was used due to the temperature limitations of the Combi-Pal auto-sampler (standard configuration). Our conditions are in agreement with most of the HS-SPME methods found nowadays in the literature (Table 2.3) Regarding VNAs, as can be seen in Figure 3-B in Paper III, the selection of extraction temperature was more critical since the maximum extraction efficiencies of NDMA, NMEA and NDEA are achieved at a temperature of 60 ºC while for the rest of the VNAs the highest extraction efficiencies were obtained at 80 ºC. As a compromise we propose using a temperature of 70 ºC for all VNAs.

The modification of the sample matrix such as the ionic strength and the pH may improve the HS-SPME efficiency. So, NaCl at a concentration of 20% (w/w) was selected as salting-out reagent for furan HS-SPME (Fig 1B, Paper I) and sodium sulphate (30%, w/w) instead of sodium chloride was used for VNAs since higher extraction efficiencies (c.a. 2-fold) were obtained. With regard to the pH, it is not expected to produce any important effect on the HS-SPME extraction efficiency since furan is a neutral compound and the studied VNAs are very weak bases that undergo protonation only in super acid conditions while, in alkaline solution, they exhibit very weak acidity of the hydrogen at α-carbon positions (Challis &Challis, 1982). Even though it was found that the addition of 0.1 g of NaOH slight increased the extraction of

VNAs from meat products which as indicated in Paper III is the liberation of VNAs from the meat sample is favoured at basic conditions.

Another parameter of the HS-SPME method optimization is the ratio between headspace and sample volumes ($\beta = V_h/V_s$). As it is well known a small HS volume increases the concentration of analytes in headspace and as a consequence an improvement in sensitivity is achieved the equilibrium time is reduced. In this thesis for the automatic HS-SPME methods using a 20-ml HS-vial, low volume rations were proposed, $\beta$ of 1.67 for furan analysis and 0.67 for VNAs. A smaller value was needed for VNAs because of the lower volatility of these compounds. The HS-SPME extraction time which is defined as the time of the fibre exposure into the headspace phase of the sample in the vial was also optimized. As can be seen in Figure 1-D, Paper I, the equilibrium of furan is achieved after an extraction time of 30 min. However, in the automated method, Paper II, the time needed to reach the equilibrium was only 20 min because smaller HS vials (20 ml) than in the manual method (40 ml) were used. For VNAs, the extracted amounts by the fibre were still increasing after 120 min which is due to the lower volatility of these compounds. It has been indicated in the literature (Pérez et al., 2008) that very long times (200h) are required to achieve the equilibrium for the analysis of NDMA in beer. In order to propose a method able to be applied in routine analyses an extraction time of 45 min at non-equilibrium conditions, was selected as optimum. Finally, desorption temperature and time for the SPME-fibre were optimized ensuring that no carryover (memory effect) occurred. For both methods, it was found that exposing the 75-μm CAR/PDMS fibre in the injector port at a temperature of 275 ºC for 2 min were enough for complete and efficient desorption of furan (Paper I) and also VNAs (Paper III).

To increase throughput in the developed automated methods (Papers II and III) a fibre-conditioning station (a special configuration of CTC Combi-Pal auto-sampler) was used for a rapid (1 min at 275 ºC) cleaning of the fibre between each extraction cycle. Besides, we also used a high temperature-resistant Merlin Micro-Seal septum (a micro valve sealing) instead of the conventional GC-silicon-septum that require a frequent maintenance. In addition, SPME desorption process is also affect by the GC injector-port design and the inner diameter (ID) of the glass liner. For rapid transfer of desorbed

analytes from the SPME fibre to GC-column with sharp injection bands, the volume of the gas liner must be reduced. Therefore, in all our studies, the smallest commercially available SPME glass inlet liner (I.D., 0.75 mm, SGE) was fitted to the GC-injector port instead of conventional split/spliless glass liner (I.D., 3 mm). This allowed increasing the peak height about 3-times and thus the method sensitivity improved.

### 2.5.2. GC-MS optimisation and methods validation

In this thesis, for the analysis of furan, instead of a porous layer open tubular GC-column such as HP-Plot-Q, proposed by US FDA, we used a wall coated open tubular column because the adsorption at the head of the column is reduced providing higher efficiency. The best results were obtained with a cyanopropylphenyl-polysiloxane stationary phase of 1.4 μ. film thickness (BPX-volatiles column) that allowed to obtain an adequate retention time for furan. The suitability of this column for the analysis of furan was later confirmed by other authors (La-Pera, et al., 2009; JRC-IRMM, 2008a). A 5% phenyl-, 95% dimethylpolysiloxane column (Rtx-5Sil MS) was selected for the GC separation of VNAs as recommended by US EPA for the VNAs analysis of these compounds in water (US EPA method 521, 2004). This column provided an adequate separation of VNAs (only NDPA and NMOR were not baseline completely separated) Table 1 in Paper 3 shows the retention times of VNAs obtained under the optimal GC conditions which are described in the experimental section (Paper III).

Regarding ion-trap MS, two instruments, a GCQ/Polaris ion-trap MS (Paper I) with an external ionization source and a Saturn-2200 ion trap MS (Paper II and Paper III) with internal ionisation have been used in this thesis. For the analysis of furan in food electron ionization (EI) was used and the molecular ion $[M]^{+\bullet}$ (*m/z* 68) and the fragment ion $[M-CHO]^+$ (*m/z* 39), the most abundant in the spectrum (Fig 2, Paper I), were monitored for quantitation and confirmation, respectively. Figure 3.2 shows the proposed furan ionization/fragmentation pathway.
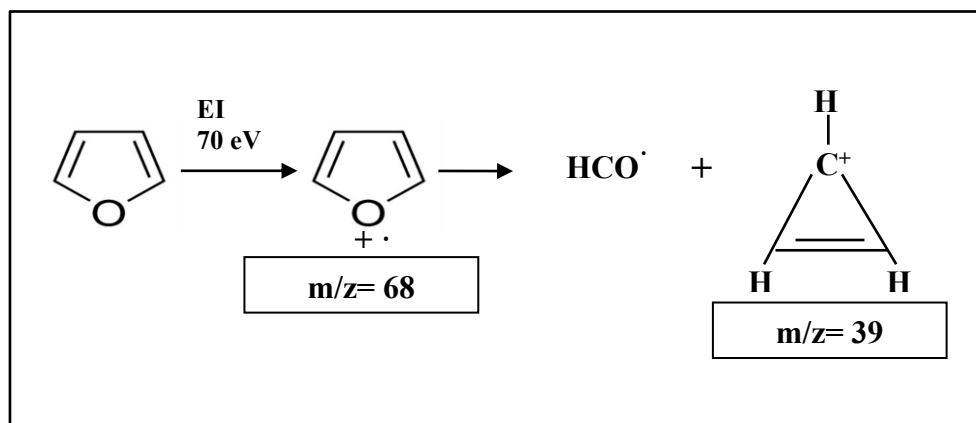
Figure 3-2: Ionisation/fragmentation pathway of furan.

As commented in the introduction of this chapter, chemical ionization (CI) provides higher sensitivity for the analysis of VNAs than electron ionisation. Moreover, in 2004, the US EPA published a method recommending the use methanol as CI reagent for the analysis of VNAs in water (EPA method 521, 2004) that has also been employed by other authors (Hung et al., 2010; Llop et al., 2010). This method was re-evaluated, in this thesis, and experiments were conducted to establish the CI conditions to achieve detection limits low enough to determine VNAs in food samples by HS-SPME GC-CI-MS/MS. The design of the Saturn-2200 ion-trap instrument with internal ionization allows liquid methanol to be fed into the ion source making the application of the CI easier and safe. For Me-OH CI-MS, the main generated reagent ions are $CH_3OH_2^+$ and $CH_3OH^{\cdot+}$ which were used to daily adjust the level of Me-OH in the ion-trap. The ionization conditions optimized in this work, ion trap temperature (175 ºC), filament emission current (80 µA), ionisation time (2000 µs) and reaction time (128 ms). (Fig 1, Paper III) and also, the use of the optimal MS/MS parameters (excitation voltage, excitation time, isolation time) allowed to improve sensitivity from 15 to 70 fold. The precursor ion was protonated molecular ion ($[M-H]^{\cdot+}$) of each compound which was the only ion obtained in the methanol CI spectra and two product ions were used as quantifier and qualifier ions. As an example, the CI-MS/MS spectra of NDMA at CID excitation voltages of 0, 0.4 and 0.62V are shown in Figure 2 (Paper III) and the fragmentation pathway is given in Fig 3.3.
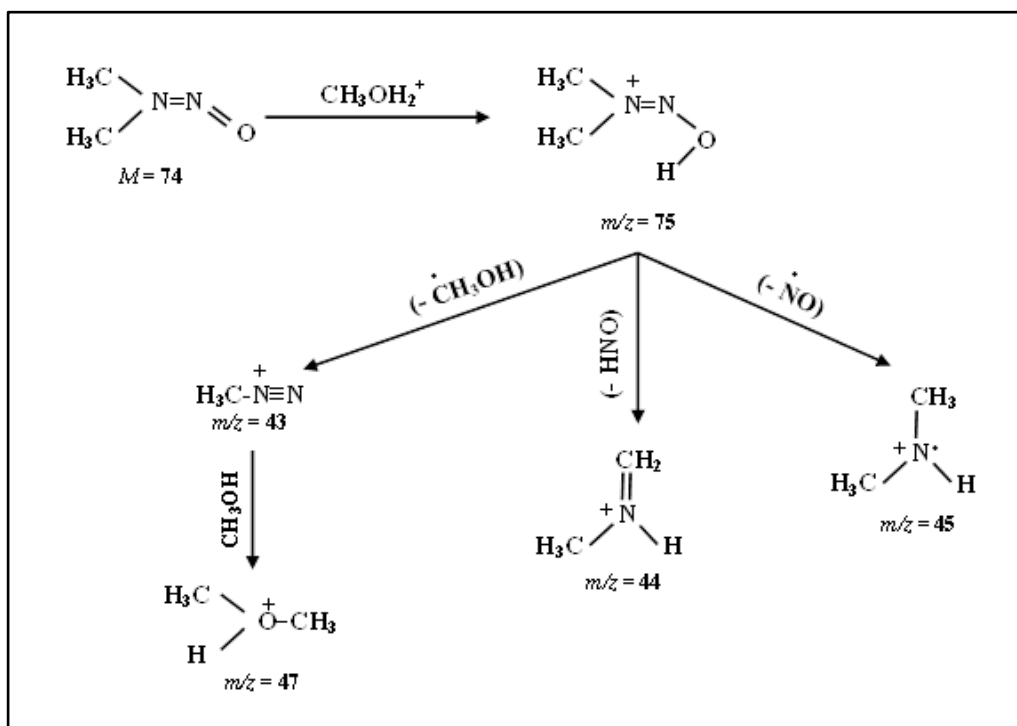
Figure 3-3: Ionisation/fragmentation pathway of NDMA.

For quantitation labelled standards have been used for both furan and VNAs analysis. For furan, isotope dilution using furan-d4 was proposed. In this thesis and since the US FDA uses standard addition (7 concentration levels for each sample) with furan-d4 as IS for calibration, the applicability of isotope dilution using water-based standard solutions with furan-d4 as IS for calibration was evaluated by comparing the results obtained with both methods. No significant differences were observed (Table 2, Paper I) and so, the isotope dilution method can be used with the advantage of reducing analysis time in routine analysis (for 4 samples, 3h less than using standard addition). For VNAs, external calibration using four deuterated standards eluting at different retention times along the chromatogram, was used. In both cases, a good linearity with determination coefficients ($r^2$) higher than 0.99, were obtained.

With regard quality parameters of the HS-SPME-GC-MS methods established in this thesis, good specificity, trueness, precision and limits of quantification were obtained. First, as the HS-SPME-GC-MS methods have been developed to be used in

food control laboratories, it was demonstrated that the use of different 75-µm CAR/PDMS fibres obtained from different lots, and also the durability of the fibre (life-time up-to 200 analyses), have no significant effects on the performance of the methods. To evaluate limits of quantification (LOQs) blank food samples, when available, spiked at low concentration levels were used. For furan and since no blank samples can be found, LOQs were estimated from food samples contain very low concentration levels and also adding furan-d4 at a low concentration to the samples. Good agreement between both approaches was obtained and the LOQs that were sample-depended ranged from 0.05 to 0.4 ng g$^{-1}$ (Table 2, Paper II). Our obtained LOQs for furan in food were about 10-times lower than those reported in the literature for HS-SPME (Table 2.3) and also than those obtained when using direct HS methods (Table 2.2). In addition, no significant differences were found between the automated HS-SPME method (Table 1, Paper II) and the manual one (Table 2, Paper I) although slightly lower LOQs were obtained with this last method. LOQs and also the decision limits (CCα) and detection capabilities (CCβ) were evaluated for VNAs analysis in food (Table 2 of Paper III) when using HS-SPME coupled to GC-CI-MS/MS. Satisfactory LOQs (0.1-0.38 ng g$^{-1}$) were obtained which are from 7 to 25-fold lower than those reported in the literature using HS-SPME and similar to the values found using exhaustive extraction methods such as LLE and SPE (Table 2.4) which are labor intensive and require large amounts of organic solvents. In addition, these LOQs are lower than the maximum admissible values established in several countries for levels of VNAs in cured meat products (3 ng g$^{-1}$- 10 ng g$^{-1}$) and beer (0.5 ng g$^{-1}$- 5 ng g$^{-1}$). In relation to the precision of the HS-SPME-GC-MS methods developed in this thesis, for the analysis of furan in different food samples the relative standard deviation (%RSD) of the results does not exceed 10% (Table 1 Paper I) and a similar value was found for the VNAs (12%) (Table 2, Paper III).With respect to trueness, different approaches were used. For furan it was assessed participating in an interlaboratory study on furan in baby food organized by the Institute for Reference Materials and Measurements (IRMM) of the European Commission's Joint Research Centre (JRC). Our results obtained using the developed automated method, were satisfactory with a z-score < 0.6 (JRC-IRMM, 2008b). Moreover, the proposed HS-SPME-GC-MS method was compared to the direct HS-GC-MS which was proposed by US FDA and considered as

reference for furan analysis in food. As can be seen in Table 2 of Paper II, no significant differences between the results obtained by both methods was found (P-value < 0.05 at 95% confidence level) although the HS-SPME showed better precision (5- 8%) than direct HS (9- 12%) and it is up to 20-fold more sensitive (LOD; 0.02- 0.12 ng g-1) than direct HS which is mainly due to the higher pre-concentration capacity of HS-SPME than HS and also to the use of splitless injection mode instead of the split injection used in direct HS. For VNAs, since no certificated reference material is available, trueness was assessed through a quantitative recovery study. The mean quantitative recoveries (Table 2 of Paper III) were within the values recommended by the Commission Decision 2002/657/EC.

### 2.5.3. Analysis of furan and volatile N-nitrosamines in food

To ensure the applicability of the developed fully-automated HS-SPME-GC-MS methods, several foodstuffs of different characteristics, liquid, semi-solid and solid commercialized in Barcelona were analyzed to determinate furan and VNAs content. As can be seen in Table 3 of Paper II, furan was found in all the samples analysed at a concentration ranging from 0.1-0.3 ng $g^{-1}$ in sauces and soups, to 40-70 ng $g^{-1}$ in baby food and brewed coffee, being the highest the values found for powdered coffee (820-1100 ng $g^{-1}$). These concentration levels were in accordance with data previously reported in the literature (Tables 2.2; 2.3 and 2.4). For instance, concentrations of furan in fruit juices (1 to 6 ng $g^{-1}$) reported by the Swiss Federal Office of Public Health (SFOPH) and the US FDA (SFOPH, 2004; FDA, 2004a) are in agreement with our values 0.7-2.3 ng $g^{-1}$ and the values reported by EFSA and FDA for honey (3- 10 ng $g^{-1}$) and soup (3- 125 ng $g^{-1}$) were also in the same concentration range that the samples commercialized in Spain. In this context it is important to mention that there are two food categories, baby food and coffee, that have a high level of furan and in addition, are highly consumed. For this reason, additional work regarding the presence of furan and also the risk to furan exposure associated to the consumption of these foodstuffs was performed in this thesis. The results obtained are included in Chapter III. Regarding to VNAs as can be seen in Table 3 of Paper III, no positive beer samples were found (12 samples) while for meat products, the percentage of positive samples was very low

(16%) and only some VNAs, were quantified at low concentration levels, NDMA (2.4-3.7 ng g$^{-1}$) and NPYR (2.6- 3 ng g$^{-1}$), in Spanish chorizo and cured sausage. Similar results were found in the literature. For instance in beer some authors did not detected VNAs (Cárdenes et al., 2002; Pèrez et al., 2008) while other only found low concentration levels of NDMA (0.14- 0.56 ng g$^{-1}$) (Jurado-Sánchez et al., 2007; Yurchenko and Mölder, 2005). For meat product samples, only NDMA and NPYR between 0.3 and 4.1 ng g$^{-1}$ (Campillo et al., 2011, Sannino and Bolzoni, 2013) were found. These low levels of VNAs in such samples is due, as commented in Chapter 1, to the application of European legalisation regarding food additives that has reduced the permitted levels of these compounds in processed meat, and to the modification of the industrial process to reduce the formation of VNAs in food. For this reason, no additional samples were analysed and risk assessment was not performed for these compounds in this thesis.

# 3. FURAN OCCURRENCE IN SPANISH FOOD

## 3.1. INTRODUCTION

As commented in the Introduction of this thesis, furan was classified as possibly carcinogenic to humans by IARC (Group 2B) in the mid-nineties (IARC, 1995) but it is still unclear which risks are associated with its intake. This may be related to the limited data available about its occurrence in processed food and, as a consequence, also about the exposure of the population to this process-induced food carcinogen. Therefore, in the last years, extensive research activities have been launched by national authorities and research institutes to assess the potential health risks of furan. As a result of these initiatives, information about furan occurrence in food was published between 2004 and 2016. The first set of furan data in food products was released by the US FDA in 2004. This institution requested for more information on furan in food (US FDA, 2004a) and held a food advisory committee meeting (June 2004) to seek advice about data needed to assess the risk posed by furan to consumers. At that time limited information about furan occurrence in food was available and US FDA developed an action plan to increase it. As a result, the first US FDA exploratory data on furan in food that contained less than 300 samples was expanded to approximately 750 food samples in 2009 (US FDA, 2009). The results of these studies (number of samples and negatives) and the range of concentration levels of furan in foods reported by US FDA are included in Table 3.1. This survey clearly revealed that furan occurred in a broad variety of foods which underwent heat treatment, including baby food, infant formulas, coffee, beer, soup, sauces, meat, fish, canned vegetables and fruits with furan levels ranging from non-detectable to 174 ng g$^{-1}$. It must be mentioned that baby foods in cans and jars, except baby cereals, and also coffee were the only food categories that always contained furan at concentration levels up to 112 and 84 ng g$^{-1}$, respectively.

In parallel, the European Food Safety Authority (EFSA) welcomed the proposal of the panel on contaminants in the food chain (CONTAM) of establishing a working group to start collecting information on furan in food. In the initial report of the CONTAM panel issued in 2004, information about furan occurrence was only available for a narrow set of foods (EFSA, 2004).

Table 3.1: Furan occurrence in food reported by US FDA between 2004 and 2009. (US FDA, 2009)

| Food category | Sub-category | Number of samples | | Furan concentration | |
|---|---|---|---|---|---|
| | | Total | Negative | Minimum | Maximum |
| **Baby food** | Purred fruits and fruit juice | 49 | 0 | 1.3 | 31.7 |
| | Purred vegetables | 34 | 0 | 20.2 | 112 |
| | Vegetables with rice or potatoes | 29 | 0 | 14.5 | 108 |
| | Contain chicken or meat | 23 | 0 | ≥ 0.8 [a] | 87.3 |
| | Pasta with chees | 8 | 0 | 9.1 | 54 |
| | Baby cereals | 5 | 3 | 3.9 | 6.4 |
| **Infant formula** | Need domestic preparation | 23 | 10 | 8.3 | 26.9 |
| | Ready to feed | 10 | 3 | 2.5 | 21.1 |
| **Coffee** | Brewed coffee | 8 | 0 | 33.6 | 84.2 |
| | Instant coffee | 5 | 0 | ≥ 2 [a] | 7.2 |
| **Beverages** | Chocolate and vanilla | 22 | 2 | ≥ 0.4 [a] | 38 |
| | Other nutrition drink | 24 | 5 | 0.4 | 174 |
| | Beer | 8 | 4 | 0.8 | 4.4 |
| **Desserts and snakes** | Packed desserts, jams and similar products | 107 | 29 | ≥ 0.8 [a] | 37.4 |
| | Honey, molasses and syrup | 11 | 1 | 0.5 | 88.3 |
| | Breakfast cereals | 40 | 19 | 1.2 | 47.5 |
| | Snakes including potato chips | 29 | 2 | ≥ 3.2 [a] | 64.7 |
| | Concentrated milk | 3 | 3 | - | - |
| | Brown sugar | 2 | 0 | 1 | 2.2 |
| **Mixtures** | Pasta sauces | 22 | 0 | ≥ 5 [a] | 26.1 |
| | Chicken broths | 7 | 0 | 6.7 | 18.2 |
| | Soups | 12 | 0 | 10 | 125 |
| | Vegetarian soup | 16 | 0 | 16.4 | 91.2 |
| | Chilli products | 3 | 0 | 66.3 | 94.4 |
| | Prepared pasta | 10 | 0 | 9.5 | 42.9 |
| | Sauces for meat food | 11 | 0 | 5.8 | 46 |
| **Fish and meat products** | Tuna in water | 6 | 0 | ≥ 5 [a] | 7.1 |
| | Tuna in oil | 1 | 0 | 1.5 | - |
| | Other fish products | 2 | 0 | 4 | 8.1 |
| | Frankfurters | 7 | 2 | 0.7 | 7.7 |
| | Cooked Sausages | 4 | 2 | ≥ 5 [a] | 39.2 |
| | Meat gravies | 8 | 0 | 13.3 | 173.6 |
| | Other meat products | 22 | 6 | 0.3 | 31.6 |
| **Fruit and vegetables** | Vegetables only | 66 | 4 | 0.8 | 68.1 |
| | Vegetables with meat | 9 | 0 | 37.2 | 122 |
| | Fruit only including juices | 63 | 6 | 0.5 | 30.5 |
| **Miscellaneous** | Bread | 12 | 11 | ≥ 2 [a] | - |
| | Crackers and crisp-breads | 4 | 0 | 4.2 | 18.6 |
| | Dried fruits | 8 | 1 | 0.6 | 2.2 |
| | Butter | 7 | 0 | 2.1 | 7.5 |
| | Mayonnaise | 3 | 3 | - | - |
| | Ketchup | 1 | 0 | 12.3 | - |
| | Soy sauces | 5 | 0 | 17.2 | 75.6 |
| | Cheese | 6 | 1 | 0.4 | 2.9 |
| | Egg | 1 | 1 | - | - |
| | Evaporated milk | 3 | 0 | 10.9 | 15.3 |

Furan concentration (ppb) in positive samples.
Values ≤ LOQ.

Therefore, the panel envisaged the need for future analyses and collection of furan occurrence data in a wider spectrum of food commodities. In addition, a call for data on furan in food and beverages was issued in 2006 (EFSA, 2006) in conjunction with the European Commission recommendation published in 2007, on the monitoring of the presence of furan in foodstuffs. As a result, a total of approximately five thousand analytical results from samples analysed between 2004 and 2010, for furan content in food, submitted by 20 European countries, were reported by EFSA (EFSA, 2011). The analytical results including the number of samples, percentage of positives, furan maximum concentrations and median and mean values for lower and upper bounds obtained are indicated in Table 3.2. To calculate values in a lower bound scenario, concentrations below LOD and between LOD and LOQ were set to zero while in an upper bound scenario, values below LOD and values between LOD and LOQ were set to the LOD and LOQ respectively. EFSA results also showed, in agreement with the US FDA results, that a considerable part of the occurrence of furan in food corresponds to baby food and coffee samples. In this database, the maximum reported concentration among all food samples was for roasted coffee beans with a mean concentration of 3660 ng g$^{-1}$ while the minimum concentration was for infant formula samples with a mean concentration of 3.2 ng g$^{-1}$.

Several independent surveys on furan in a wide variety of food commodities have also been conducted in the last years in various European countries including Finland (Jestoi et al., 2009), Belgium (Scholl et al., 2012a; 2012b; 2012c; 2013), Germany (Lachenmeier et al., 2009; Waizenegger et al., 2012), The Netherlands (Wegener and López-Sánchez, 2010), Spain (Mesías and Morales, 2014; Ruiz et al., 2010; Mesías et al., 2013) Italy (Bononi and Tateo, 2009; Bianchi et al., 2006; La Pera et al., 2009), Switzerland (Zoller et al., 2007), Lithuania (Pugajeva et al., 2016) and UK (Robert et al., 2008; Pye and Crewes, 2013). Moreover, data on furan concentrations in many food products from Canada (Becalski et al., 2005b; 2010; 2016) as well as from Australia (Food Standards Australia New Zealand (FSANZ) 2010) have been published.

Table 3.2: Results of furan content in food (the range indicates the results for the lower and upper bounds) (EFSA, 2011).

| Food category | Sample number | | Reported LOQs range (ng g⁻¹) | Furan concentration (ng g⁻¹)[b] | | | |
|---|---|---|---|---|---|---|---|
| | Total | Samples (%) > LOQ | | $P_{25}$ | Median | Mean | Maximum |
| Coffee instant (a) | 109 | 96 | 0.2 - 28 | 58 | 132 | 394 | 2200 |
| Coffee roasted bean (a) | 30 | 100 | 0.2 - 10 | 2160 | 3789 | 3660 | 11000 |
| Coffee roasted ground (a) | 110 | 99 | 0.1 - 100 | 476 | 1770 | 1936 | 6900 |
| Coffee brew | 89 | 57 | 5 - 10 | 0 - 5 | 13 | 42 - 45 | 360 |
| Coffee, not specified (a) | 596 | 99 | 0.1 - 28 | 1040 | 1932 | 2016 | 6588 |
| Baby food | 1617 | 83 | 0.04 - 10 | 5 – 8.6 | 24 | 31 - 32 | 233 |
| Infant formula | 11 | 9 | 1 - 10 | 0 – 2.5 | 0 – 2.5 | 0.2 – 3.2 | 10 |
| Vegetables | 192 | 42 | 0.2 - 10 | 0 - 3 | 0 - 5 | 6.9 – 9.6 | 74 |
| Vegetable juices | 80 | 16 | 0.6 - 10 | 0 – 3.6 | 0 – 9.1 | 2.9 – 9 | 60 |
| Fruits | 142 | 29 | 0.18 - 10 | 0 - 3 | 0 - 5 | 2 – 6.4 | 36 |
| Fruit juices | 250 | 34 | 0.6 - 10 | 0 – 1.5 | 0 – 2.1 | 2.2 – 4.6 | 90 |
| Cereal products | 190 | 58 | 0.3 - 40 | 0 – 3.3 | 4 - 10 | 15 – 18 | 168 |
| Fish products | 47 | 75 | 0.32 - 10 | 0 – 1.2 | 2.7 – 4.3 | 17 | 172 |
| Meat products | 174 | 46 | 0.18 - 20 | 0 - 4 | 0 - 10 | 13 - 17 | 160 |
| Milk products | 64 | 50 | 0.18 - 10 | 0 – 0.3 | 0.3 – 0.9 | 5 – 5.6 | 80 |
| Beer | 102 | 50 | 0.18 – 9.1 | 0 - 2 | 0.1 - 3 | 3.3 – 5.2 | 28 |
| Soy sauces | 94 | 90 | 0.2 - 10 | 14 | 24 | 27 | 78 |
| Soups | 270 | 80 | 0.18 - 10 | 2.5 - 5 | 18 | 23 - 24 | 225 |
| Sauces | 271 | 52 | 0.18 - 10 | 0 - 4 | 2.4 – 8.5 | 8.3 - 11 | 175 |
| Baked beans | 57 | 77 | 0.2 - 10 | 2.7 - 10 | 20 | 22 - 24 | 80 |
| Other products | 552 | 76 | 0.18 - 20 | 0.3 - 3 | 7.2 – 8.9 | 14 - 15 | 164 |

(a) Furan content in solid coffee.
(b) $P_{25}$: The furan content of 25% of the considered samples was found below this numeric value.

In addition, surveys on furan in food from Brazil (Arisseto et al., 2010; 2011; 2012), Chile (Mariotti et al., 2013) and also some Asian countries including China (Nie et al., 2013; Shen et al., 2016), Taiwan (Liu and Tsai, 2010) and Korea (Kim et al., 2009; 2010; Kim et al., 2016) have been reported. In general, the main food samples included vegetables, fruit, meat and fish, sauces, soups and broths, cereal products, drinks and beers, desserts, coffee and coffee drinks and baby foods. In order to summarise and give an overview of the furan occurrence data in foods found in the literature from independent surveys, the percentage contribution of food categories and furan concentration are shown in Figure 3.1 and Table 3.3, respectively. As can be seen in Figure 3.1, baby food (32%) and coffee (17%) are the most studied food categories for furan content. For baby food (Table 3.3), a high furan concentration range has been found (0.5 - 331 ng g$^{-1}$) with a median concentration of 15 ng g$^{-1}$ while for coffee brews the range is between 7 and 352 ng g$^{-1}$ with a median concentration of 46 ng g$^{-1}$.
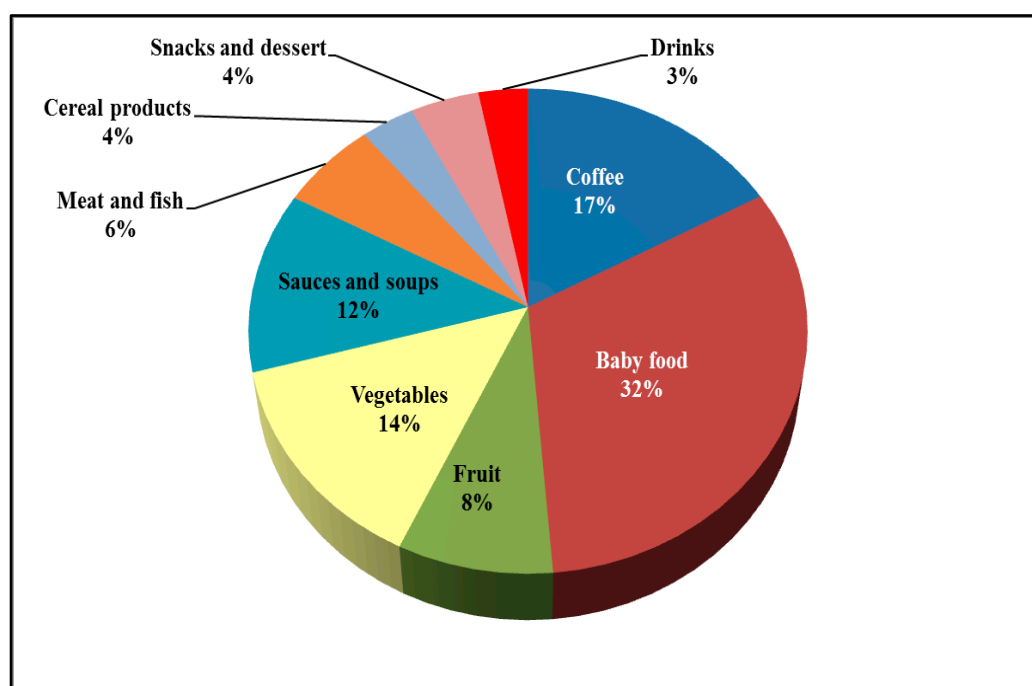


Figure 3.1:  Contribution percentage of studied food categories for furan content.

Table 3.3: Furan concentration levels in several food categories found in the literature (2004-2016).

| Food category | Furan concentration (ng g$^{-1}$ or ng ml$^{-1}$) | | | | |
| --- | --- | --- | --- | --- | --- |
| | Min | P$_{25}$ | Median | P$_{75}$ | Max |
| Soups and broths | 13 | 26 | 39 | 87 | 120 |
| Snacks and dessert | 1 | 6 | 14 | 25 | 180 |
| Meat and fish products | 3 | 14 | 30 | 134 | 195 |
| Fruit products | 0.5 | 4 | 9 | 16 | 51 |
| Cereals products for adults | 13 | 22 | 30 | 41 | 65 |
| Beer and another drinks | 0.4 | 1 | 2 | 10 | 33 |
| Sauces products | 2 | 16 | 26 | 58 | 286 |
| Coffee brews | 7 | 29 | 46 | 85 | 352 |
| Baby-foods | 0.5 | 5 | 15 | 35 | 331 |
| Vegetables products | 1 | 5 | 16 | 35 | 338 |

In this table we have included the first and third quartiles of furan concentration in addition to median, minimum and maximum concentration values to provide a better picture of furan occurrence in food.

From the data included in these tables (Table 3.1, Table 3.2, and Table 3.3) it can be deduced that furan is present in most heat treated food categories and its concentration level varies over wide range (not detected up to 11000 ng g$^{-1}$). However, by far the occurrence data of furan in food shows that two categories of foods, coffee and baby food accumulate always important amounts of furan.

## 3.2. RATIONALE OF THE STUDY

As commented in the previous section, one of the main sources of exposure to furan for adults is the intake of coffee while for children, the presence of furan in baby-food can be a problem since frequently it is almost their sole diet. The high furan levels

found in most foods that underwent a heat treatment in a sealed container such as baby food is related to the fact that the generated furan is almost completely trapped. In the case of coffee, the high levels of furan found in coffee may be related to the roasting process of the beans where high temperature, exceeding most of the other food processing procedures, is used. Analysing data of furan in coffee and baby food samples reported in the literature we found that the percentage of coffee samples reported in the US FDA survey (Table 3.1) was very small (less than 2%, 13 samples) while for baby food and infant formula were about 19% and 4%, respectively, of total studied food (750 food samples). With regard to Spain, the reported data of furan occurrence in baby food (17 samples), infant formula (0 samples) and coffee (27 samples) are small (EFSA, 2011). Moreover, it must be mentioned that most furan data of coffee samples reported by EFSA (Table 3.2) were obtained from beans and/or ground coffee (solid coffee) and coffee brews are less than 2% of the total studied samples (5050 samples) although, from a consumption point of view, data on furan content in brewed coffee is of higher interest. In relation to baby food data it must be mentioned that generally most of the samples have been analysed as purchased. Although the occurrence of furan in such food samples is mainly influenced by the physical and chemical conditions of the industrial processes (ingredients, temperature and time), the way of sample preparation for the food to be ready to eat could affect the final furan intake. In fact, EFSA claimed the need for information on furan in food samples analysed as purchased and also as consumed indicating the influence of preparation processes on the furan content in ready-to-feed samples (EFSA, 2011).

So, we thought that additional research was needed to expand the database on furan levels in coffee and baby foods from Spain and to evaluate the effects of sample preparation, ready-to-feed, on furan levels which is of high interest for risk assessment evaluation. The works included in this thesis represent a detailed investigation on the presence of furan in coffee and baby food in Spain and have been published simultaneously with the results of several European projects, in which Spain has been involved, such as PROMETHEUS, FURA-RA and HEATOX (our research group was a partner in this last project) all studying furan in food (Mesías and Morales, 2014;

Mesías et al., 2013). Nevertheless, there are no studies on the occurrence of furan in different coffee brews neither in infant formula and cereals baby food commercialised in Spain and the rare available dada on furan in jarred or canned baby food are not representative of the different baby food groups (Ruiz et al., 2010; Mesías et al., 2013). In addition, the exposure estimations are scarce and no furan risk assessment from coffee and baby food for Spanish population has been published.

In this thesis, two studies were carried out aiming to provide data on the occurrence of furan in coffee brews **(Paper IV)** and baby foods **(Paper V)** commercialised in Spain. Moreover, the exposure to furan and its risk assessment in Spain was evaluated using the obtained data from each study and consumption data for those samples.

## 3.3. FURAN IN COFFEE AND BABY-FOOD FROM THE SPANISH MARKET

The results of the studies performed in this thesis related to the analysis of furan in coffee brews and baby food are included in **Paper IV** and **Paper V**. In addition, the influence of several factors such as roasting conditions, brewing methods, processing steps or consumer handling on the final furan level in coffee brews are evaluated in **Paper IV**. Similarly, the effects of baby-food treatment before consumption, such as preparation, reheating and handling, on the furan content of the final food are also discussed in (**Paper V**).The estimations of furan intake for babies from baby food consumption are included in **Paper V**, while for adults furan intake by coffee consumption is commented in the section 3.4 of this chapter.

# 3.3.1. PAPER IV

## OCCURRENCE OF FURAN IN COFFEE FROM SPANISH MARKET: CONTRIBUTION OF BREWING AND ROASTING.

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# Occurrence of furan in coffee from Spanish market: Contribution of brewing and roasting

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A R T I C L E   I N F O

A B S T R A C T

In this work, we evaluated the occurrence of furan in brews obtained from regular, decaffeinated, and instant coffee and commercial packed capsules. For this purpose, a previously developed automated headspace solid-phase microextraction method coupled to gas chromatography–mass spectrometry (HS-SPME-GC–MS) was used. Initially, the influence of HS-SPME conditions on furan formation was evaluated. In addition, the effect of roasting conditions (temperature and time) used for coffee beans on furan formation was also studied. We found that low temperature and long roasting time (140 °C and 20 min) decreases the final furan content. Furan concentrations in regular ground coffee brews from an espresso coffee machine were higher (43–146 ng/ml) than those obtained from a home drip coffee maker (20 and 78 ng/ml), while decaffeinated coffee brews from a home drip coffee maker (14–65 ng/ml) showed a furan concentration similar to that obtained from regular coffee. Relatively low concentrations of this compound (12–35 ng/ml) were found in instant coffee brews, while commercial packed coffee capsules showed the highest concentrations (117–244 ng/ml). Finally, the daily intake of furan through coffee consumption in Barcelona (Spain) (0.03–0.38 μg/kg of body weight) was estimated.

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## 1. Introduction

In recent years considerable attention has been given to the study of heat-induced toxicants in food as a result of their potential harmful effects on human health (Wenzl, Lachenmeier, & Gökmen, 2007). Among these chemicals, furan ($C_4H_4O$) is one of the main undesirable contaminants formed during thermal treatment of food and drink. Although the presence of furan in foods has been known for years (Maga, 1979), interest in this compound has increased recently because of its classification as a possible human carcinogen (Group 2B) by the international Agency for Research on Cancer (IARC) (FDA, 2004; IARC, 1995; NTP, 2004). Therefore, monitoring programs have been launched by several food organisations such as the US Food and Drug Administration (FDA), the Swiss Federal Office of Public Health (SFOPH) and the European Food Safety Authority (EFSA) (EFSA, 2004; FDA, 2008; SFOPH, 2004), to determine the occurrence of furan in food commodities. Several mechanisms that can explain furan formation in foods are well documented. These mechanisms are related mainly to thermal degradation of carbohydrates, pyrolysis of sugars at high temperature, oxidation of polyunsaturated fatty acids and decomposition of ascorbic acid and its derivatives (Crews & Castle, 2007).

Coffee contains higher concentrations of furan (up to 199 ng/ml) than other processed foods (EFSA, 2004; FDA, 2008; SFOPH, 2004; Zoller, Sager, & Reinhard, 2007). Moreover, given that coffee is highly consumed (ICO, 2008), exposure to furan may be a potential risk to human health. Furan in coffee is generated during the roasting of green coffee beans, which are rich in carbohydrates and amino acids (Murkovic & Derler, 2006). The temperature and time used during roasting are the main factors responsible for furan formation in coffee. In addition, the brewing procedure can also affect the furan content. However, there is little information about the influence of roasting and brewing processes on furan formation in coffee (Kuballa, Stier, & Strichow, 2005; Zoller et al., 2007). Moreover, the measurement of human exposure to furan is hampered by a scarce data about its occurrence in this beverage (EFSA, 2007), which is the main source of furan for adults. In order to obtain reliable data to assess the exposure of humans to furan through coffee consumption, more information is required.

The analysis of furan is currently performed by headspace (HS) (Crews, Roberts, Lauryssen, & Kramerc, 2009; FDA, 2006; Morehouse, Nyman, Mcneal, Dinovi, & Perfetti, 2008; Zoller et al., 2007) or headspace solid-phase microextraction (HS-SPME) (Altaki, Santos, & Galceran, 2007, 2009; Bianchi, Careri, Mangia, & Musci, 2006; Ho, Yoo, & Tefera, 2005; La Pera et al., 2009) both coupled to gas chromatography–mass spectrometry (GC–MS). However, furan can be generated during the analysis and the extraction temperature and time must be controlled to prevent its formation (Crews & Castle, 2007). For instance, furan formation

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has been reported during HS analysis of ground green coffee beans at a temperature as low as 40 °C (Senyuva & Goekmen, 2005). Moreover, the HS method proposed by the FDA in 2004 was updated in 2006, decreasing sampling temperature from 80 to 60 °C because of the observation of furan formation at the former temperature (Nyman, Morehouse, McNeal, Perfetti, & Diachenko, 2006, 2008). One advantage of using HS-SPME instead of HS is that lower extraction temperatures (~30 °C) can be commonly used and consequently the generation of furan can be reduced.

Here we examined the furan content in brews obtained from regular, decaffeinated and instant coffee commercialised in Spain. We used a fully automated HS-SPME-GC–MS method. In addition, the occurrence of furan in coffee brews from commercially packed capsules was evaluated for the first time. The influence of HS-SPME conditions, such as temperature and time, on furan formation during the analysis was also studied. Moreover, the effect of roasting conditions and brewing procedures on the furan content of the final brews was examined. By means of a 24-h recall questionnaire, we also estimated furan intake from coffee consumption in a small population sample in Barcelona (Spain).

## 2. Materials and methods

### 2.1. Chemicals and standards

Furan and $[^2H_4]$ furan (furan-$d_4$) at a purity >99% were purchased from Sigma–Aldrich (Munich, Germany). Water of organic trace grade, methanol of GC grade and sodium chloride of analytical grade were all obtained from Merck (Darmstadt, Germany). Individual stock standard solutions of furan and furan-$d_4$ at a concentration of 12 mg/g in methanol were prepared by transferring 20 µl of pure compounds to a 2-ml amber-sealed vial previously filled with methanol. Intermediate standard solutions of furan and furan-$d_4$ were prepared by weight at a concentration of 90 µg/g from stock standard solutions by appropriate dilution with methanol. All these solutions were stored at 0 °C and prepared weekly. Aqueous working standard solutions of furan and furan-$d_4$ at a concentration of 90 ng/g were individually prepared daily by spiking 20 ml of water with appropriate volumes of their intermediate standard solutions. For furan determination, eight-calibration standard solutions at concentrations ranging from 0.05 to 10 ng/g were prepared by adding in weight, through the septum, an appropriate amount of the aqueous working standard solution of furan into a 20-ml HS vial. In addition, 100 µl of the furan-$d_4$ aqueous working solution was added to each calibration solution to achieve a concentration of 0.9 ng/g.

### 2.2. Coffee sample preparation

A total of 23 samples of ground coffee (15 caffeinated and 8 decaffeinated) and five samples of instant coffee, selected from the most consumed brands in Spain, were obtained from the major markets in Barcelona (Spain). In addition, 12 Nespresso® coffee capsules (Nestlé Nespresso® SA, Paudex, Switzerland) (*Ristretto, Arpeggio, Roma, Livanto, Capriccio, Volluto, Cosi, Decaffeinato, Decaffeinato intense, Vivalto Lungo, Decaffeinato Lungo and Finezzo Lungo*) were purchased. Before use, commercial coffee samples were stored at 4 °C in their sealed packages to prevent loss of furan. To study the effect of roasting on furan formation, green coffee beans from Brazil were purchased from a local roasting factory in Barcelona, Spain. Portions of these beans (ca. 100 g) were then individually roasted at temperatures of 140, 170 and 200 °C for 6 min in an electric rotating laboratory roaster (Probat-Werke Pré 1Z, Probat-Werke, Germany). Moreover, to determine the influence of temperature and time on the formation of furan, several

portions of the beans were roasted to the same degree under three temperature–time conditions: high temperature-short roast (200 °C, 6 min), intermediate temperature-intermediate roast (170 °C, 12 min) and low temperature-long roast (140 °C, 20 min). All coffee samples were then ground at room temperature to a commonly commercialised particle size (200–250 µm). To determine the degree of roasting, the international standard colour space parameters were used. These parameters, proposed by the International Commission on Illumination (CIE) (Papadakis, Abdul-Malek, Kamdem, & Yam, 2000), allow the characterisation of coffee colour by means of $L^*a^*b^*$ parameters, where $L^*$ is the luminance or lightness component and $a^*$ and $b^*$ are the chromaticity coordinates. These measurements were performed using a Chromameter CR-410 spectrophotometer (Konica Minolta, Osaka, Japan).

The regular ground coffee samples (9 g) were brewed using an espresso coffee machine and a home drip coffee maker to obtain approximately 60 ml of brew in both cases. For the coffee roasted in the laboratory, only the automatic espresso machine was used to obtain brews. Decaffeinated ground coffees were prepared with a home drip coffee maker using the same coffee/water ratio as that used for the brewed regular ground coffee. Instant coffee was obtained following the manufacturer's recommendations, mixing 2 g of powder with 60 g of boiling water. For preparation of Nespresso® coffee, all espresso blends, ca. 5.5 g of coffee, were brewed with an Essenza Nespresso coffee machine to obtain 40 ml of coffee, while for the Lungo coffee capsules, 110 ml of brew was obtained from ca. 7 g of coffee. After preparation, all brews were placed immediately in a closed vial with no headspace and then stored at 4 °C until analysis.

### 2.3. GC–MS conditions

GC–MS analyses were performed on a Varian 3800 gas chromatograph coupled to a Saturn 2200 ion-trap MS system (Varian, Mississauga, Canada) and equipped with a CTC Combi-Pal autosampler (CTC Analytics AG, Zwingen, Switzerland), which is configured with a sample tray for 32 vials of 20 ml, a SPME fibre conditioning station, and a temperature-controlled single magnet mixer tray. Separation was performed on a BX-volatile (cyanopropylphenyl polysilphenylene-siloxane), 60 m × 0.25 mm i.d., fused-silica capillary column (SGE Europe, Villebon, France) with a film thickness of 1.4 µm. The temperature programme was 35 °C (held for 2 min) to 230 °C at 20 °C/min (held for 5 min). The injector was fitted with an SPME inlet liner (i.d., 0.75 mm, SGE Europe) and a 23-gauge Merlin Micro-seal septum (Supelco, Bellefonte, PA, USA) and operated in splitless injection mode (3 min). Helium was used as carrier gas at a constant flow-rate of 1.7 ml/min. The ion trap mass spectrometer was tuned using perfluorotributylamine (FC-43) following the manufacturer's recommendations. Electron ionisation (70 eV) with 30 µA of emission current and temperatures of 200, 80 and 280 °C were set for the trap, manifold and transfer line, respectively. For MS acquisition, EI full-scan mode was used over the mass range $m/z$ 35–100 at 0.75 s/scan (7 µscan per scan). Quantification of furan was performed by isotope dilution using $m/z$ 68 and $m/z$ 72 for furan and furan-$d_4$, respectively. For confirmation, $m/z$ 39 $[M–CHO]^+$ for furan was monitored. A Varian Saturn Workstation software (Version 6.42) was used for control, general operation and data acquisition of the GC–MS system, while the Cycle composer software (Version 1.5.3) was used to control and program the CTC Combi-Pal autosampler.

### 2.4. Automated HS-SPME method

Furan analysis in coffee samples was performed using an automated HS-SPME method that was previously developed and

validated (Altaki et al., 2009). Briefly, an aliquot (0.5 ml) of brewed coffee was quickly transferred from the storage vial, immersed in an ice/water bath (4 °C) to a 20-ml headspace vial containing a PTFE-coated stir bar, 2 g of sodium chloride and an adequate amount of water (up to 8 ml). The vial was immediately sealed and then spiked with 100 μl of furan-$d_4$ aqueous working standard solution. For HS-SPME analysis, a 75-μm carboxen–poly-dimethylsiloxane fibre (CAR/PDMS) (Supelco, Bellefonte, PA, USA), which was conditioned following the manufacturer's recommendations (300 °C under helium flow for 1 h) was used. Before analysis, the sample vial was equilibrated for 5 min in the single magnet mixer tray at 30 °C and 750 rpm. The fibre was cleaned for 1 min at 275 °C in a conditioning station and then immediately exposed to the headspace of sample vial for 20 min at 30 °C. Thermal desorption of furan was accomplished by exposing the fibre in the GC injector port at 275 °C for 2 min.

### 2.5. Quality control and quality criteria

Quality control of the HS-SPME method was performed through the routine analysis of procedural blanks and quality control standards and samples to ensure the absence of contaminants and possible carryover between samples, and to assess the quality of the results. In addition, a daily sensitivity test was carried out to check the possible changes in the absorption capacity of the SPME fibre and the GC–MS response. Although no certified reference materials are available, the method was assessed in an interlaboratory study on furan in baby food (z-score < 0.6), organised by the Institute for Reference Materials and Measurements (IRMM) of the European Commission's Joint Research Centre (JRC) (JRC, 2008). The identification criteria for furan were based on its retention time and the intensity ratios of furan and furan-$d_4$ ions. A deviation of the ion intensity ratios within 20% of the mean values of the calibration standards was considered acceptable.

### 2.6. Coffee consumption data and furan intake

Data on coffee consumption habits in Spain were obtained from the Spanish Coffee Federation (FEC) and the Spanish Ministry of Agriculture, Fisheries and Food (MAPA) (FEC, 2007; MAPA, 2007). Furthermore, coffee consumption data were obtained using a 24-h recall questionnaire on 488 participants (219 females and 269 males) aged from 19 to 58 years from five zones in the city of Barcelona. Each participant was asked about the number of cups of coffee had in the previous 24 h. Furan intake per day and capita from brewed coffee was estimated from the average amount of coffee consumed and the minimum and maximum concentration of furan detected in espresso and drip coffee brews.

## 3. Results and discussion

### 3.1. Evaluation of furan formation during HS-SPME analysis

Here we used an automated HS-SPME-GC–MS method previously developed for the analysis of furan in food (Altaki et al., 2009). The method provided good linearity ($r^2 > 0.999$) over a wide range of concentrations (0.01–10 ng/g) and low limits of detection (LODs, 0.02–0.12 ng/g) with good precision (RSD% ⩽ 10%). However, since the formation of furan from green coffee has been reported during headspace analysis at an extraction temperature of 40 °C (Senyuva & Goekmen, 2005), experiments were conducted to check whether furan is effectively formed during the HS-SPME method. The effects of extraction time and the sample size on furan formation were evaluated at the HS-SPME extraction temperature (30 °C). For this purpose, two aliquots (0.5 and 2 ml) of a natural

coffee brew obtained using an espresso coffee machine were analysed in triplicate using the proposed HS-SPME method (Section 2.4). No significant differences between the furan concentration in the two aliquots (57 ± 4 ng/ml for 0.5 ml of coffee and 55 ± 3 ng/ml for 2 ml) were observed, thereby demonstrating that the amount of precursor, which is directly related to the amount of coffee, did not affect the formation of this compound at 30 °C. In addition, an aliquot of 0.5 ml of the same coffee brew was analysed using an equilibration time of 100 min and the same extraction time (20 min). In this case, the concentration of furan (58 ± 4 ng/ml) was not statistically different to that obtained previously using a 5-min equilibration at a confidence level of 95%. These findings indicate that furan is not formed during HS-SPME analysis at 30 °C during the equilibration and extraction. Moreover, the stability of the sample in the vials before analysis was examined. For this purpose, four sample vials were placed in the sample tray of the automatic injector at laboratory temperature (20 ± 2 °C) and analysed following the proposed HS-SPME method after 0, 1, 6 and 15 h. For all standing times, good agreement in the results (RSD% < 12%) was obtained, thereby demonstrating the stability of the coffee samples in the vials before analysis.

### 3.2. Influence of the roasting process on furan formation

The properties of roasted coffee, such as aroma and taste, are highly influenced by the roasting degree, which particularly depends on the temperature and time applied during the process. However, there is little information about the effect of roasting conditions on furan formation. For instance, Zoller et al. (2007) found that furan concentration increases from 22 to 1792 ng/g (semi-quantitative data) when the roasting temperature is increased from 150 to 250 °C in an open headspace vial (small-scale in the laboratory). In our study, we addressed the effect of roasting on the furan content of green beans from Brazil, which were roasted under conditions commonly used in the coffee manufacturing industry (roaster, roasting degree, cooling and grinding). First, the effect of temperature was evaluated by roasting the coffee beans in an electric rotating roaster for 6 min at 140, 170 and 200 °C. After roasting, the beans were ground and the roasting degree was measured (Section 2.2). Green coffee was not analysed because it is not directly consumed. An Espresso machine was used to prepare a brew from each sample (9 g of coffee powder in 60 ml of water) and the furan content was determined in triplicate using the proposed HS-SPME GC–MS method. Furan levels increased from 22 ± 2 to 138 ± 8 ng/ml with the roasting degree ($L^*$ from 47.4 to 23.16) (Table 1). These findings can be explained by the increase in roasting temperature.

The combined effect of both temperature and time on furan formation was evaluated by roasting several portions of Brazilian green coffee beans to a degree ($L^* = 21 ± 0.7$), commonly used for commercialised regular roasted coffees, but applying different temperature–time conditions. The following three conditions were tested: high temperature-short roast (200 °C, 6 min), intermediate

**Table 1**
Effect of roasting temperature on furan level in coffee brews[a].

| Temperature (°C) | Lightness ($L^*$) [b] | Furan level in brewed coffee | |
|---|---|---|---|
| | | Mean ± sd (ng/ml)[c] | RSD (%) |
| 140 | 47.4 | 22 ± 2 | 9 |
| 170 | 39.98 | 53 ± 3 | 6 |
| 200 | 23.16 | 138 ± 8 | 6 |

[a] Roasting time 6 min. Brewed with an automatic espresso machine at same conditions.
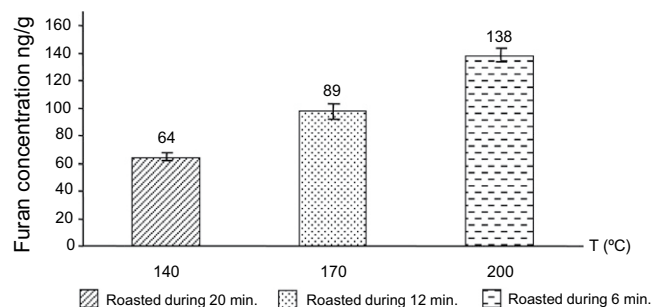[b] $L^* = 100$ (White), 0 (Black).
[c] $n = 3$.

**Fig. 1.** Influence of temperature–time roasting conditions on the furan content of brewed coffee (roasting degree, $L^* = 21 \pm 0.7$).

temperature-intermediate roast (170 °C, 12 min) and low temperature-long roast (140 °C, 20 min). After roasting and grinding, coffee powder was brewed in an espresso machine to obtain 60 ml of coffee brew which was analysed by HS-SPME-GC–MS The lowest furan concentration (64 ± 5 ng/ml) was found for low temperature and slow roasting (140 °C and 20 min) conditions, while for intermediate and quick roasting processes at higher temperatures, the concentration of this compound increased to 89 ± 7 and 138 ± 8 ng/ml, respectively (Fig. 1). These observations could be attributed to the combination of two effects, namely the decrease in furan formation at low temperature and the increase in the loss of furan by evaporation during the long roast at 140 °C.

### 3.3. Influence of coffee type and brewing procedure on furan content

The effect of the brewing procedure on the furan content of the coffee brews was studied using a professional espresso coffee machine and a home drip coffee maker, the most common machines used to prepare coffee in Europe. For this study, brews obtained from 15 commercial ground coffees selected from the most consumed brands in Spain were analysed in triplicate using the proposed HS-SPME GC–MS method. Furan concentration in brews from the two brewing methods ranged from 20 ± 3 to 78 ± 6 ng/ml for brews obtained with a home drip coffee maker and between 43 ± 4 and 146 ± 12 ng/ml for those obtained using espresso machine with a relative standard deviation lower than 15% (Table 2). These concentrations are consistent with those reported in the literature for brewed coffees of diverse origins (3–199 ng/ml) (EFSA, 2004; FDA, 2008; SFOPH, 2004; Zoller et al., 2007). Compar-

ing the results obtained using the two brewing methods and the same coffee/water ratio, the furan content in espresso brews was always higher, between 32% and 85%, than that obtained with a home drip coffee maker. These results can be explained by the higher pressure applied in the espresso machine (900–1000 kPa) than that in the home drip maker (100 kPa), which increases the extraction of furan from the coffee powder. Another factor that must be considered is the loss of furan in the cup or in the pot by evaporation during brewing. Preparation of a cup of coffee often requires 30 s using an espresso machine, while a home drip coffee maker takes about 100 s. This time difference may explain the higher furan content found in espresso coffee brews. High concentrations of furan in brews prepared with an espresso machine have been reported previously (Kuballa et al., 2005; Zoller et al., 2007).

Decaffeinated and instant coffee samples were also analysed. Decaffeinated brews were prepared using a home drip coffee maker while instant coffee samples were obtained following the manufacturer's instructions (Section 2.2). Furan was found in all samples at concentration levels ranging from 14 ng/ml to 65 ng/ml for decaffeinated coffees and between 12 ng/ml and 35 ng/ml for instant coffee (RSD% ⩽ 12%) (Table 3). These concentrations

**Table 3**
Furan concentration (ng/ml) in decaffeinated and instant coffee brews.

| Coffee sample | Furan concentration (ng/ml) | |
|---|---|---|
| | Mean ± sd[a] | RSD (%) |
| *Decaffeinated coffee*[b] | | |
| Natural decaffeinated coffee #1 | 24 ± 3 | 12 |
| Natural decaffeinated coffee #2 | 28 ± 2 | 7 |
| Natural decaffeinated coffee #3 | 20 ± 2 | 12 |
| Natural espresso coffee | 35 ± 3 | 9 |
| Natural cream espresso coffee | 65 ± 2 | 3 |
| Natural 100% Arabica coffee | 14 ± 1 | 8 |
| Natural low caffeine content coffee | 18 ± 2 | 11 |
| Coffee blend (50% natural and 50% torrefacto coffee) | 40 ± 2 | 5 |
| *Instant coffee*[c] | | |
| Natural instant coffee #1 | 12 ± 1 | 8 |
| Natural instant coffee #2 | 20 ± 2 | 10 |
| Natural decaffeinated instant coffee | 28 ± 3 | 11 |
| Natural pure 100% Colombian coffee | 35 ± 2 | 6 |
| Natural 100% Arabica coffee | 32 ± 2 | 6 |

[a] $n = 3$ replicates.
[b] Brewed coffee prepared using a drip coffee maker (9 ml/60 of boiling water).
[c] Instant coffee prepared following the manufacturer directions.

**Table 2**
Furan concentration (ng/g) in brewed regular coffees obtained by drip coffee maker (filter) and espresso coffee machine.

| Regular coffee | Furan concentration in coffee brew (ng/ml) [a] | | | |
|---|---|---|---|---|
| | Drip coffee maker | | Espresso machine | |
| | Mean ± sd | RSD (%) | Mean ± sd | RSD (%) |
| Natural coffee #1 | 40 ± 3 | 8 | 70 ± 5 | 7 |
| Natural coffee #2 | 29 ± 3 | 10 | 65 ± 5 | 8 |
| Natural coffee #3 | 28 ± 3 | 11 | 43 ± 4 | 9 |
| Natural coffee #4 | 48 ± 3 | 6 | 93 ± 6 | 6 |
| Natural espresso coffee | 30 ± 3 | 10 | 60 ± 5 | 9 |
| Natural cream espresso coffee | 39 ± 3 | 8 | 72 ± 6 | 8 |
| Natural filter coffee | 20 ± 3 | 15 | 50 ± 5 | 10 |
| Natural Italian style coffee | 43 ± 3 | 7 | 110 ± 8 | 7 |
| Natural pure 100% Colombian coffee | 66 ± 4 | 6 | 125 ± 6 | 5 |
| Natural pure 100% African coffee | 46 ± 3 | 7 | 82 ± 5 | 6 |
| Natural pure 100% Brazilian coffee | 78 ± 6 | 8 | 146 ± 12 | 8 |
| Natural 100% Arabica coffee | 24 ± 3 | 13 | 56 ± 5 | 8 |
| Natural black 100% Arabica coffee | 52 ± 3 | 6 | 101 ± 5 | 5 |
| Coffee blend (70% natural and 30% torrefacto coffee) | 35 ± 3 | 9 | 74 ± 6 | 8 |
| Coffee blend (50% natural and 50% torrefacto coffee) | 32 ± 3 | 9 | 59 ± 5 | 8 |

[a] $n = 3$.

were similar to those obtained for regular ground coffee and are in agreement with data described in the literature (Crews & Castle, 2007; FDA, 2008). For instance, the FDA reported furan concentrations from 34 to 53 ng/ml in decaffeinated coffee, while Crews et al. (2009) found levels ranging between 8 and 66 ng/ml. For instant coffee, concentrations ranging from 1 to 51 ng/ml have been reported (Crews & Castle, 2007; Crews et al., 2009; Zoller et al., 2007). Given that decaffeinated coffee is produced from green coffee, our results confirm that the roasting process is responsible for the formation of furan. In addition, the slightly lower concentrations of furan in instant coffee can be attributed to the lower coffee/water ratio (2 g coffee powder/60 ml of water) used for brew preparation.

### 3.4. Analysis of furan in Nespresso coffee capsules

Recent years have witnessed a large increase in the consumption of coffee from packed capsules worldwide. This system guarantees fresh espresso coffee brews with a high-quality aroma and taste, and protects coffee from the damaging effects of light, air and humidity until brewing. The capsules in this system are filled with ground roasted coffee and are hermetically plastic-wrapped. A specialised coffee machine is required for each capsule brand. To determine the furan content in brews obtained with this system, eleven espresso coffee capsules from Nespresso (Table 4), one of the most widely consumed coffee capsules in the world, were selected and analysed in triplicate following the proposed method. For brewing coffee, a Nespresso coffee machine (Essenza) was used to obtain 40 ml of brews for coffee blends (*Ristretto, Arpeggio, Roma, Livanto, Capriccio, Volluto, Cosi, Decaffeinato* and *Decaffeinato intense*) and 110 ml for Lungo coffees (*Vivalto Lungo, Decaffeinato Lungo* and *Finezzo Lungo*). Furan concentrations for these coffee capsules ranged from 117 ng/ml for *Capriccio* to 244 ng/ml for *Decaffeinato intenso* (Table 4). These results were higher than those obtained in espresso brews of regular ground coffee (43–146 ng/ml), although similar coffee powder/water ratios were used (5.5 g/40 ml for Nespresso capsule and 9 g/60 ml for espresso coffee). This observation could be attributable to two factors, namely prevention of furan loss during handling as a result of the hermetic seal of the capsules, and the higher pressure applied by the Nespresso coffee machine (1850 kPa), which favours the extraction of this compound from the ground coffee. Regarding the *Lungo* coffee capsules, furan concentrations ranged from 68 to 102 ng/ml and were lower than those obtained with espresso capsules. This finding may be due to the higher dilution of the coffee brew (7 g coffee powder/110 ml of water) and the longer time period of brewing, which may increase the loss of furan.

### 3.5. Furan intake from coffee consumption

At present, the average coffee consumption in Spain reported by the Spanish Coffee Federation is 599 cups of coffee per person and year (FEC, 2007), which corresponds to 1.64 cups of coffee per person and day. These values are consistent with data provided by the Spanish Government (MAPA, 2007) and the International Coffee Organization (ICO, 2008), which reported an average coffee consumption of 4.5 kg of coffee beans per person and year for the last three years in Spain. On the basis of these data and on the furan content obtained in this study for espresso and drip coffee brews, we estimated the daily furan intake to be from 1.97 to 14.4 μg/person, which implies exposure of between 0.03 and 0.21 μg/kg body weight (bw)/day of this compound, assuming an average adult weight of 70 kg. Although these data are useful to establish the overall exposure of the total population to furan, we considered it of interest to obtain a more detailed picture of coffee consumption habits of people living and working in a large city. For this purpose, a 24-h recall questionnaire was completed from a representative sample of 488 participants aged from 19 to 58 years from five districts of Barcelona (Spain) (219 females and 269 males). Each participant was asked about his/her coffee drinking habits, such as the amount of coffee (cups of coffee/day) ingested in the previous 24 h, type of coffee and brewing procedure, and also some personal information, such as age, sex, address, citizenship and body weight. Fig. 2 shows the results of the coffee consumption survey. Around 20% of the participants were not habitual coffee drinkers. The average daily coffee consumption was 3.1 and 2.1 cups of coffee for males and females, respectively. This consumption rate implies a daily furan intake for males from 3.6 to 26.3 μg/person and for females between 2.4 and 17.5 μg/person. These findings indicate exposure to furan of 0.05 to 0.38 μg/kg bw/day for males and between 0.03 and 0.25 μg/kg bw/day for females. These data are only slightly higher than those obtained using the official Spanish average consumption data. These results agree with those reported by the US FDA (0.15 μg/kg bw/day) (Morehouse et al., 2008) and EFSA (<0.03–1.65 μg/kg bw/day) (EFSA, 2004) and are much lower than the non-observed adverse effect level (NOAEL), which was established at 2 mg/kg bw/day for experimental animals (Moser, Foley, Burnett, Goldsworthy, & Maronpot, 2009). Since the acceptable daily intake (ADI) was estimated to be 2 μg/kg bw/day within a safety factor of 1000 (Kuballa et al., 2005), the maximum furan intake found in this study (0.38 μg/kg bw/day) was around five times lower than this value. Consequently, we conclude that the estimated risk from coffee consumption, which is the biggest source of furan exposure for adults, is low.

**Table 4**
Furan levels (ng/ml) in brewed coffee of Nesspreso capsules.

| Coffee blend | Nesspreso capsule | Furan concentration (ng/ml) | |
|---|---|---|---|
| | | Mean ± sd [a] | RSD (%) |
| Espresso blend[b] | *Ristretto* | 230 ± 7 | 3 |
| | *Arpeggio* | 127 ± 8 | 6 |
| | *Roma* | 149 ± 8 | 5 |
| | *Livanto* | 193 ± 8 | 4 |
| | *Capriccio* | 117 ± 5 | 4 |
| | *Volluto* | 146 ± 6 | 4 |
| | *Cosi* | 132 ± 8 | 6 |
| | *Decaffeinato* | 158 ± 8 | 5 |
| | *Decaffeinato intense* | 244 ± 12 | 5 |
| Lungo blend[c] | *Vivalto Lungo* | 83 ± 5 | 6 |
| | *Decaffeinato Lungo* | 102 ± 6 | 6 |
| | *Finezzo Lungo* | 68 ± 4 | 6 |

[a] *n* = 3 replicates.
[b] Final volume of brewed coffee: 40 ml.
[c] Volume of coffee brew 110 ml.



**Fig. 2.** Coffee consumption in Barcelona (Spain) obtained from a 24-h recall questionnaire (488 participants).

## 4. Conclusions

Furan formation was not observed during the analysis of brewed coffee by automatic HS-SPME combined with GC–MS using a low extraction temperature (30 °C) and a short time (20 min). The roasting time and temperature applied to green coffee beans affect the final furan content of coffee. Therefore, we recommend gently roasting conditions (140 °C for 20 min) to reduce furan formation. The furan concentration levels found in regular ground coffee (20–146 ng/ml), decaffeinated (14–65 ng/ml) and instant coffee (12–35 ng/ml) brews commercialised in Spain were in agreement with those reported in the literature, showing the highest concentrations in espresso brews. Nevertheless, it must be mentioned that furan concentrations in brews from Nespresso coffee capsules (117–244 ng/ml) were higher than those obtained for regular ground coffee. The daily furan intake from coffee consumption was estimated to be 0.25 and 0.38 μg/kg bw for males and females, respectively. These values are lower than the established acceptable daily intake of 2 μg/kg bw.

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# PAPER V

## FURAN IN COMMERCIAL BABY FOOD FROM THE SPANISH MARKET: ESTIMATION OF DAILY INTAKE AND RISK ASSESSMENT

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# Furan in commercial baby foods from the Spanish market: estimation of daily intake and risk assessment

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**ABSTRACT**

The occurrence of furan in commercial baby food samples from the Spanish market was evaluated using an automated headspace solid-phase microextraction method coupled to gas chromatography-mass spectrometry (HS-SPME-GC-MS). A total of 76 baby food samples including infant formula, baby cereals, fruit in cans and/or jars, vegetables, meat, and fish, were surveyed for furan content. The lowest concentration of this compound was found in infant formula ($<0.02$–0.33 ng ml$^{-1}$), and cereal-based food (0.15–2.1 ng g$^{-1}$) while baby food containing fish showed the highest concentrations (19–84 ng g$^{-1}$). Following recommendation of the European Food Safety Authority (EFSA), the effect on furan content was evaluated of consumer home preparation of foods, heating and handling. Furan concentrations were reduced by up to 35% when samples were heated in a dish using microwave oven and by up to 53% when a hot water bath was used. Finally, we estimated the furan intake from baby food consumption (0.002–1.18 µg kg$^{-1}$ body weight day$^{-1}$) and we calculated the margin of exposure (MOE) from samples as purchased and also after home preparation of the food. For infant formula and cereal baby foods, the MOEs (26,278–412,776) indicated no infant health concern or priority, while for meat and fish-based baby foods the values pointed to a potential public health risk, even considering the furan losses during preparation at home.

## Introduction

Furan ($C_4H_4O$) is known to exhibit carcinogenic activity in animals and is suspected to cause harmful effects on human health. Thus, the IARC included this compound in group 2B as a possible carcinogen to humans (IARC 1995), while the US Department of Health and Human Services listed it as a human pathogen (NTP 2014). The presence of furan in food has been documented since the 1960s as one of the Maillard reaction products (Maga 1979); however, public health concern about furan in food arose in 2004 when the USFDA detected it at relatively high concentrations (up to 174 ng g$^{-1}$) in a wide range of highly consumed foodstuffs (EFSA 2004). Shortly thereafter, the EFSA devoted special efforts to the study of furan in food (EFSA 2004, 2008, 2010a, 2010b). Nowadays, it is well known that furan is formed during the heat treatment of food via mechanisms that may involve the thermal-induced degradation of carbohydrates, the oxidation of polyunsaturated fatty acids and the decomposition of

ascorbic acid or its derivatives (Crews & Castle 2007).

In addition to being a highly volatile compound (32°C, b.p.), furan has a relatively low octanol–water partition coefficient (log $P_{ow}$ = 1.3). Consequently, headspace (HS) techniques are a suitable approach for its analysis. Several methods based on HS alone and HS-SPME have been developed for the determination of furan in food (USFDA 2006; Bianchi et al. 2006; Altaki et al. 2007, 2009; Zoller et al. 2007; Morehouse et al. 2008; La Pera et al. 2009). The determination of furan in food is hindered by the possible losses of furan during sample preparation, but also by its generation during HS sampling or analysis (Senyuva & Gökmen 2005). For instance, the HS method originally proposed by the USFDA in 2004 was updated in 2006 to reduce furan formation during analysis, decreasing the sampling temperature from 80 to 60°C (Nyman et al. 2006, 2008). An advantage of HS-SPME over HS is the use of a lower extraction temperature (approximately 30°C),

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thereby minimising possible furan formation (Altaki et al. 2011). Generally, for each sample type it must be checked that the applied analytical method does not cause extra formation of furan (Crews & Castle 2007).

Furan in baby food is of particular concern because it has been found at relatively high concentrations (up to 224 µg kg$^{-1}$) (EFSA 2009) and infants are more susceptible to its adverse effects than adults. In addition, since baby food, especially varieties in cans and jars, is often the sole diet of infants, furan intake can be high in relation to their body weight. According to EFSA (2004), there is only a slight difference between the amounts of furan to which humans may be exposed and the doses that are carcinogenic to experimental animals. In addition, recently, the EFSA stated that more information is required not only about the furan content in commercial food as purchased but also about the effect of domestic preparation (EFSA 2010a, 2010b). To date, the impact of in-home food preparation on furan content in final meal is not clear. For instance, Roberts et al. (2008) reported a decrease of furan levels in most foods in cans or jars after heating due to simple evaporation of furan. In contrast, Hasnip et al. (2006) showed that it is not significantly lost when food is warmed to be ready to eat. This emphasises the need for more accurate information about the influence of domestic preparation of commercial baby food on furan level. To our knowledge, limited information has been reported regarding the exposure to furan from baby food consumption with respect to domestic preparation and it is, from a consumer point of view, of interest to know the real infant dietary exposure to furan. At present there are no studies on the occurrence of furan in infant formula and baby cereals commercialised in Spain and data available on baby foods in jars are limited (Ruiz et al. 2010; Mesías et al. 2013) and not representative of the different baby food groups. Therefore, there is a need to extend the amount of data on furan levels in Spanish baby foods to have a more realistic dietary exposure assessment.

The aim of this work was to evaluate the occurrence of furan in the most common baby foods which are commercialised in Spain, including infant formula, cereals, vegetables, chicken, meat and fish, in order to obtain a clearer picture of furan intake from baby food.

In addition, the influence of common sample preparation at home, such as heating and handling, on furan concentration in commercial baby foods was also investigated. Finally, an exposure assessment was conducted to estimate the risk associated with the consumption of Spanish commercial baby foods as purchased and also after preparation at home.

## Methods and materials

### Chemicals and standards

Furan and [$^2$H$_4$]-furan (furan-d$_4$) at purity > 99% were purchased from Sigma-Aldrich (Munich, Germany). Individual stock standard solutions of furan and furan-d$_4$ were prepared by weight at a concentration of 12 mg g$^{-1}$ by introducing an appropriate amount of pure compound into a 2-ml amber-sealed vial previously filled with methanol. Similarly, intermediate standard solutions (2 ml) of furan and furan-d$_4$ were prepared individually from their stock standard solutions in methanol at a concentration of 75 µg g$^{-1}$ using a chilled GC syringe. All these intermediate solutions were prepared weekly and stored at 0ºC before use. Individual aqueous working standard solutions (20 ml) of furan and furan-d$_4$ at 35 and 75 ng g$^{-1}$ were prepared daily from their intermediate standard solutions, respectively. For furan determination, nine calibration solutions at furan concentrations ranging from 0.02 to 10 ng g$^{-1}$ and furan-d$_4$ at 0.375 ng g$^{-1}$ were prepared by adding an appropriate amount of working standard solutions using a GC syringe through the septum of 20-ml sealed headspace vials, containing a PTFE-coated stir bar, 2 g of sodium chloride and a suitable amount of water (up to 8 ml) (Altaki et al. 2007, 2009). Water of organic-trace grade, methanol of GC grade and sodium chloride of analytical grade were obtained from Merck (Darmstadt, Germany).

### Sample treatment

A total of 76 commercial baby food samples were collected randomly from local supermarkets in Barcelona, Spain. These samples were classified into the following categories on the basis of their ingredients: milk, cereal porridge, fruit, vegetable, meat and fish. Baby food samples were cooled in their sealed container to 4ºC to prevent any losses of

furan during sample preparation. Semi-solid samples were homogenised for 1 min at 4ºC by immersing the pot in an ice/water bath and using a mixer and an Ultra-Turrax T25 basic disperser (IKA-Werke, Staufen, Germany), while liquid samples were homogenised by manual shaking. Powder infant formula and cereal porridge samples were prepared from solid products following the instructions provided by the manufacturer. For milk samples, 37 g of powdered milk and 240 ml of water (40ºC) previously boiled were mixed in a baby bottle that was shaken until homogenisation, while for cereal porridge samples a portion of solid product (32 g) was mixed in a dish with 200 ml of the previously prepared milk (30–40ºC) and stirred until a homogenised sample was obtained. After preparation, all samples were kept in 40-ml screw-cap glass vials without headspace at 4ºC.

### Headspace-solid-phase microextraction

For HS-SPME analysis, a portion of 1–2 g of each homogenised baby food and 5 g of already prepared milk and baby cereal porridge were transferred to a 20-ml headspace vial immersed in an ice/water bath (4ºC) containing a PTFE-coated stir bar, 2 g of sodium chloride and an appropriate amount of water (up to 8 ml). The vial was immediately sealed, spiked with 50 µl of furan-$d_4$ aqueous working standard (75 ng $g^{-1}$) and then vortexed for 2 min. A 75-µm carboxen-polydimethylsiloxane fibre, CAR/PDMS (Supelco, Bellefonte, PA, USA), previously conditioned for 1 h at 300ºC, was used for HS-SPME analysis. Before extraction, the vial was equilibrated for 5 min in the magnetic mixer tray at 30ºC and 750 rpm and the fibre was cleaned for 1 min at 275ºC using the SPME fibre-conditioning station. The sample was then extracted from the headspace for 20 min at 30ºC and an agitation rate of 750 rpm. Thermal desorption of furan was accomplished by exposing the fibre in the GC injector port to 275ºC for 2 min.

### Gas chromatography-mass spectrometry determination

Furan determination by GC-MS was carried out on a Varian model CP-3800 gas chromatograph coupled to a Saturn 2200 ion-trap MS system

(Varian, Mississauga, Canada) equipped with a CTC Combi-Pal autosampler (CTC Analytics AG, Zwingen, Switzerland). Chromatographic separation was accomplished on a BPX-volatile fused-silica capillary column (SGE Europe, Villebon, France), 60 m × 0.25 mm I.D. and 1.4 µm film thickness. Helium was used as carrier gas (1.7 ml $min^{-1}$) and the oven temperature was programmed from 35ºC (held for 2 min) to 230ºC (held for 5 min) at 20ºC $min^{-1}$. Splitless injection mode (3 min) at 275ºC was used for GC analysis, and transfer line, ion-trap manifold and ion-trap source temperatures were set at 280, 80 and 200ºC, respectively. MS acquisition in electron ionisation full-scan mode over the mass range of $m/z$ 35–100 at 0.75 s/scan (7µscan per scan) was used (Altaki et al. 2009). Furan quantification was performed by isotope dilution, monitoring the $m/z$ 68 $[M]^{+\cdot}$ and $m/z$ 39 $[M - CHO]^+$ for furan and $m/z$ 72 $[M]^{+\cdot}$ and $m/z$ 42 $[M - C^2HO]^+$ for furan-$d_4$ as quantifying and qualifier ions, respectively.

### Quality control and quality criteria

Quality control of the HS-SPME method was performed through the routine analysis of procedural blanks and quality control standards and samples to ensure the absence of contaminants and possible carryover between samples, and to assess the quality of the results. LODs and LOQs based on a signal-to-noise ratio ($S/N$) of 3 and 10, respectively, were determined using baby food samples spiked at low concentrations of furan-$d_4$, since no blank samples were found. LODs were periodically tested and typically ranged from 20 to 60 pg $g^{-1}$ (or pg $ml^{-1}$) depending of the sample, while LOQs were between 40 and 110 pg $g^{-1}$ (or pg $ml^{-1}$). Within-day (run-to-run) precision of the HS-SPME-GC-MS method, expressed as RSDs, was 7%, obtained from six replicate analyses of a carrot and potato purée sample (furan content = 50.2 ng $g^{-1}$) performed on the same day, while the between-day (day-to-day) precision was 10%, obtained from 18 replicate analyses performed on 3 consecutive days (six replicates per day). In addition, a daily sensitivity test using a quality control sample (follow-on milk infant formula with a furan content of 0.15 ng $ml^{-1}$) was carried out to check possible changes in the

absorption capacity of the SPME fibre and the GC-MS response.

## Estimation of risk assessment from baby food consumption

Furan intake was calculated considering the levels found in the present study and the daily Spanish consumption data of baby food available from MERCASA (2015), which is a public company of the Spanish government whose shareholders are the state industrial holding company (SEPI) and the Ministry of Agriculture, Food and Environment through the Spanish Agricultural Guarantee Fund (FEGA). The estimation of furan intake, EDI ($\mu g \ kg^{-1}$ body weight $day^{-1}$), was carried out using the following equation:

$$EDI = C \times Q/b.w.,$$

where $C$ is the furan concentration found in baby food ($\mu g \ g^{-1}$); $Q$ is the daily consumption of baby food ($g \ day^{-1}$); and b.w. is the average body weight (kg). In addition, the risk assessment was conducted according to the harmonised approach of the EFSA for substances which are genotoxic and carcinogenic (EFSA 2005). The EFSA has developed and recommends an approach known as the margin of exposure (MOE). This approach uses the ratio between the doses of substances that have been observed to cause low but measurably harmful responses in animals as reference and the substance-specific dietary intake estimates in humans. The MOE is estimated using the following equation:

$$MOE = BMDL_{10}/EDI,$$

where $BMDL_{10}$ is the benchmark dose lower confidence limit that correspond to excess health risk of 10% above background concentration; and EDI is the estimated daily intake. The $BMDL_{10}$ value used in the present work for calculating the margin of exposure

was $0.96 \ mg \ kg^{-1}$ b.w. $day^{-1}$, which has been recommended by the FAO/WHO Expert Committee on Food Additives for Furan (FAO/WHO 2011). MOEs of 10,000 or higher would be considered as a low public health concern, while MOEs lower than 10,000 represent a potential health concern and a high priority for risk-management actions.

## Results and discussion

For the analysis of furan in the baby food samples we used an automated HS-SPME-GC-MS method previously developed (Altaki et al. 2009). In a first step, the influence of HS-SPME conditions on furan formation during analysis of samples with different ingredients and potential furan precursors (proteins, carbohydrates and lipids) were analysed. The selected samples included follow-on infant formula, porridge made of eight cereals and fruits prepared with milk, three-fruit purées, chicken and vegetable purée, sole fish with vegetables, and green beans with steamed potato and beef (Table 1). Equilibration times of 5, 30 and 100 min at 30ºC were tested. The results of three replicate analysis at each equilibration time were statistically compared by analysis of variance (ANOVA) at a confidence level of 95%. No significant differences ($p > 0.05$) in furan concentration were observed using the different equilibration times (Table 1), indicating that furan was not formed from baby food during HS-SPME analysis. In addition, since furan formation during SPME fibre thermal desorption at high temperatures in the GC injector port has also been reported (Adams et al. 2012), several experiments were carried out to assess its possible formation. Two desorption temperatures (250 and 275ºC) and

**Table 1.** Effect of the equilibration time of the HS-SPME method on the furan concentration of baby food samples.

| Food sample | Furan concentration (mean ± SD) (ng g⁻¹)[a] | | | |
| | Equilibration time (min) | | | |
| | 5 | 30 | 100 | *p*-value[b] |
|---|---|---|---|---|
| Follow-on milk infant formula (37 g powder milk and 240 ml of water) | 0.11 ± 0.01 | 0.09 ± 0.01 | 0.13 ± 0.02 | 0.15 |
| Porridge of eight cereals and fruits prepared in milk (32 g powder and 200 ml of milk). | 0.52 ± 0.06 | 0.48 ± 0.05 | 0.43 ± 0.04 | 0.18 |
| Three fruits (apple, orange and banana) | 1.86 ± 0.08 | 1.9 ± 0.1 | 1.8 ± 0.2 | 0.45 |
| Purée of chicken and vegetables (carrot, tomato, potato, green bean, onion, pea) | 12.6 ± 0.6 | 12 ± 1 | 11.6 ± 0.6 | 0.30 |
| Sole fish with vegetables (carrot, potato, green bean) | 43 ± 2 | 43 ± 2 | 42 ± 2 | 0.87 |
| Green beans with steamed potato and beef | 21 ± 2 | 19.6 ± 0.8 | 18.7 ± 0.7 | 0.13 |

Notes: [a]$n = 3$.
[b]*p*-value < 0.05 (at a 95% confidence level) indicates significant differences between equilibration times.

desorption times of 2, 3 and 4 min were tested using the above-mentioned baby food samples and no significant differences in furan responses were observed, indicating that furan was not generated at the optimal SPME desorption conditions.

### Furan levels in baby food

The furan levels in 76 samples of infant formula and jarred baby food were determined in triplicate, and the results are given in Table 2. The lowest furan concentration was found in infant formula (up to $0.33 \pm 0.04$ ng ml$^{-1}$) and cereal porridge (0.15–2.1 ng g$^{-1}$) samples with a mean of 0.2 and 0.8 ng g$^{-1}$, respectively. Slightly higher concentrations (0.7–2.7 ng g$^{-1}$) were found in fruit-based baby foods with a mean of 1.6 ng g$^{-1}$, while for samples containing vegetables, furan concentrations increased up to 29 ng g$^{-1}$ (mean = 10 ng g$^{-1}$). These results agree with those reported by the EFSA in fruit-based (n.d.–58 ng g$^{-1}$) and vegetable-based (9.3–233 ng g$^{-1}$) baby foods in jars collected around Europe during 2004–10 (EFSA 2011) and are two to 10 times lower than those reported for similar baby food groups in samples commercialised in Spain during 2010 (4.0–9.0 ng g$^{-1}$ for fruit-based and 22.2–53.4 ng g$^{-1}$ for vegetable-based foods) (Ruiz et al. 2010) and 2011 (7.7–32.1 ng g$^{-1}$ for fruit-based and 10.9–143.0 for vegetable-based baby foods) (Mesías et al. 2013). This significant decrease of furan content in these groups of baby foods could be related to changes in the thermal processing conditions and/or the use of additives for controlling the furan formation from potential precursors (Soak et al. 2015). The significantly higher furan levels in vegetable-based foods than those found in fruit baby foods are consistent with data reported in the literature (Zoller et al. 2007; Jestoi et al. 2009; Lachenmeier et al. 2009; Ruiz et al. 2010; Mesías et al. 2013) and can be attributed to ascorbic acid degradation, which is favoured at higher pH (vegetable-based pH approximately 5.7; fruit-based pH about 3.9) (Becalski et al. 2010; Mesías-García et al. 2010; Palmers et al. 2016b). In addition, the industrial treatment of the product, pasteurisation (short time) for fruit and sterilisation (long time) for vegetables may also influence the different furan content in these food samples (Bianchi et al. 2006; Palmers et al. 2016a).

For samples of baby foods from jars containing meat as a main ingredient, furan concentrations ranging between 7.9 and 64 ng g$^{-1}$ with a mean of 35 ng g$^{-1}$ were found. These results are within the ranges reported in previous studies by Ruiz et al. (2010) (n.d.–64.6 ng g$^{-1}$) and Lachenmeier et al. (2009) (2–50 ng g$^{-1}$). The higher furan levels found in the meat-based baby food compared with fruit and vegetable-based foods may be related to the differences in the origin and nature of proteins, carbohydrates and fats, which are the main precursors of furan. In this survey, the highest furan concentration was found in fish-based baby foods (19–84 ng g$^{-1}$), with a mean concentration of 49 ng g$^{-1}$, which is consistent with data reported in the literature (USFDA 2006; Zoller et al. 2007; EFSA 2009; FAO/WHO 2011). The high furan levels found in fish-based baby foods can be attributed to the oxidation and/or degradation of the highly unsaturated fatty acid composition of fish muscle (Yasuhara & Shibamoto 1995) that can be produced during the thermal treatment of the food. These findings recommend the implementation of modifications in heat-processing during production to eliminate or minimise the formation of furan in fish-based baby foods.

### Influence of baby food preparation on furan concentration

Commercial baby food is commonly heated and handled in-home before its consumption. Since furan is a high volatile compound and its formation is related to heat treatment, the final furan content in the ready-to-eat baby food is expected to be influenced by these procedures. To study this effect, several commercial baby food samples were warmed using either a kitchen microwave oven (800 W) or a hot water bath to reach a final temperature of 40ºC. The furan content in the heated samples was determined and compared with the concentration in the original sample (as purchased). For microwave handling, two approaches were used: (1) the samples were directly heated for 1 min in their original open jars; and (2) the samples were placed in a dish. In both cases they were stirred for 1 min after heating. For the hot water bath, uncapped jars were immersed in a bowl containing hot water (60–70ºC) and stirred for 5 min. After that each sample was analysed in triplicate; the results are given in Table 3. It is

**Table 2.** Mean furan concentration (ng g$^{-1}$) in commercial baby foods from the Spanish market.

| Category | Code | Description | Furan concentration (mean ± SD)[a] |
|---|---|---|---|
| Infant formula | 01 | Prepared continued milk (from 6 month)[c] | 0.08 ± 0.01 |
| | 02 | Prepared continued milk (from 6 months) [c] | 0.11 ± 0.01 |
| | 03 | Prepared continued milk (from 6 months) [c] | 0.33 ± 0.04 |
| | 04 | Prepared continued milk (from 6 months) [c] | n.d.[b] |
| | 05 | Prepared continued milk (from 6 months)[c] | n.d.[b] |
| | 06 | Prepared continued milk (from 6 months)[c] | n.d.[b] |
| | 07 | Prepared continued milk (from 12 months)[c] | n.d.[b] |
| | 08 | Liquid continued milk (from 12 months)[c] | 0.24 ± 0.03 |
| Baby cereals | 09 | Porridge of gluten-free cereals prepared with milk | 0.15 ± 0.02 |
| | 10 | Porridge of eight cereals with cacao prepared with milk | 0.24 ± 0.02 |
| | 11 | Porridge of eight cereals with honey prepared with milk | 0.46 ± 0.03 |
| | 12 | Porridge of eight cereals with fruits prepared with milk | 0.52 ± 0.03 |
| | 13 | Ready-to-eat porridge of gluten-free cereals (brand 1) | 1.1 ± 0.1 |
| | 14 | Ready-to-eat porridge of gluten-free cereals (brand 2) | 1.25 ± 0.09 |
| | 15 | Ready-to-eat porridge of cereals with honey (brand 1) | 0.90 ± 0.09 |
| | 16 | Ready-to-eat porridge of cereals with honey (brand 2) | 1.4 ± 0.1 |
| | 17 | Ready-to-feed porridge of cereals with fruits (brand 1) | 0.40 ± 0.06 |
| | 18 | Ready-to-feed porridge of cereals with fruits (brand 2) | 0.70 ± 0.05 |
| | 19 | Ready-to-feed porridge of cereals with cacao | 2.1 ± 0.1 |
| Fruit-based product | 20 | Orange juice (concentrated base) | 0.70 ± 0.08 |
| | 21 | Apple juice (concentrated base) | 1.3 ± 0.1 |
| | 22 | Juice of assorted fruit (brand 1) | 1.94 ± 0.09 |
| | 23 | Juice of assorted fruits (brand 2) | 2.17 ± 0.09 |
| | 24 | Juice of grape and carrot | 2.68 ± 0.08 |
| | 25 | Three fruits with lemon juice | 1.86 ± 0.08 |
| | 26 | Apple jar | 0.76 ± 0.09 |
| | 27 | Banana and apple jar | 1.08 ± 0.09 |
| Vegetables-based product | 28 | Varied vegetables | 7.3 ± 0.6 |
| | 29 | Vegetables cream with pasta | 28.8 ± 0.9 |
| | 30 | Vegetables with rice | 2.2 ± 0.2 |
| | 31 | Cream of seven vegetables | 1.7 ± 0.2 |
| | 32 | Cream of zucchini | 17 ± 1 |
| | 33 | Cream of green beans and potato | 3.7 ± 0.3 |
| Meat-based product | 34 | Chicken and rice jar | 47 ± 4 |
| | 35 | Pureed vegetables and chicken jar | 12.6 ± 0.6 |
| | 36 | Vegetable soup with chicken and white sauce | 32 ± 2 |
| | 37 | Chunky chicken stew jar | 49 ± 3 |
| | 38 | Chicken stew with vegetables | 38 ± 2 |
| | 39 | Chicken with rice | 16.4 ± 0.7 |
| | 40 | Chicken with chunky with vegetables and pasta | 15 ± 2 |
| | 41 | Chicken with vegetables | 32 ± 3 |
| | 42 | Chicken with rice and vegetables | 7.9 ± 0.7 |
| | 43 | Chicken with various vegetables | 17 ± 2 |
| | 44 | Chicken stock with carrot and rice | 30 ± 3 |
| | 45 | Beef and rice jar | 20 ± 2 |
| | 46 | Pureed vegetables with chicken and beef jar | 27 ± 2 |
| | 47 | Mashed vegetables and turkey | 37 ± 3 |
| | 48 | Lamb stew jar | 56 ± 6 |
| | 49 | Beef jardinière jar | 46 ± 4 |
| | 50 | Chunky and beef stew jar | 31 ± 2 |
| | 51 | Green beans with steamed potato and beef | 21 ± 2 |
| | 52 | Pea with pork meat | 53 ± 3 |
| | 53 | Vegetables with pork meat and pasta | 49 ± 2 |
| | 54 | Beef meat with rice | 44 ± 3 |
| | 55 | Jardinière beef meat | 51 ± 3 |
| | 56 | Lamb meat stew (brand 1) | 34 ± 2 |
| | 57 | Lamb meat stew (brand 2) | 43 ± 2 |
| | 58 | Beef meat with carrot | 37 ± 2 |
| | 59 | Pork and beef meat with vegetables | 42 ± 3 |
| | 60 | Lentils with beef | 33 ± 2 |
| | 61 | Tender peas with pork meat | 12 ± 1 |
| | 62 | Stew of beef meat | 27 ± 1 |
| | 63 | Stew of pork meat and vegetables | 64 ± 3 |
| | 64 | Beef and pork meat with spaghetti | 40 ± 3 |
| | 65 | Beef meat with vegetables and pasta | 52 ± 3 |
| | 66 | Potage of chickpeas | 49 ± 3 |
| Fish-based product | 67 | Monkfish with potato | 84 ± 4 |
| | 68 | Hake with rice | 69 ± 3 |
| | 69 | Sole fish with vegetables | 43 ± 2 |
| | 70 | Sole fish with selected vegetables | 30 ± 2 |
| | 71 | Selected vegetables and small sea bass dinner | 38 ± 2 |

(*Continued*)

**Table2.** (Continued).

| Category | Code | Description | Furan concentration (mean ± SD)[a] |
|---|---|---|---|
| | 72 | Cream of hake and with sauce | 51 ± 5 |
| | 73 | Selected vegetables and rape jar | 52 ± 5 |
| | 74 | Pureed peas and rice with hake jar | 75 ± 5 |
| | 75 | Cream of vegetables with whiting | 19 ± 3 |
| | 76 | Cream of vegetables with rice and hake | 28 ± 2 |

Notes: [a] $n = 3$ replicate analyses.
[b] n.d., Not detected: < LOD (0.02 ng ml$^{-1}$).
[c] Concentration = ng ml$^{-1}$ of reconstituted milk.

interesting to note that a marked decrease in furan content ranging from 19% to 35% was observed when samples were heated in a dish using a microwave oven while an even higher reduction on furan content, between 31% and 53%, was obtained when using a hot water bath instead of a microwave oven. Since the most common procedure used by consumers is to heat the samples in their open jars using a microwave oven, differences in furan concentration between the samples as purchased and after heating were evaluated using one-way ANOVA at a significant level of 0.05. A slight increase in concentration ($p < 0.05$), as a result of furan formation, was found for some meat and fish-based baby food (codes E and L). In contrast, samples containing as main components vegetables (codes A and B) and also vegetables with chicken or lamb meat stew (codes C and J) showed a decrease in concentration as a result of furan evaporation ($p < 0.05$). These results showed that furan evaporation is the dominant process during sample heating, although furan formation can also occur but to a much lesser extent. These results are consistent with those published in the literature. For instance, losses of

furan up to 55% have been found during the heating of vegetable purée using a microwave oven (Zoller et al. 2007), while an increase in furan concentration in freshly food cooked at home has been observed when heated for a long time (1 h at high 80ºC) using a baby food warmer (Lachenmeier et al. 2009).

Given the high volatility of furan, the standing and handling of samples is also expected to reduce the content of this compound. To study this effect, several baby foods belonging to various categories were analysed after standing times of 0, 2 and 5 min in a dish for solid samples and in a baby bottle for milk and juice (Figure 1). Longer standing or handling times were not studied because they are uncommon when preparing baby food at home. Moreover, manual stirring was also applied in order to simulate common domestic preparation. For samples of apple juice, three-fruit purée, cream of vegetables with pasta, chicken with rice, and beef with rice, furan concentration decreased from 22% to 47% after a standing time of 2 min and from 45% to 70% when the time was increased to 5 min. Therefore,

**Table 3.** Effect of sample heating on furan concentration (ng g$^{-1}$) in selected baby food samples.

| Food | Code | Main ingredients | As purchased | Open jar (*p*-value)[b] | Dish | Open jar |
|---|---|---|---|---|---|---|
| | | | | Furan concentration (mean ± SD) (ng g$^{-1}$)[a] | | |
| | | | | After heating | | |
| | | | | Microwave oven | | Hot water bath |
| Vegetables | A | Varied vegetables (potato, green bean, tomato, carrot, celery and onion) | 7.3 ± 0.6 | 6.0 ± 0.3 (0.03) | 5.2 ± 0.2 | 4.0 ± 0.2 |
| | B | Vegetables cream (green bean, carrot, pea, onion) with pasta | 29 ± 1 | 25 ± 1 (0.01) | 22 ± 1 | 17 ± 1 |
| Meat based | C | Chicken stew with vegetables (potato, carrot, pea, leek, onion, tomato, celery) | 38 ± 2 | 32 ± 2 (0.02) | 25 ± 2 | 18 ± 1 |
| | D | Chicken with rice (carrot, tomato, celery and onion) | 16 ± 1 | 15 ± 1 (0.09) | 13 ± 1 | 10 ± 1 |
| | E | Pea with pork meat | 53 ± 3 | 60 ± 3 (0.03) | 41 ± 2 | 32 ± 1 |
| | F | Pork meat with pasta and vegetables (carrot and tomato) | 49 ± 2 | 46 ± 2 (0.18) | 32 ± 3 | 23 ± 2 |
| | G | Beef meat with rice (potato, carrot, tomato and leek) | 44 ± 3 | 46 ± 2 (0.35) | 34 ± 2 | 25 ± 2 |
| | H | Jardinière beef meat (potato, carrot, tomato, pea, green bean and onion | 51 ± 3 | 56 ± 2 (0.08) | 41 ± 2 | 35 ± 2 |
| | I | Lamb meat stew (potato, carrot, pea and anion) | 34 ± 2 | 37 ± 2 (0.09) | 25 ± 2 | 22 ± 2 |
| | J | Lamb meat stew-02 (carrot, green bean, onion and pea) | 43 ± 2 | 36 ± 3 (0.04) | 30 ± 1 | 23 ± 1 |
| Fish based | K | Monkfish with potato (tomato, carrot and onion) | 84 ± 4 | 85 ± 1 (0.71) | 65 ± 3 | 45 ± 3 |
| | L | Hake with rice (potato, onion and celery) | 69 ± 3 | 76 ± 2 (0.02) | 55 ± 2 | 45 ± 2 |

Notes: [a] $n = 3$.
[b] *p*-value < 0.05 (at a 95% confidence level) indicates significant differences between furan concentrations in baby food as purchased and after heating.
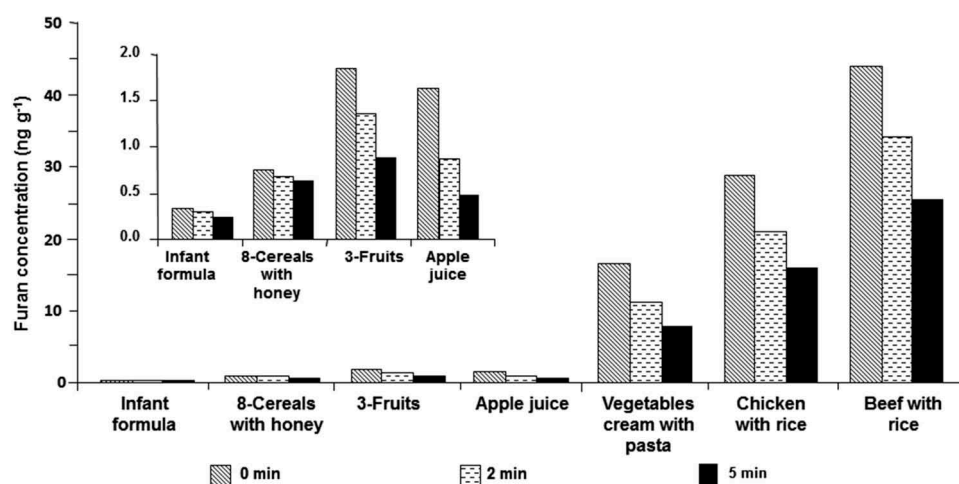
**Figure 1.** Effect of handling time during sample preparation on furan content in baby food samples.

the use of a standing-time step during food preparation favours the reduction of furan content in baby food. However, for samples of follow-on infant formula and eight-cereal porridge with honey, a lower decrease in furan concentration was found (from 9% to 11% and from 17% to 26% for a 2- and 5-min standing time, respectively). The minor loss of furan in these samples could be explained because it has been previously lost during the preparation of the food following the recommendations of the manufacturers (see the second section). To confirm this hypothesis, we evaluated the loss of furan during the preparation of two follow-on milk samples and two cereal porridges which were prepared with special care to prevent furan losses. For this purpose, a chilled powdered cereal (5.6 g) and a powdered milk (5.4 g) were quickly transferred to 40-ml vials filled with 35 ml of cold water and the vial was then sealed and stirred until homogeneity using a stir bar previously placed into the vial. These samples were analysed and the results compared with those obtained preparing the samples following the manufacturer's recommendations. A significant decrease (34–62%) on furan concentrations was observed in samples prepared as recommended by the manufacturer (Figure 2), indicating that about 50% of the furan content is lost during sample handling.

### Estimation of daily intake and risk assessment

Infant furan intake per day was first estimated using available data on the daily consumption of Spanish baby food (MERCASA 2015). The average daily consumption of baby food in jars, regardless of category, is 117.8 g day$^{-1}$ per infant, while for infant formula and baby cereal porridge the average consumption 81.4 and 94.3 g of dry food day$^{-1}$ per infant, respectively. These consumption amounts correspond to babies between 6 and 9 months old, since they often consume foods containing fruits, vegetables, meat and/or fish ingredients. Assuming an average infant b.w. of 8.4 kg for 6–9-month-old babies (range = 7.9–8.9 kg) (WHO 2006), the furan intake from the daily consumption of infant formula and cereal-based foods ranged between 0.002 and 0.037 µg kg$^{-1}$ b.w. day$^{-1}$ (mean = 0.010–0.016 µg kg$^{-1}$ b.w. day$^{-1}$), while for canned and/or jarred baby foods the furan intake varied from 0.01 to 1.18 µg kg$^{-1}$ b.w. day$^{-1}$ (mean = 0.02–0.69 µg kg$^{-1}$ b.w. day$^{-1}$) (Table 4). These findings are in agreement with data reported in the literature. For instance, means of furan intake for 6-month-old infants in Germany were estimated to be between 0.5 and 1.9 µg kg$^{-1}$ b.w. day$^{-1}$ (Lachenmeier et al. 2009), while in Finland the furan exposure was between 0.1 and 2.1 µg kg$^{-1}$ b.w. day$^{-1}$, although intakes up to 10.6 µg kg$^{-1}$ b.w. day$^{-1}$ were estimated in a worse-case scenario (Jestoi et al. 2009). Also, in Spain values of furan exposure for fruit and vegetable-based baby foods ranging from 0.1 to 2.1 µg kg$^{-1}$ b.w. day$^{-1}$ were found (Mesías et al. 2013). Moreover, our results are in agreement with those published by EFSA (2009), where the average furan daily intake from infants aged from 3 to 12 months old was estimated to be between 0.13 and 0.97 µg kg$^{-1}$ b.w. day$^{-1}$ and data from the United
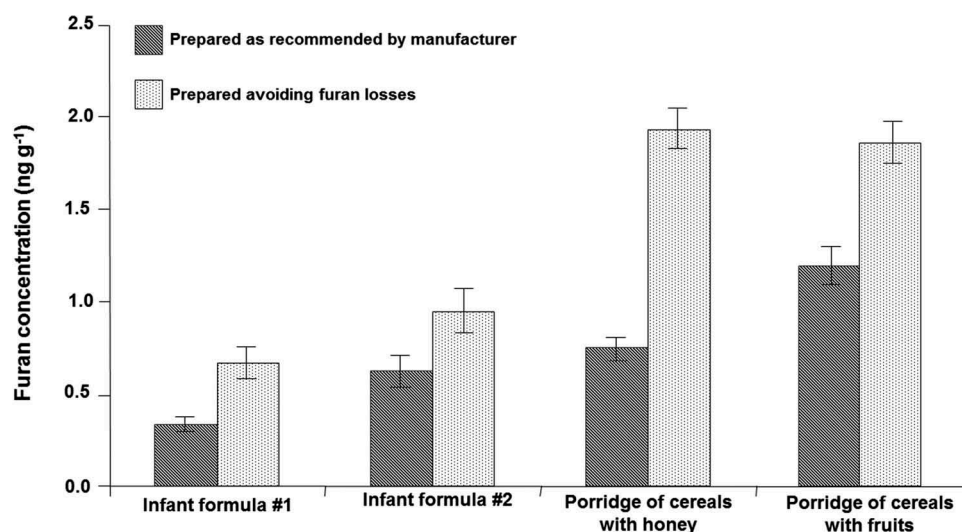
**Figure 2.** Furan concentration in baby food after in-home preparation avoiding furan losses and following the procedure recommended by the manufacturers.

**Table 4.** Furan concentration, estimation of daily intake and margins of exposure (MOEs) for commercial baby foods from the Spanish market.

| Baby food | Furan concentration (ng g$^{-1}$ or ng ml$^{-1}$) | | | Estimation daily intake (µg kg$^{-1}$ b.w. day$^{-1}$) | | | MOE | | |
|---|---|---|---|---|---|---|---|---|---|
| | Range | Median | Mean | Range | Median | Mean | Range | Median | Mean |
| Infant formula | 0.08–0.33 | 0.2 | 0.2 | 0.002–0.024 | 0.007 | 0.010 | 40,099–412,776 | 139,291 | 95,879 |
| Cereal porridge | 0.15–2.1 | 0.7 | 0.8 | 0.004–0.037 | 0.012 | 0.016 | 26,278–247,666 | 79,253 | 61,419 |
| | | | | | | | | | |
| *Jarred baby food* | | | | | | | | | |
| Fruit based | 0.7–2.7 | 1.6 | 1.6 | 0.01–0.04 | 0.02 | 0.02 | 25,543–97,793 | 43,053 | 43,776 |
| Vegetable based | 1.7–29 | 5.5 | 10 | 0.02–0.40 | 0.08 | 0.14 | 2377–39,342 | 12,538 | 6727 |
| Meat based | 7.9–64 | 37 | 35 | 0.11–0.90 | 0.52 | 0.50 | 1065–8643 | 1839 | 1941 |
| Fish based | 19–84 | 47 | 49 | 0.27–1.18 | 0.66 | 0.69 | 813–3592 | 1458 | 1372 |

States where the furan exposure varied from 0.4 to 1.0 µg kg$^{-1}$ b.w. day$^{-1}$ for babies aged from 0 to 12 months (USFDA 2008).

In order to evaluate the risk associated with furan intake from baby food consumption, MOEs were calculated using the estimation daily intake obtained in this study. Table 4 shows the range, mean and median for MOEs obtained in the present study. For infant formula and cereal porridge, MOEs ranged from 26,278 to 412,776 (mean = 61,419 and 95,879, respectively). These findings suggest that furan intakes from infant formula and cereal porridge represent a low concern for public health and that a low priority for risk-management actions is required, since MOEs were higher than the threshold of 10,000 (EFSA 2005). For all categories of baby foods in jars, MOEs ranged from 813 to 97,793 (mean = 1372–43,776) (Table 4). Among them, fruit-based baby foods showed the highest values

(range = 25,543–97,793) with MOEs higher than 10,000. In contrast, MOE means ranging between 1372 and 6727 were obtained for vegetable, meat and fish-based foods, which represent a potential public health concern for infants. These results are consistent with those reported in the literature. In Germany, Lachenmeier et al. (2009) reported MOEs for 6-month-old infants of 2692 and 997 for the mean and 95th percentile, respectively. In Brazil, Arisseto et al. (2010) determined means of 1702 for infants fed exclusively with commercial baby food, while for 9-month-old Chilean babies MOE was estimated as 4922 (Mariotti et al. 2013). For a more realistic intake assessment we also estimated MOEs considering in-home preparation. As already seen, the presence of furan in baby food samples is generally reduced after home preparation. However, complete elimination of furan via evaporation from samples was not possible even after a long standing

**Table 5.** Margin of exposure (MOE) after heating baby food using a microwave oven and a hot water bath.

| | MOE | | | | | |
| | Microwave oven[a] | | | Hot water bath[b] | | |
| Baby food | Range | Median | Mean | Range | Median | Mean |
|---|---|---|---|---|---|---|
| Vegetable based | 3234–53,526 | 17,059 | 9152 | 4192–69,386 | 22,113 | 11,864 |
| Meat based | 1443–11,711 | 2492 | 2630 | 1878–15,243 | 3243 | 3423 |
| Fish based | 1036–4576 | 1857 | 1748 | 1369–6047 | 2455 | 2310 |

Notes: [a]After reheating the sample in a dish for 1 min using a microwave oven.
[b]After reheating the uncapped jar using a hot water bath and stirring for 5 min.

time, probably due to a slow diffusion and a high trapping of furan in the baby food matrix. Considering the determined percentages of furan lost during sample heating (26% using a microwave oven and 42% by means of hot water bath), the MOEs were recalculated, and the results obtained are given in Table 5. As can be seen, the MOEs are still lower than 10,000 for meat and fish-based baby foods, while for vegetable-based foods the median and mean were now 22,112 and 11,864, respectively, using a hot water bath for heating. Taking into account that furan content can be additionally reduced by a mean percentage of 48% (34–62%) after a standing time of 5 min once heated, which is a common procedure during home preparation, it is recommended to keep the sample in a dish during this time before eating to reduce the exposure risk of infants to furan.

## Conclusions

HS-SPME-GC-MS has been shown to be a fast method for the determination of furan in baby foods avoiding furan formation during analysis and providing low limits of detection and good precision. In general, the heating procedure used for baby food preparation affects the final furan content. A mean reduction of 26% and 42% in the furan content of baby food was observed using a microwave oven and a hot water bath, respectively. Moreover, a standing time of 5 min after heating promoted the loss of furan by evaporation to about 50%. Therefore, we recommend the use of a gently heating process using a hot water bath followed by a standing time of 5 min with stirring to reduce significantly the furan content and, therefore, the daily intake. The furan concentrations found in the selected baby food samples retailed in Spain agree with those reported in the literature, achieving the highest values for meat-based (7.9–61 ng g$^{-1}$) and fish-based baby foods

(19–84 ng g$^{-1}$). These finding could be attributed to the oxidation and/or degradation of the highly unsaturated fatty acids present in these matrices. The estimation of the daily intake and MOE from baby food consumption showed a potential public health concern for fish- and meat-based baby foods, although the handling time that favours the loss of furan by evaporation reduces the furan intake for these matrices. In contrast, the consumption of infant formula and jarred cereal baby foods presents a low public health concern and a low priority for risk management actions.

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## 3.4 DISCUSSION OF THE RESULTS

### 3.4.1. Occurrence of furan in coffee and baby food samples

The survey of furan occurrence performed in this thesis includes 131 products (55 coffee and 76 baby food samples) commonly highly-consumed in Spain that were purchased from food producers or from trade establishments, including pharmacies and supermarkets, selected randomly in different areas of Barcelona. Several sub-categories of coffee brews such as instant, decaffeinated, 100% natural, blend of natural and *torrefacto* (roasted with a small portion of sugar), 100% Arabica and a mixture of Arabica and Robusta were analysed (**Paper IV**). For infant food, three classes of samples were mainly considered, namely infant formula, baby cereals and baby foods which were classified according to the main ingredients: fruit, vegetables, chicken, meat and fish (**Paper V**).

The proposed automated HS-SPME-GC-MS method (Chapter 2) was used for furan determination in coffee and baby food samples. However, as the formation of furan is linked to heat treatment, there were concerns about the possible formation of furan during analysis. In fact, there are evidences that in some food matrices furan can be formed at temperatures as low as 40 ºC (Senyuva and Gokmen, 2005). Therefore the analytical procedure was checked for each studied sample type to ensure that the formation of extra furan does not occur. To this end, the influence of temperature and time on furan formation during HS-SPME analysis was studied (Papers IV and V) using samples with different ingredients to cover all studied coffee, infant formula and baby food categories. For baby foods, no significant differences (p-values > 0.05) in furan content were observed when using HS-SPME at a temperature of 30 ºC even when the heating time increased up to 100 min (Table 1, Paper V). Similar results were obtained for coffee brews where in addition, it was demonstrated that increasing the precursors in the vial (higher sample size) does not yield to an enhancement of furan generation (Paper IV). Therefore, it was concluded that under the conditions proposed in this thesis for the HS-SPME method, no extra furan is formed during the analysis of coffee and baby food samples.

The study performed in this thesis about the occurrence of furan in baby food products and coffee from the Spanish market was undertaken as described in Papers V and IV respectively. The aim of the study was to obtain data on the occurrence of furan in both sample types as purchased and also after typical at home sample preparation. The obtained results are summarized in Table 3.4 and are given in detail in Tables 2, 3 and 4 of paper IV and Table 2 of paper V.

Table 3.4: Furan concentrations in coffee and baby food analysed samples.

| Sample | Number of samples | Positive samples | Furan concentration | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Min. | $P_{25}$ | Median | Mean | $P_{75}$ | Max. |
| Coffee brew [a] | 55 | 100 % | 12 | 32 | 56 | 72 | 97 | 244 |
| Infant formula [a] | 8 | 50 % | 0.08 | 0.1 | 0.2 | 0.2 | 0.26 | 0.33 |
| Baby cereals [b] | 11 | 100 % | 0.15 | 0.43 | 0.7 | 0.8 | 1.17 | 2.1 |
| Baby food [b] | 56 | 100 % | 0.7 | 12.6 | 31 | 30.3 | 46 | 84 |

(a) Furan concentration in (ng $ml^{-1}$).
(b) Furan concentration in (ng $g^{-1}$).

Regarding the presence of furan in coffee, EFSA reported high concentration levels of furan (up to 11000 ng $g^{-1}$) in solid coffee such beans and/or ground coffee (Table 3.2). In this thesis we have evaluated furan concentration in brewed coffee (ready to consumption) since from a human exposure point of view, furan concentration in brewed coffee is more important than in roasted beans or ground coffee. Moreover, the contribution of coffee to the furan daily intake can only be assessed using occurrence data in coffee brews. All the analysed coffee brews (Table 3.4) contained furan as happens in the study performed by US FDA (Table 3.1) although the concentration levels were slightly higher (2-3 times) than in USA and similar to that found in the surveys performed in different countries between 2004 and 2016 (Table 3.3) that ranges from 10 to 352 ng $ml^{-1}$. With regard to our results (Paper IV), it is important to mention that lower furan contents were found in coffee brews prepared with instant coffee (12– 35 ng $ml^{-1}$) and decaffeinated coffee (14 – 65 ng $ml^{-1}$) than those prepared from regular coffee (68- 146 ng $ml^{-1}$) and from coffee capsules (68- 244

ng ml$^{-1}$) (Tables 2; 3; and 4, Paper IV). The lower concentration of furan in decaffeinated coffee is probably due to the decaffeination process while for instant coffee brew in addition to the manufacturing process of this type of coffee it can also be attributed to a dilution factor (2 g for 60 ml of brewed coffee as recommended by the manufacturers). What is clear is that furan level is coffee type dependent although it can also be influenced by other factors as it is discussed in the next section.

In infant food products, furan was found in 50% of total studied infant formula samples while for cereal porridge and baby foods the percentage of positives was 100% (Table 3.4). This widespread occurrence of furan in baby food is probably related to the high number of different reactions that can generate this product that follow multiple routes and involve different precursors and intermediates although at the present state of knowledge it is not clear which ingredients are more prone to generate this compound. As can be seen in Table 3.4, furan concentration in baby food samples (0.7- 84 ng g$^{-1}$, median concentration: 31 ng g$^{-1}$) obtained in our study is in agreement with the values reported by EFSA (median concentration. 24 ng g$^{-1}$). In contrast, the concentrations in infant formula are about 10 times lower (median concentration: 0.2 ng ml$^{-1}$) than those reported by EFSA (median concentration up to 2.5 ng ml$^{-1}$) and moreover, the percentage of positive samples is higher, 50 % in front of 9 % in EFSA survey. This difference can be explained by the higher LOQs (1 – 10 ng g$^{-1}$) of the methods used by the laboratories included in the EFSA report which are 10 to 25 times higher than those of the HS-SPME method developed in this thesis. Moreover, our results (Table 2, Paper V) show that the nature of the baby food has an important influence in the concentration of furan. For instance, an increase in furan concentration (3-4 times) was observed in products where cereals are added to milk (Table 3.4). Out of baby food, fruit based products presented a relatively low furan concentration (0.7- 2.68 ng g$^{-1}$) that increased in vegetable baby foods (1.7- 28.8 ng g$^{-1}$), arriving to the highest concentration levels in chicken and meat (7.9 – 64 ng g$^{-1}$) and fish (19.1 – 84.2 ng g$^{-1}$) based products. These results are in agreement with literature where it is reported that fruit-based baby foods contain lower amounts of furan than meat and vegetable based products (Paper V). This difference in furan content can be due to the different heating treatment as fruit samples

are generally pasteurised (70 – 80 ºC) whereas the vegetables are sterilized (110 – 120 ºC) and the fish and meat are cooked. In addition, the high furan concentration in meat and fish based baby foods can be related to the different nature of proteins, carbohydrates and fats and to the presence of unsaturated fatty acids susceptible to oxidation and degradation during thermal treatments. Finally it must be mentioned that the results of furan concentration in baby foods from samples as purchased are of interest for industrials and manufactures but from a health perspective it must be taken into account that in-home preparation could affect furan content in ready-to-eat baby food samples as it is discussed in the next section.

## 3.4.2. Factors affecting furan content in coffee and baby food.

Coffee and most of baby foods are not consumed as purchased but they underwent to several preparation steps before consumption. Brewing coffee using different coffee brewers, heating baby food in home using different approaches and also the handling of both categories of samples can influence the furan content in the final ready-to-eat sample. The little information about this influence which is of great importance to reduce uncertainty in the furan exposure estimation, prompted us to study the effect of the preparation of the food on the furan concentration in coffee and baby food samples. In addition, it must be mentioned that from an industrial point of view, the influence of coffee type and roasting conditions, as well as the ingredients and industrial heat treatment of baby food on furan concentration in the final product, is also of great interest.

Regarding furan in coffee, our first attempt was addressed to evaluate the influence of coffee type on furan concentration in brewed coffee. It is well known that both, Arabica coffee and Canephor (Robusta) coffee are almost the unique types of coffee used in coffee industry. Arabica accounts for approximately 64% while Robusta accounts for about 35% of the world's production. Figure 3.2 shows the results obtained from the analysis of furan in brewed coffee prepared with Robusta coffee and Arabica coffee from different origins all of them purchased from the same local roasting factory

and roasted up to the same roasting degree using the same temperature-time programme.
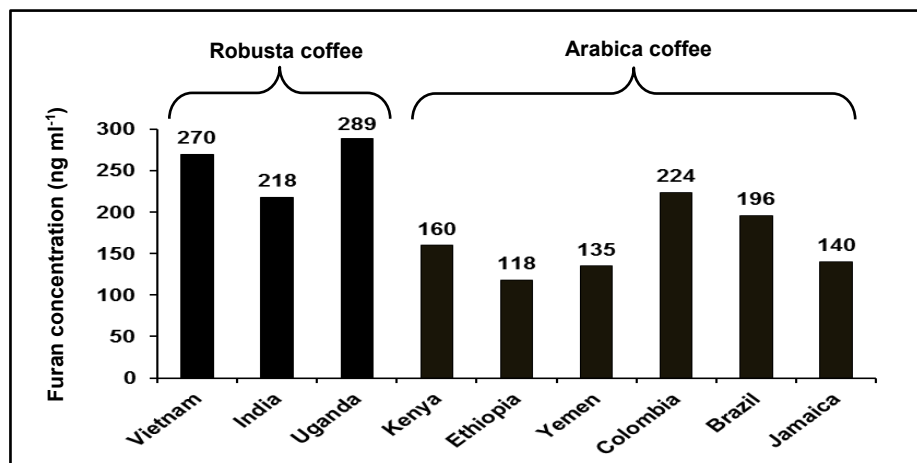


Figure 3.2: Influence of coffee origin on furan content in brewed coffee

As can be seen, in general, slightly higher content of furan was found in brews prepared from Robusta coffee in agreement with the results obtained by Arisseto et al. 2011. However, these higher values do not seem to be correlated with potential precursors since the concentrations of sucrose and lipids are commonly higher in Arabica than in Robusta coffee while the concentration of polysaccharides and amino acids are similar. In fact, other authors (Guenther et al. 2010) did not find differences in furan concentrations in Robusta and Arabica coffee samples. Probably the roasting process of green coffee is more important in the generation of furan than the type of coffee.

Coffee roasting is a complex heat transfer process where coffee beans are subjected to a given temperature for a scheduled time until a desired roasting degree is obtained. The degree to which coffee beans are roasted is one of the most important factors that determine the development of flavour and aroma. The most common way to describe coffee roasted levels is by the colour of the roasted beans, ranging from light to dark. Undoubtedly, furan is undesirably formed during coffee roasting but few studies are available in the literature on the relationship between coffee roasting conditions and resulting furan formation. Here we studied the influence of the most important roasting

conditions, namely temperature and time, on furan occurrence in coffee. Real industrial coffee roasting conditions were simulated and green coffee beans from Brazil (the largest grower and exporter of green coffee beans in the world) were used (Paper IV) to obtain light, medium and dark roasted coffee. The results of furan content (Table 1 and Figure 1 in Paper IV) indicated that furan concentration increases with roasting temperature. Besides, lower furan concentrations were found if coffee beans roasted to the same roasting degree (dark coffee), at low temperature (lower formation yield) using long roasting time (the loss of furan increases). Considering these results, we recommend using low temperature and long roasting time (140 ºC, 20 min) to decrease the level of furan.

Another important factor that affects the furan content in brewed coffee is the brewing process. Among the different brewing procedures currently used to prepare coffee beverages the drip-filter and espresso brewing are the most popular, although nowadays, Nespresso brewer (based on espresso method) is widely used in European countries. These procedures operated under different parameters such as pressure and time of brewing and as a result, it is expected to affect furan concentration in the final brew. The effect of these brewing methods on furan occurrence in coffee brews has been studied in this thesis (Paper IV) and the results obtained are summarized in Table 3.5.

Table 3.5: Furan concentration in coffee brews obtained using different brewing procedures.

| Brewing procedure | Furan concentration (ng ml$^{-1}$) | | | | |
|---|---|---|---|---|---|
| | Min | $P_{25}$ | Median | $P_{75}$ | Max. |
| Drip-filter | 20 | 30 | 39 | 47 | 78 |
| Expresso | 43 | 60 | 72 | 97 | 146 |
| Nespresso | 117 | 132 | 149 | 193 | 244 |

Two factors could principally affect the furan content in coffee brew: extraction efficiency of furan from the solid coffee and the loss of furan during brew preparation.

For Espresso and Nespresso coffee the pressure used for coffee brewing is higher than in home drip filter thus enhancing the extraction of furan. In addition, losses of furan can increase using the drip filter brewer because the required brewing times (100 – 120 s) are higher than for Expresso and Nespresso (25 – 30 s). Moreover, the higher content of furan in Nespresso than Espresso brew can be due to the higher pressure used in Nespresso machine (1850 KPa) than in Espresso (900 KPa) and also to the reduction of furan loss during handling because of the hermetic seal of the capsules used for Nespresso coffee.

The effect of handling on furan concentration in brewed coffee (time after brewing and before consumption) was also evaluated in this thesis. Due to the volatility of furan, its concentration in brewed coffee is evidently reduced by evaporation during the handling of the coffee at high temperature (80 – 70 ºC). For instance (Paper IV), an important reduction of furan concentration, 23% after one minute (at 70 ºC) and 50% after 5 min (at 57 – 55 ºC) was observed. So, a useful way to reduce the possible risk of furan to humans from coffee consumption is to wait one or two minutes before drinking the coffee.

Regarding to baby food, an objective of this thesis was to evaluate the levels of furan in baby foods as consumed after domestic preparation. In fact, few studies have been conducted to monitor changes in levels of furan during preparation and handling of baby foods. A first indication of the effect of consumer preparation on furan in baby food samples showed that the furan levels decreased in most canned and jarred foods after preparation due to evaporation (Roberts et al. 2008). However, the described results were insufficient to make a final conclusion regarding furan content in baby food after in-home preparation and moreover, they cannot provide enough data for the evaluation of furan risk assessment. In this thesis, we studied the effect of sample preparation as well as the effect of sample handling on furan level in baby foods (Paper V). In addition, several heating practises (commonly used for domestic baby food preparation) were evaluated for their effect on furan level.

For baby food samples that need preparation to be ready for consumption such as powder milk and porridge, our results (Figure 2 Paper V) indicated that furan level determined after preparation according to manufacturer's recommendations were (34 % - 62 %) lower than those prepared from the same samples but under conditions that limit the loss of furan (Experimental section, Paper V). This reduction of furan is due to its evaporation during sample preparation. Therefore, it is recommended to follow the preparation protocols proposed by manufacturers to decrease the risk of furan.

Another domestic preparation practice commonly used before the consumption of baby foods is sample heating. Heating is normally applied to samples that contain vegetables, rice, pasta, meat and/or fish. In our study we evaluated the effect of heating baby foods in opened sample jars using both a microwave oven and a hot water bath and also after sample platting using a microwave oven. The furan levels found in baby foods as purchased and after heating using different approaches are included in Table 3 Paper V, and here are summarised in Figure 3.3. An important decrease in furan content ranging from 31% to 53% was observed when samples were heated in a hot water bath at a temperature of 65 ºC and stirred for 5 min. Similarly, furan concentration levels in baby food samples heated using microwave after their platting were clearly lower (19% - 35%) than in the same samples analysed as purchased. In contrast, the change in furan concentration was not as evident for samples heated in open jars in a microwave. In this case, for most of the samples, mainly those containing vegetables as main components, furan level decreased after their heating ($p$-value $< 0.05$) while for other samples (meat and fish based) the furan level slightly increased ($p$-value $< 0.05$). These results indicate that though furan evaporation is the main important process during heating, there is a competition between furan formation and furan loss and the final content of furan in a given sample is determined by which process takes place in a higher extension. So, it would be better to heat the baby food samples using a hot-water bath instead of the most common actually used approach (microwave-oven in opened jar sample) in order to reduce furan concentration (up-to 53%) and decrease the risk of furan.

Finally and as described in paper V, the effect of sample handling on the decrease of furan concentration in baby food samples due to its losses via evaporation was studied (Figure 1 paper V). When using long sample handling times, a higher amount of furan is lost and the final concentration in baby food samples depended on sample ingredients (different retention and/or diffusion of furan in the sample) and also on the physical state (liquid or semi-solid). For instance, the furan concentration reduction for a semi solid fruit baby food was 44% (5 min in a dish) while for samples containing vegetables and/or meat (also semi-solid), the decrease of furan concentration after the same handling time was lower, between 25% and 33%. With respect to the effect of the physical state (liquid or semi-solid) in furan reduction, liquid samples showed in general, higher losses of furan. For instance, the highest loss of furan (57%) was found for a sample of apple juice after 5 min handling in a baby bottle. So, we recommend handling baby food samples before consumption for a period of time between 2- 5 min to reduce the concentration level of furan and minimise its possible health risk.

### 3.4.3. Estimation of daily intake and risk assessment

In this thesis, the exposure to furan from baby food and brewed coffee consumption was evaluated by the estimated daily intake (EDI) calculated using the obtained analytical results (concentration in the samples), the consumption data (Q) for each food category and the average consumer body weight (EDI = C × Q / B.W). For coffee, consumption data  obtained from the Spanish Coffee Federation (FEC, 2007) was used to obtain overall exposure information but in addition, a small scale study (a 24-h recall questionnaire) on coffee consumption in Barcelona was performed by our research group (Paper IV) in order to obtain a more elaborated picture of consumption habits in large cities. The results from this last evaluation indicated that females consume less coffee than males, 31% of the total female participants were not coffee consumers (Fig 2 paper IV) in front of 10% for males and moreover, the average coffee consumption is also lower (2.1 *versus* 3.1 cups per day).
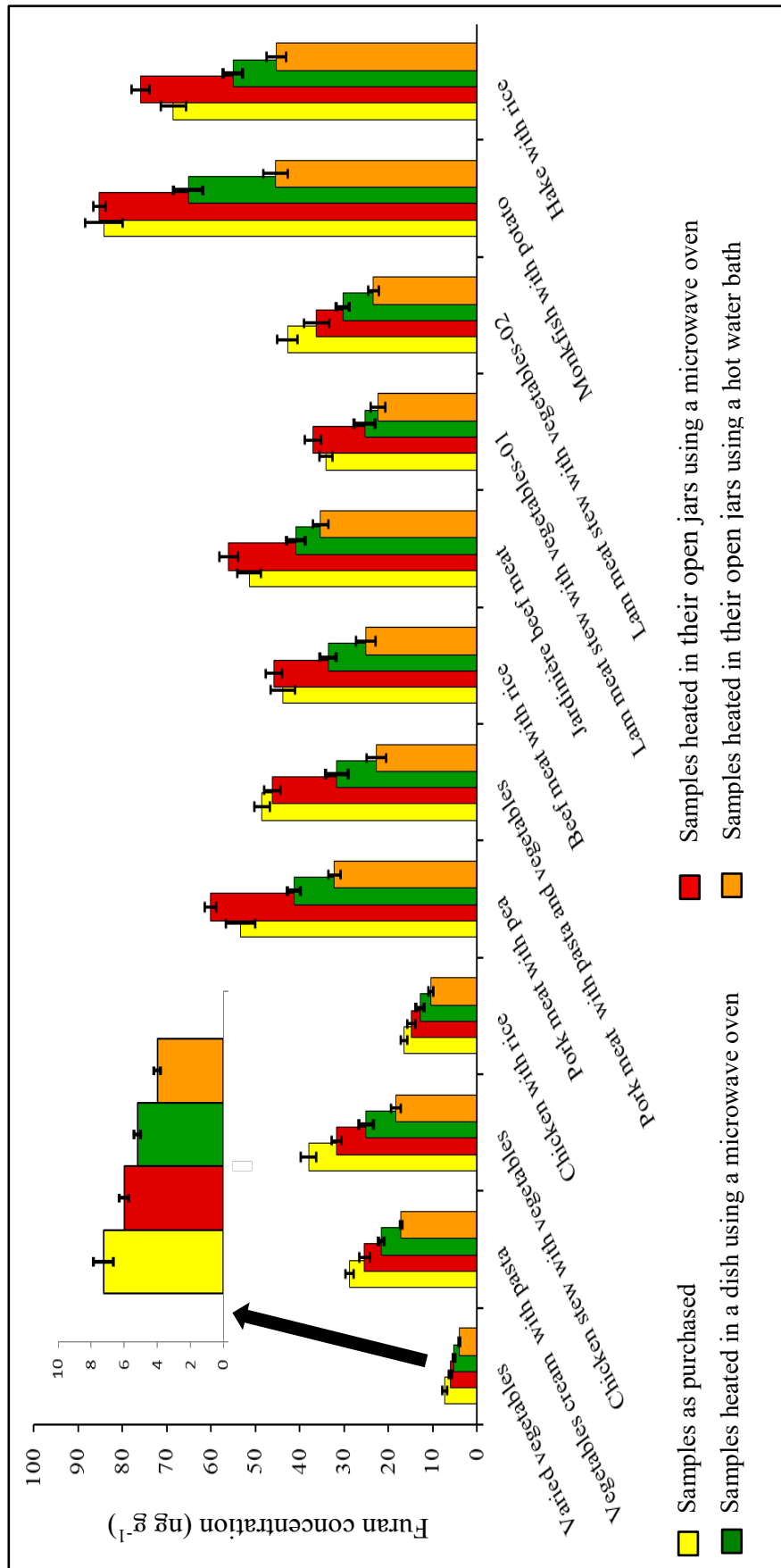
Figure 3-3: Furan concentration in baby food samples analysed as purchased and also after heating using different domestic approaches.

These values are slightly higher than those reported by the Spanish Coffee Federation (FEC, 2007), with 1.64 cups of coffee per person and day, indicating that the coffee consumption is probably higher in large cities than in small villages. The EDI values obtained in this thesis are summarized in Table 3.6. The lower exposure to furan from coffee consumption found for females (0.02 to 0.34 µg kg$^{-1}$ b.w. day$^{-1}$) than for males (0.03 to 0.50 µg kg$^{-1}$ b.w. day$^{-1}$) can be directly related to the female lower coffee consumption. With regard to the brewing procedure it is clear from Table 3.6 that the EDI of furan from coffee brews prepared by expresso machine is higher than those prepared with a drop-filter brewer and that the highest EDI of furan is obtained from the consumption of coffee brews prepared by Nespresso machine. Therefore, from a point of view of furan intake drop-filter brews must be recommended in front of Expresso and Nespresso ones.

Table 3.6: Exposure to furan from coffee brews consumption.

| Coffee sample | Sex [a] | Estimation daily intake (EDI) (µg kg$^{-1}$ b.w. day$^{-1}$) [b] | | | |
| --- | --- | --- | --- | --- | --- |
| | | Min | Median | Mean | Max |
| All categories | F | 0.02 | 0.1 | 0.12 | 0.34 |
| | M | 0.03 | 0.15 | 0.18 | 0.50 |
| Drop-filter brews | F | 0.04 | 0.07 | 0.07 | 0.14 |
| | M | 0.05 | 0.1 | 0.11 | 0.21 |
| Expresso brews | F | 0.08 | 0.13 | 0.14 | 0.26 |
| | M | 0.11 | 0.19 | 0.21 | 0.39 |
| Nespresso | F | 0.14 | 0.21 | 0.22 | 0.34 |
| | M | 0.21 | 0.31 | 0.32 | 0.50 |

a: F= Female, M= Male.
b: Adult body weight= 70 kg.

The mean EDI of furan from coffee consumption found in this thesis (0.12 and 0.18 µg kg$^{-1}$ b.w. day$^{-1}$ for male and female respectively) is in agreement with the value (0.15 µg kg$^{-1}$ b.w. day$^{-1}$) reported by FDA (2007) and lower than the mean EDI (0.5- 0.6 µg kg$^{-1}$ b.w. day$^{-1}$) reported by EFSA (2009) although these last values corresponded to the combined consumption of coffee, tea and cocoa. With regard to data from Spain, our EDI values are similar to those reported by Mesías et al. (2014) that found EDI values

up to 0.43 µg kg$^{-1}$ b.w. day$^{-1}$ analysing coffee from automatic vending coffee machines which are closed system, similar to Nespresso coffee brewer.

For baby food, the average daily consumption data (117.8 g per day and infant, for jarred baby food and 81.4 g and 94.3 g per day and infant, for infant formula and baby cereal porridge) used to calculate EDI were obtained from *Mercasa* (Mercasa, 2015) which is a public company of the Spanish Government. Since it was not possible to obtain baby food consumption data based on the ingredients (fruit, vegetables, meat, etc.) and also the recommended age for each product, we considered that the infants between six and nine months old consume baby foods of all type and ingredients. Estimated infant furan daily intake due to baby food calculated in this thesis is given in Table 4, Paper V, and in Table 3.7 there is a summary of the EDI values taking into account the main categories of baby foods (canned and/or jarred baby food, infant formula and cereal porridge).

Table 3.7: Exposure to furan from commercial baby foods.

| Sample | Estimation daily intake (EDI) (µg kg$^{-1}$ b.w. day$^{-1}$) [a] | | | |
|---|---|---|---|---|
| | Min | Median | Mean | Max |
| All categories | 0.016 | 0.45 | 0.45 | 1.24 |
| Infant formula | 0.002 | 0.007 | 0.01 | 0.024 |
| Cereal porridge | 0.004 | 0.012 | 0.016 | 0.037 |
| Baby food | 0.01 | 0.43 | 0.42 | 1.18 |

a: Baby body weight= 8.4 kg.

As can be seen in Table 3.7, the furan intake for infants is mainly due to the consumption of canned or jarred baby food since cereals porridge and infant formula only contributed with a small percentage, lower than 5% to EDI. The EDI values found in this thesis considering all food categories, are similar to those reported in European countries (0.13 and 0.97 µg kg $^{-1}$ b.w. day $^{-1}$) for infants aged from 3 to12 months (EFSA 2009) and slightly lower than those of USA, 0.4 to 1.0 µg kg $^{-1}$ b.w. day $^{-1}$ for babies aged from zero to 12 months (US FDA, 2007). The low values for infant formula found in this thesis are in agreement with those reported (0.01- 0.03 µg kg$^{-1}$ b.w. day$^{-1}$) for Norwegian infants (VKM, 2012) and lower than EFSA (2009) values (0.05- 0.09 µg

kg$^{-1}$ b.w. day$^{-1}$). With regard to the baby food ingredients (Table 4 Paper V) the contribution to the EDI of furan follows the same pattern that the furan concentration being the contribution of fruit based baby food (0.01- 0.04 μg kg$^{-1}$ b.w. day$^{-1}$) the smallest and the fish based baby food the highest (0.27- 1.18 μg kg$^{-1}$ b.w. day$^{-1}$).

In order to discuss the risks associated to furan intake due to consume of coffee by adults and baby food by infants, in this thesis the margin of exposure approach (MoE) has been applied. The use of MoE as a tool to consider possible safety concerns arising from the presence of both genotoxic and carcinogenic substances (such furan) in food is recommended by the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO) expert committee on food additives (JECFA), the European Food Safety Authority (EFSA) and the International Life Sciences Institute–European branch (ILSI Europe). Two factors are taken into account to calculate the MoE, the dose at which a small but measurable adverse effect is first observed (Benchmark Response, BMR) for a given population and the theoretical, predicted, or estimated exposure dose (EDI) to the considered substance (MoE =BMDL$_{10}$ / EDI). A benchmark response (BMR) of 10% was used since the modelling of lower responses generally results in greater uncertainty. As commented in the Introduction of this thesis, the FAO/WHO expert committee on food additives (JECFA) set the BMDL$_{10}$ for furan at 0.96 mg kg $^{-1}$ b.w. day $^{-1}$ and this value was used to calculate the MoEs. In addition, we followed the indication of EFSA that considers a MoE value of 10,000 or higher of low concern from a public health point of view while a value lower than 10,000 means that it may be a concern and represents a high priority for risk-management actions (EFSA 2005).

The results of the estimated MoE from baby food consumption are given in Table 4 in Paper V and they are also summarised in Table 3.8 where MoE values for coffee consumption for all coffee brews independently of the type of brew and for Espresso and Nespresso brews are also included. As can be seen in Table 3.8, the obtained MoE values due to baby food consumption are higher than 10,000 for infant formula and cereal porridge indicating low concern (no public health risk).

Table 3.8: Estimated MoE to furan for Spanish population.

| Population | Sex[a] | Margin of exposure (MoE)[b, c] | | | |
|---|---|---|---|---|---|
| | | Min. | Median | Mean | Max. |
| **Infant 6- 9 months** | | | | | |
| Infant formula | | 412,776 | 139,291 | 95,879 | 40,099 |
| Cereal porridge | | 247,666 | 79,253 | 61,419 | 26,278 |
| Baby food | | 97,793 | 2,233 | 2,286 | 813 |
| Total | | 80,000 | 2,133 | 2,182 | 793 |
| | | | | | |
| **Adults > 18 years** | | | | | |
| Espresso brew coffee | F | 12,000 | 7,385 | 6,857 | 3,692 |
| | M | 8,727 | 5,053 | 4,571 | 2,462 |
| Nespresso brew coffee | F | 6,857 | 4,571 | 4,364 | 2,824 |
| | M | 5,421 | 3,097 | 3,000 | 1,920 |
| Total coffee brews | F | 48,000 | 9,600 | 8,000 | 2,823 |
| | M | 32,000 | 6,400 | 5,333 | 1,920 |

a: F; female, M; male.
b: BMDL10 = 0.96 (mg kg $^{-1}$ b.w. day $^{-1}$).
c: EDI at min, median, mean and Max obtained from Table 3.6 and 3.7.

In contrast, the MoE values obtained for as purchased jarred and canned baby food or considering all categories of baby food, except values correspond to the lowest EDI values (minimum), are all lower than 10,000 indicating a possible concern for public health and the requirement of risk-management actions. It must be mentioned that the MoE values lower than 10,000 are mainly due to the contribution of the high furan concentration in meat and fish based baby food (MoE; 8,643- 813) while for only fruit based baby food MoE values (25,543 to 97,793) higher than 10,000 (Table 4, Paper V) were obtained. The MoE values found for vegetables based baby food (Table 4, Paper V), between 39,342 and 2,377 with a median value at 12,538 (higher than 10,000) but with a mean value at 6,727 (lower than 10,000) indicate that in some cases they can represent a potential health concern. Therefore, form an industrial point of view, baby food manufacturers must put especial attention to reduce the furan concentration especially in meat, fish and also vegetables based baby foods. For coffee, it is clear that consumption of Espresso and/or Nespresso brews (which are highly consumed in Spain) may present a high concern for public health as most MoE values, except at minimum EDI for female, are lower than 10,000. Considering all type of coffee brews (Drop-filter

and instant coffee in addition to Espresso and Nespresso brews) the MoE values increased but the median and mean MoEs are lower than 10,000 resulting a possible concern for public health that would require a risk-management action. In this context, coffee factories must consider this fact and modify coffee roasting process in order to eliminate or at least reduce furan concentration in coffee. It is interesting to indicate that in a study on furan risk assessment performed by the Norwegian scientific committee of food safety (Vitenskapskomiteen for mattrygghet VKM, 2012) the $BMDL_{10}$ for furan is set at 0.14 mg kg $^{-1}$ b.w. day $^{-1}$ instead of 0.96 mg kg $^{-1}$ b.w. day $^{-1}$ and 0.02 mg kg $^{-1}$ b.w. day $^{-1}$ is taken as the point of departure dose. Considering this new value in our MoE estimation, a 48-fold reduction of the values of Table 3.8 is obtained placing brewed coffee as well as baby food (jarred/canned baby food) as foods to be considered for public health concern in Spain. For baby food, other researchers (Arisseto et al., 2010, Lachenmeier et al., 2012; Scholl et al., 2013) have also found MoE values lower than 10,000 suggesting that furan exposure in infants could be considered as a possible public health risk. With regard coffee drinkers, a study performed in Germany (Waizenegger et al., 2011) reported that the MOE for instant coffee was above 10,000 (14,565 to 193,578) but for other brewed coffees values lower than 10,000 were obtained suggesting that the human exposure to furan from coffee consumption could be considered as a possible public health risk. Nevertheless, in this thesis we have shown that the concentrations of furan in coffee brews and baby foods are highly influenced by in home preparation and/or handling, which can drastically reduce furan occurrence. As we discussed in the previous section, furan concentration in coffee brews is approximately 50% reduced after handling the cup for 5 min before the consumption. This reduction in furan concentration will decrease the EDI (0.011- 0.17 for female, 0.016- 0.25 for male) and increase the MoE values to values between 88,889 and 5,704 for female and 60,215-3,864 for male being only MoE at maximum EDI lower than 10,000. So, holding the coffee cup for some minutes before consumption can be recommended to reduce the possible health effect of furan intake from coffee brews consumption Similarly, for baby foods which do not need re-heating, the reduction of furan concentration after a handling time of 5 min was 57% for liquid samples (juice and also milk) and 44% for semi-solid samples (fruit puree and also porridge). To follow these directions is important since, as already commented; the MoE values for

such samples as purchased were lower than 10,000. For baby foods that require re-heating, the use of a hot water bath or a microwave pouring the food in a plate is recommended since at these conditions an average reduction in furan concentration of 42% and 27%, respectively is obtained and as a consequence an increase in MoE values occur (Table 5, Paper V), although even following these recommendations MoE values are in some cases still lower than 10,000. To further increase these values a sample handling of 5 min is recommended as a good practice. This will allow reducing health concern for infants until manufactory companies found the way to use more safety processes to prepare canned and jarred baby food instead of conventional thermal processing.

# CONCLUSIONS

**CONCLUSIONS**

This thesis was conducted to study furan and volatile *N*-nitrosamines as process-induced food toxicants from the development of analytical methodologies to the evaluation of their presence in food and risk assessment estimation for Spanish population.

The main results of the work are summarized in the following conclusions:

**Regarding analytical methods developed for furan and *N*-nitrosamines**

➢ The feasibility of HS-SPME coupled to GC- ion trap MS for the analysis of furan in food commodities has been demonstrated. In this context, a fully-automated method has been developed and proposed as an alternative to the reference HS-GC-MS FDA method.

➢ For HS-SPME analysis of furan, the use of a carboxen/polydimethylsiloxane (CAR/PDMS, 75 μm) fibre is proposed because it is the most effective coating among the tested fibres with single or mixed stationary phases. Maximum responses for furan were obtained working at 30 ºC for 20 min using a headspace/aqueous phase volume ratio of 1.5 (12 ml/8 ml) and adding 20% NaCl (w/w). It is important to mention that at these conditions, furan formation, a problem frequently found when analysing furan with HS methods, does not occur.

➢ The automatic HS-SPME GC-MS method provided low limits of quantitation (0.05-0.17 ng $g^{-1}$) and good precision (run-to-run: RSD% ≤ 6% and day-to-day: RSD% ≤ 11%) for furan determination. These LOQs (5 to 20-fold lower) and precisions (two times higher) were better than those found with the HS method proposed by the FDA. To increase sample throughput for routine analysis the use of isotope dilution for furan quantitation is proposed instead of the standard addition method recommended by US FDA.

➢ For routine analysis of *N*-nitrosamines a fully automated HS-SPME method combined with GC-CI-MS/MS, using methanol as reagent gas, is proposed. This method has demonstrated to be applicable for the analysis of VNAs in beer and processed meat products at low concentration levels (μg $kg^{-1}$).

➢ CI-MS/MS parameters, such as ion-trap temperature, filament emission current, CI reaction time and isolation time of precursor ions, were found to be critical to improve sensitivity for VNA determination. The accurate optimization of these instrumental parameters allowed achieving limits of detection from 15 to 70-fold better than those reported using the methods based on US EPA method 521. For HS-SPME extraction, the use of CAR/PDMS fibre, at a temperature of 70 ºC in alkaline medium with a high ionic strength under non-equilibrium conditions (45 min) is proposed.

➢ The developed HS-SPME GC-CI-MS/MS method provided good selectivity and sensitivity for the determination of VNAs in beer and meat processed foods, with limits of quantification (0.10–0.38 $\mu g \ kg^{-1}$) lower than the maximum levels established by international regulations. In addition, the developed method showed good trueness (recoveries: 70–119%) and precision (RSD% < 12%) with decision limits (CCα: 0.40–0.44 $ng \ g^{-1}$) and detection capabilities (CCβ: 0.44–0.50 $\mu g \ kg^{-1}$) low enough for an adequate VNA determination in beer and foods.

➢ The applicability of both HS-SPME-GC-MS methods for the determination of furan and VNAs has been demonstrated analysing several selected foods commercialised in Spain. Furan was detected in all samples at concentrations ranging from 0.1 $ng \ g^{-1}$ to 1.1 $\mu g \ g^{-1}$, being baby food and coffee the products with the highest furan content. In contrast, VNAs were not detected in any of the analysed beer samples at concentrations levels above LOQs, and the presence of VNAs (NDMA and NPYR) were only found in 3 out of 18 processed meat product samples analysed (one sample of cured sausage and two of Spanish chorizo) at concentrations ranging from 2.4 to 3.7 $ng \ g^{-1}$. These results indicate the effectiveness of the measures adopted by the manufactures to avoid the formation of VNAs.

**Concerning to the presence of furan in coffee and baby food**

➢ Furan concentration found in coffee brews, between 12 and 244 $ng \ mL^{-1}$, is in agreement with that found in other countries (5–360 $ng \ mL^{-1}$). Among all studied samples, instant coffee showed the lowest furan concentration (12–35 $ng \ mL^{-1}$) which is directly related to the lower coffee/water ratio used for this preparation.

➢ It has been proved that the origin of the coffee beans has little influence in furan concentration. Similar values were found in brews prepared from Arabica coffee (118–224 ng mL$^{-1}$) and Robusta coffee (218–289 ng mL$^{-1}$), both roasted under the same conditions and at the same roasting degree. In addition, the process of coffee decaffeination does not seem to affect furan generation since decaffeinated and regular coffee brews (both prepared using the same coffee/water ratio and brewing method), showed similar mean furan concentrations (regular coffee, 40.6 ng mL$^{-1}$, decaffeinated coffee, 30.50 ng mL$^{-1}$).

➢ The industrial roasting conditions of the coffee beans (temperature and time) have demonstrated to be important factors in furan generation. Brews obtained using coffee beans roasted at the same roasting degree using high temperatures and short roasting times showed a higher furan concentration (138 ng mL$^{-1}$) than those obtained using lower temperatures and longer roasting times (64 ng mL$^{-1}$). So, the use of a relatively low temperature (140 ºC) and roasting times of 20 min is recommended to decrease furan formation in coffee.

➢ The results obtained in this thesis also shown that furan content in coffee brews highly depends on the brewing procedure. The higher pressure and the use of a close system that reduce furan evaporation in Nespresso brewing machine, lend to obtain coffee brews with high furan content (117–244 ng mL$^{-1}$). In contrast, lower furan concentration in coffee brews was found when using open systems such as drip-filters (20–78 ng mL$^{-1}$).

➢ Regarding baby food, furan has proven to be a common heat-induced food contaminant in the baby food samples evaluated. It has been found in 100% of the canned and/or jarred baby food (0.7–84 ng g$^{-1}$) and baby cereals (0.15–2.1 ng g$^{-1}$) but only in 50% of infant formula (0.08–2.1 ng g$^{-1}$) samples.

➢ The highest furan concentration was found for fish (19.1–84.2 ng g$^{-1}$) and meat based baby foods (7.9–64 ng g$^{-1}$) which is probably related to the presence of unsaturated fatty acids in this samples. In fruit based products (0.7–2.68 ng g$^{-1}$) furan concentrations were lower than in vegetable based ones (1.7–28.8 ng g$^{-1}$) probably due to the higher stability of the precursors in the original product and to the lower temperature used in industrial treatments.

➢ It has been confirmed that in-home preparation influences the content of furan in the final ready-to-eat food. For powder milk and cereals porridge, the sample must be prepared, to be ready-to-eat, following the manufacturers recommendations as the concentration of furan is reduced (34–62%) by evaporation. With respect to jarred and/or baby foods, the results obtained proved that furan evaporation is the dominant process during heating, although its formation can also occur but in much less extension. These results prompted us to recommend: (i) heating the products before consumption since furan concentration can be reduced from 19 to 53% in comparison to the product as purchased and (ii) using a hot water bath (highest reduction of furan concentration, 31–53%) for heating instead of a microwave oven, although this is the most fast and common procedure used by consumers.

**Related to furan daily intake and risk assessment**

➢ The exposure to furan from coffee brews consumption, (0.02 to 0.34 $\mu$g kg$^{-1}$ b.w. day$^{-1}$ for females and from 0.03 to 0.5 $\mu$g kg$^{-1}$ b.w. day$^{-1}$ for males), is important and indicates a possible concern for public health. Moreover, the mean values of margin of exposure (MoE) considering all type of coffee brews, 8,000 for female and 5,333 for male (MoE < 10,000), obtained in this work signify that there is a need of taking some safety measures. These MoE values are considerably lower for Espresso and Nespresso consumers (down to 3,000 for male) suggesting that actions must be taken to reduce the health risk for consumers drinking always Espresso and/or Nespresso coffee brews.

➢ For Spanish infant with ages between 6 and 9 months, the mean value of margin of exposure (MoE) resulted from the estimated daily intake of furan found in this thesis (from 0.01 $\mu$g kg$^{-1}$ b.w. day$^{-1}$ for infant formula, to 0.42 $\mu$g kg$^{-1}$ b.w. day$^{-1}$ for jarred and/or canned baby food) was 2,182 indicating a possible concern for infant health (MoE < 10,000). Additionally, we proved that (i) the contribution of jarred and canned baby food on the infant health risk (MoE= 2,286) is higher than that of infant formula (MoE= 95,879) and cereal porridge (MoE= 61,419), (ii) in-home preparation (heating) reduces the margin of exposure, reduction that for vegetables based baby food indicated no health concerns and (iii) the intake of furan from

infant formula and cereal porridge consumption do not present a public health risk (mean values of MoE higher than 10,000).

➢ The MoE results obtained in this thesis indicated that actions must be taken to advise manufacturers of baby food to take actions able to minimize furan generation especially in meat, and fish based baby foods. Similarly, the implementation of coffee roasting conditions, such those proposed in this thesis (low temperature for long time), would help to reduce the occurrence of furan in coffee.

➢ To minimize the risk of furan intake, we recommend coffee consumers to handle the cup for one or two minutes before consumption. Also, handling baby foods for 5 min after heating and before consumption is proposed as an additional safety factor to reduce children exposure to furan due to baby food.

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# ABBREVIATION AND ACRONYMS

## ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| **ADI** | Acceptable daily intake |
| **BDA** | cis-2-butene-1, 4-dial |
| **BMDL$_{10}$** | Lower 95% confidence limit benchmark response |
| **CCα** | Decision limit |
| **CCβ** | Detection capability |
| **CID** | Collision induced dissociation |
| **EC** | European Commission |
| **EFSA** | European Food Safety Authority |
| **FAO/WHO** | Food and Agriculture Organization/World Health Organization |
| **FDA** | Food and Drug Administration |
| **HS** | Headspace |
| **HS-SPME** | Headspace solid phase microextraction |
| **IARC** | International Agency for Research on Cancer |
| **JECFA** | Joint Expert Committee on Food Additives |
| **MoE** | Margin of exposure |
| **NAs** | Nitrosamines |
| **NDBA** | Nitrosodibutylamine |
| **NDEA** | Nitrosodiethylamine |
| **NDMA** | Nitrosodimethylamine |
| **NDPA** | Nitrosodipropylamine |
| **NDPhA** | Nitrosodiphynylamine |
| **NMEA** | Nitrosomethylethylamine |
| **NMOR** | Nitrosomorpholine |

| | |
|---|---|
| **NOAEL** | Non-observed adverse effect level |
| **NOC** | N-nitroso compounds |
| **NPIP** | Nitrosopiperidine |
| **NPYR** | Nitrosopyrrolidine |
| **NTP** | National Toxicology Program |
| **PCI** | Positive chemical ionisation |
| **PUFAs** | Polyunsaturated fatty acids |
| **US EPA** | United states Environmental Protection Agency |
| **VNAs** | Volatile nitrosamines |