CRISTINA MARIA DIAS SOARES
Mestre em Química Analítica Ambiental

Assessment of the dietary intake of acrylamide in Portugal. Development and evaluation of strategies for reduction of acrylamide formation in thermally processed foods.

Tese do 3º ciclo de Estudos Conducente ao Grau de Doutoramento em Ciências Farmacéuticas, Especialidade de Nutrição e Química dos Alimentos apresentada à Faculdade de Farmácia da Universidade do Porto

Trabalho orientado por

Professor Doutor José de Oliveira Fernandes
Professor Doutor José Luís Fontes da Costa Lima

Porto
Fevereiro 2015

Agradecimentos

Hora

Sinto que hoje novamente embarco
Para as grandes aventuras,
Passam no ar palavras obscuras
E o meu desejo canta --- por isso marco
Nos meus sentidos a imagem desta hora.

Sonoro e profundo
Aquele mundo
Que eu sonhara e perdera
Espera
O peso dos meus gestos.

E dormem mil gestos nos meus dedos.

Desligadas dos círculos funestos
Das mentiras alheias,
Finalmente solitárias,
As minhas mãos estão cheias
De expectativa e de segredos
Como os negros arvoredos
Que baloiçam na noite murmurando.

Ao longe por mim oiço chamando
A voz das coisas que eu sei amar.

E de novo caminho para o mar.

Sophia de Mello Breyner Andresen

Citando a autora do poema “sinto que hoje novamente embarco para as grandes aventuras (…) e por isso marco nos meus sentidos a imagem desta hora” e não podia deixar de agradecer a todas as pessoas que de uma forma ou de outra contribuíram para a realização dos trabalhos apresentados nesta tese.

Estou assim grata ao Professor Doutor José de Oliveira Fernandes por me ter admitido como sua aluna, pela utilidade das suas recomendações, a sua disponibilidade e a sua simpatia. Ao Professor Doutor José Luís da Costa Lima, agradeço o apoio científico e os conselhos, que contribuiram de forma imprescindível para a realização deste trabalho.

À Faculdade de Farmácia da Universidade do Porto e, em especial ao Departamento de Ciências Químicas, Laboratório de Bromatologia e Hidrologia, pela admissão como aluna do curso de Doutoramento em Ciências Farmacéuticas.

À Dra Susana Casal e à Dra Sara Cunha pela disponibilidade, pelas sugestões e críticas pertinentes a este trabalho que muito contribuíram para a sua finalização.

A todos os meus colegas do laboratório de Bromatologia em particular à Olga, à Elsa, à Isabel, à Joana Santos, à Ivone, à Rita, à Anabela Costa e ao Armindo pela amizade, pelo apoio e pelas suas preciosas sugestões e ótimo ambiente de trabalho.

À minha família, a quem dedico esta tese, muito especialmente aos meus pais, pelo seu apoio incondicional e por sempre me terem ensinado que ter tudo o que queremos depende de nós próprios.

E por último não posso deixar de referir que a realização deste trabalho foi possível graças à concessão de uma Bolsa de Doutoramento (SFRH/BD/39360/2007) pela Fundação para a Ciência e a Tecnologia (FCT), financiada pelo Programa Operacional Potencial Humano (POPH) – Quadro de Referência Estratégico Nacional (QREN) - Tipologia 4.1 - Formação Avançada, comparticipado pelo Fundo Social Europeu (FSE) e por fundos nacionais do Ministério da Ciência, Tecnologia e Ensino Superior (MCTES) e no âmbito do projecto POCI/AGR/61543/2004. Em associação à Bolsa de Doutoramento, a candidata contou ainda com subsídios para deslocamento a congressos internacionais.

Resumo

A presença de acrilamida em alimentos tem sido uma preocupação de saúde pública desde o anúncio de sua descoberta em alimentos em 2002. O principal objetivo deste trabalho foi avaliar a exposição da população Portuguesa à acrilamida e apresentar algumas estratégias possíveis de mitigação para reduzir a formação do composto em comida caseira.

A fim de contextualizaros resultados experimentais apresentados neste trabalho, no primeiro capítulo desta tese apresenta-se uma revisão teórica sobre o actual estado da arte no que respeita àpresença de acrilamida nos alimentos, com destaque para a toxicidade, exposição, mecanismos de formação, estratégias de mitigação e metodologias analíticas.

Para calcular os níveis de acrilamida presente em alimentos comerciais e preparados em casa, consumidos em Portugal, foram desenvolvidas e validadas duas novas metodologias baseadas no processo de Extracção da Matriz em Fase Sólida (MSPD) acoplado à cromatografia gasosa-espectrometria de massa (GC-MS).Estas metodologias foram aplicadas na análise de acrilamida em batatas fritas e assadas, produtos de cereais (pão, torradas, cereais de pequeno-almoço, bolos, biscoitos e bolachas), chocolates e alimentos para bebé (limite de detecção: 5,2 μg/kg; limite de quantificação: 15,7 μg/kg), e na análise de acrilamidaamostras de café e sucedâneos, uma matriz mais complexa, (limite de detecção: 5 μg/kg;limite de quantificação: 10 μg/kg).

Para avaliar a influência da espécie de café, do grau de torra e do volume da bebida no conteúdo em acrilamida procedeu-se à análise de amostras de cafés espresso. A quantidade média de acrilamida em “expressos” de torra média (30 ml) foram de 1,16 ± 0,25 e 2,31 ± 0,43 μg para amostras de espécie Arábica e Robusta puros, respectivamente. Espressos preparados a partir de misturas comerciais apresentaram um nível médio de acrilamida de 1,26 ± 0,28 μg. Uma redução de 25% foi observada quando se compararam espressos preparados com café de torra média e de torra prolongada. Verificou-se que o grau de extracção de acrilamida em amostras padrão deespressos de 30 mL, foi de cerca de 80%, só sendo afectado pelo volume de bebida, com espressos longos (70mL) a apresentar praticamente toda a acrilamida do bolo de café (99%), quase o dobro do observado em expressos curtos (20 mL). Quando comparado com outras bebidas de café comum, a concentração de acrilamida em café espressofoi maior. No entanto, devido ao pequeno volume por copo, a contribuição para a ingestão de acrilamida é menor.

A influência do processamento também foi avaliada em café natural produzido no Brasil onde o descasque de frutos imaturos é visto como um método promissor de minimizar o impacto negativo de grãos com defeito na qualidade e valor comercial de café. Verificou-se que o descasque de café verde permite uma redução nos níveis de asparagina, um precursor conhecido

de acrilamida, e uma consequente redução da formação de acrilamida durante a torrefação. Diferentes condições de torra também foram avaliadas mantendo a temperatura e o tempo de torrefacção constante e o fluxo de ar quente num torrador de leito fluidizado configurado para três velocidades diferentes. Os resultados obtidos mostraram claramente uma correlação positiva entre o teor de acrilamida no produto final e a velocidade do ar quente.

Ainda no que respeita a estratégias de mitigação de acrilamida, duas abordagens diferentes para reduzir a formação de acrilamida na preparação caseira de alimentos foram testadas com sucesso em amostras de carne e batatas fritas. Para a carne, o efeito de marinadas com vinho branco, cerveja ou chá verde, isoladamente ou adicionadas com ervas e especiarias, foram estudados em carne grelhada, usando carne não marinada como controle. Os melhores resultados foram obtidos com marinada decerveja ou cerveja com ervas durante quatro horas, com uma redução de 72% e 59%, respectivamente. Em batatas, foi avaliada a importância da variedade de batata usada para fritar. Amostras de cinco diferentes cultivares disponíveis no mercado Português foram fritas sob condições de tempo e temperatura controladas, tendo os menores níveis de acrilamida sido obtidos com as variedades Agria e Marabel, o que pode ser justificado pela sua menor quantidade de açúcares redutores. Os resultados obtidos sugerem que a seleçãode variedades de batata adequadas para fritar juntamente com o procedimento de marinar a carne antes de a cozinhar pode contribuir significativamente para reduzir a ingestão de acrilamida a partir de comida caseira.

Estimou-se a ingestão média de acrilamida na população adulta (n = 2.398; idades entre 18 e 92 anos de idade) da cidade do Porto, Portugal, com base no rastreio dos níveis de acrilamida em alimentos portugueses seleccionados, preparados em casa ou de origem industrial, e nos resultados de uminquérito dietético publicado no âmbito do projecto EpiPorto. O teor médio de acrilamida em alimentos variou entre 13 e 810 μg/kg, sendo os valores mais elevados presentes em batatas fritas e substitutos de café. A média de consumo diário estimado para adultosda população do Porto foi de 41,5 μg/dia, variando de 0,60-0,70 ug/kg de peso corporal para mulheres e homens, respectivamente. As principais fontes de exposição à acrilamida foram os produtos de batata (36%), carne (25%) e pão (12%), enquanto o café apresentou menor importância do que normalmente relatado em estudos semelhantes, devido aos baixos níveis de consumo reportados.

Palavras-chave: Acrilamida, alimentos processados, asparagina, açúcares redutores, mitigação, exposição, avaliação

Abstract

Acrylamide presence in foods has been a health concern since the announcement of its finding in foods in 2002. The main purpose of this work was to assess the exposure of the Portuguese population to acrylamide and discuss some possible mitigation strategies to reduce the formation of the compound in home-made food.

In order to support the experimental results reported in this work, the first chapter of this thesis intends to present a review about the state of the art regarding toxicity, exposure, mechanisms of formation, mitigation strategies and analytical methodologies regarding acrylamide in foods.

In order to calculate the levels of acrylamide present in commercial and home –made-foods consumed in Portugal, some analytical methods were developed and validated. Methodologies based on an optimized Matrix Solid Phase Dispersion (MSPD) extraction procedure was developed for the analysis of acrylamide in a variety of food matrices: processed cereal products (bread, toasts, breakfast cereals, snacks, cookies and biscuits), chocolates and baby-foods. Final extracts were analysed by GC-MS. The MSPD-GC/MS method presented a LOD of 5.2 μg/kg and a LOQ of 15.7 μg/kg.A similar procedure was also developed for coffee and coffee substitutes, a more challenging matrix and the MSPD-GC/MS method adapted for coffee analysis presented a LOD of 5 μg/kg and a LOQ of 10 μg/kg. Meat and fish samples were also analysed.

Espresso coffees were analysed to assess the influence of coffee species, roast degree, and brew length on acrylamide formation. Mean acrylamide contents of medium roasted espressos (30 mL) were 1.16 ± 0.25 and 2.31 ± 0.43 μg for pure arabica and robusta samples, respectively. Espressos prepared from commercial blends contained an average acrylamide level of 1.26 ± 0.28 μg. A 25% decrease was observed when comparing espressos prepared with medium and dark roasted coffee. The extraction efficacy of acrylamide for standard espressos of 30 mL was near 80%, being only affected by brew volume, with long espressos (70 mL) containing practically all acrylamide of the coffee cake (99%), almost double that of short ones (20 mL). When compared with other common coffee beverages, espresso acrylamide concentration (μg/L) was higher. However, due to the small volume per cup, it may contribute less to acrylamide ingestion.

The influence of processing was also evaluated in natural coffee produced in Brazil were peeling of immature fruit is viewed as a promising method of minimize the negative impact of defective grains in the quality and commercial value of coffee. Peeling of unripe coffee and

pulping of immature coffee grainsenables a reduction in asparagine levels, a known precursor of acrylamide, and a consequent reduction of acrylamide formation during roasting.

Different roasting conditions were also assessed, keeping the temperature and roasting time constant and the flow of hot air in a fluidized bed roaster was set to three different velocities. The results obtained so far, clearly show that the content of acrylamide increases as the velocity of hot air also increases in all samples analysed.

Regarding mitigation studies, two different approaches to reduce acrylamide formation during home-cooking of meat and fried potatoes were tested. For meat, the effect of marinades with white wine, beer, or green tea, alone or added with herbs and spices, were studied in pan-fried beef, using non-marinated beef as control. Best results were obtained with beer or beer with herbs marinade during 4 hours, with a reduction of 72 % and 59 %, respectively. For potatoes, the importance of potato cultivar for frying was evaluated. Samples of 5 different cultivars available in the Portuguese market were fried under controlled time and temperature conditions. Lowest levels of acrylamide were obtained with Agria and Marabel varieties, which could be justified by its lower amounts of reducing sugars. The results obtained here suggests that selecting adequate potato varieties for frying and marinating meat before cooking can contribute significantly to reduced acrylamide ingestion from home-made food cooking.

Average intake of acrylamide was estimated in the adult population (n=2398; ages between 18 to 92 years old) of Porto, Portugal, based on a screening assay of acrylamide levels in targeted Portuguese foods, cooked at home or from industrial origin, and a published dietary survey elaborated under the framework of EpiPorto project. Average acrylamide content in foods ranged between 13 to 810 μg per kg, being the highest amounts present in fried potatoes and coffee surrogates. An estimated average daily intake for the adult sub-sample of Porto population of 41. 5 μg/day was obtained, ranging from 0.60 to 0.70 μg/kg of body weight for women and men, respectively. The main sources of acrylamide consumption were potato products (36%), meat (25%) and bread (12%), while coffee showed less importance than usually reported in similar studies, due to low levels of consumption reported.

Keywords: Acrylamide, processed food, asparagine, reducing sugars, mitigation, exposure, screening

Table of Contents

List of Figures

Abbreviations and acronyms

2,3-DBPA	2,3-Dibromopropionamide
24-HDR	24 Hour Dietary Recalls
3-APA	3-aminopropionamide
AA	Acrylamide
AAMA	Acrylamide Mercapturic Acids metabolites
ACN	Acetonitrile
ANOVA	Analysis of variance
Asn	Asparagine
Asp	Aspartic acid
ATSDR	Agency for Toxic Substances and Disease Registry
aw	Water activity
BA	Biogenic amines
C	Concentration
CAS	Chemical Abstracts Service
CIAA	Confederation of the Food and Drink Industry of the EU
CV	Coefficient of variation
CZE	Capillary zone electro phoresis
DAD	Diode array detector
DCM	Dichloromethane
DCM	Dicholoromethane
DNA	Deoxyribonucleic acid
EC	Expresso Coffee
ECD	Electron Capture Detector
EFSA	European Food Safety Authority
EPIC	European Prospective Investigation into Cancer
ERM	European Reference Materials
ESI	Electrospray ionization
EtAc	Ethyl acetate
EtAc	Ethyl acetate
EtOH	Ethanol
EU	European Union
exc	Excitation
FAO	Food and Agriculture Organisation
FAPAS	Food Analysis Performance Assessment Scheme
FDA	Food and Drug Administration
FDE	Food drink Europe
FFQ	Food Frequency Questionnaires

FLD	Lluorescence detection
FMOC-Cl	9-fluorenylmethylchloroformate
fw	Fresh weight
g	Gravitational acceleration
GA	Glicidamide
GAMA	Glycidamide Mercapturic Acids metabolites
GC	Gas Chromatography
GC	Gas chromatography
GLM	General Linear Models
Gln	Glutamine
Glu	Glutamic acid
HAAs	Heterocyclic Aromatic Amines
HbAA	Hemoglobin Acrylamide adducts
HEATOX	Heat-Generated Food Toxicants
HEPA	Heptylamine
HMF	Hydroxi methylfurfural
HPLC	High Performance Liquid Chromatography
hx	Hexane
hx	n-hexane
IARC	International Agency for Research on Cancer
ICO	International Coffee Organization
IR	Infrared
IS, I.S.	Internal standard
IUPAC	International Union of Pure and Applied Chemistry
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC	Liquid Chromatography
LE	Liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
m/v	Mass/volume
m/z	Mass-to-charge ratio
MeCN	Acetonitrile
MeOH	Methanol
MeOH	Methanol
min	Minutes
MS	Mass spectrometry, espectroscopia de massa
MS/MS	Tandem mass spectrometry
MSD	mass selective detector
MSPD	Matrix solid phase dispersion
NIR	Near Infra-red

NOEL	No Observable Effect Level
ODS	Octadecylsilane
OPA	o-Phthaldialdehyde
ORL	Organic roast loss
p	Probability
PA	Picric acid
PCA	Perchloric acid
PFE	Pressurized fluid extraction
pH	Hydrogen ion potential
PTA	Phosphotungstic acid
PVA	Preto (black), Verde (green), Azedo (sour)
r	Correlation coefficient
RSD	Relative Standard Deviation
s	Standard deviation of the intercept or standard deviation of the blank
S	Slope
S	Statistical significance
SAS	Statistical Analytical System
SD, S.D.	Standard deviation
SIM	Selected ion monitoring
SNFA	Swedish National Food Administration
SPE	Solid phase extraction
SSA	Sulfosalicylic acid
TCA	Trichloroacetic acid
TCI	Tokyo Chemical Industry
TOF	Time of Flight
UFLA	Universidade Federal de Lavras
UPLC	Ultra Performance Liquid Chromatography
UV	Ultraviolet
UV / VIS	Ultraviolet-visible
v/v	Volume/volume
WHO	World Health Organization
y	years
λ	Wavelength

General Introduction

1. General introduction

1.1. Introduction. The discovery of acrylamide in foodstuff

Acrylamide is a synthetic compound widely used in several industries but particularly into the production of polyacrylamide.The polymer has several applications from wastewater treatment to gel electrophoresis for research (Figure 1.1).

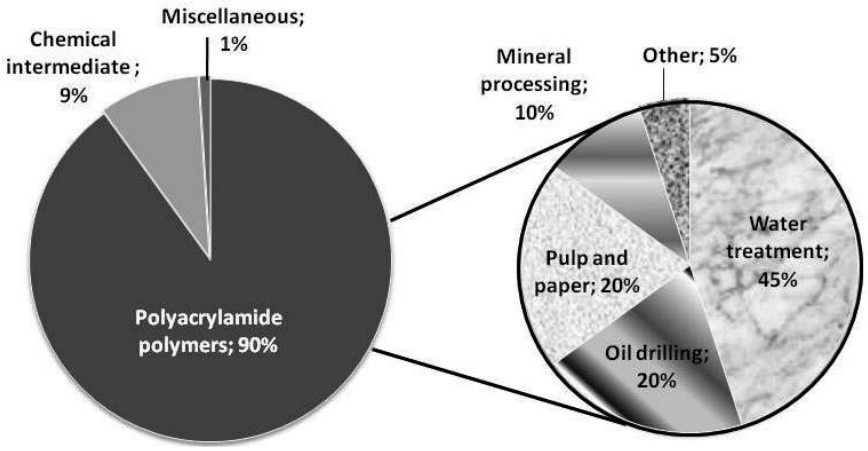

Figure 1.1. Industrial usage of acrylamide (adapted from: http://enhs.umn.edu/current/5103/acryl/uses.html)

When in 1997 cows suddenly became paralyzed and died, and dead fish were found floating in breeding pools in south-western Sweden it was discovered that the cause was the use of a sealant called Rhoca-Gil that had been injected into cracks in the tunnel walls that was being constructed in the region and contaminated the ground waters. For fear of human contamination, animal and vegetable products from the region were destroyed. And although no human deaths were reported, tunnel workers suffered mainly neurotoxic effects and people feared over possible long-term health effects of acrylamide, which is classified by the International Agency for Research on Cancer (IARC) as a probable human carcinogen (Reynolds, 2002).

While estimating the damages provoked by this accident on the workers, scientists discovered that all the population tested presented haemoglobin adducts with acrylamide including the control group not exposed to the contaminant. The connection between this

contaminant and food was made and the compound was found on widely consumed food groups like bread, fried potato products and coffee. This discovery soon raised a cancer scare based on obvious evidence that the public was being exposed to acrylamide for ages and little could be done to avoid the ingestion of the foodstuffs like potatos, bread and coffee. Some of the foods, such as bread, are so nearly universally eaten that identifying populations with widely different exposures will be difficult. Besides professional and food exposure, acrylamide is also a constituent of tobacco smoke, and the body even seems to makes some of the chemical naturally when subject to long term oxidative stress (Reynolds, 2002, Tareke et al., 2008, 2009).

Although all the studies already done about this subject, including about acrylamide health effects, the information that reaches final consumers is still being discussed in several journals, magazines, websites and food communities in a more or less sensationalist way increasing the cancer scare and the public distrust on food industry. News like "Frying potatoes to make chips or French fries produces a potent chemical carcinogen called acrylamide","consuming acrylamide boost kidney cancer rate by 59 percent", "consuming acrylamide from cooked foods boost ovarian cancer risk by 78 percent" or "dried fruit warning: prunes and pears found to contain high levels of acrylamide chemicals" (news retrieved from the website www.naturalnews.com, accessed February, 2015).

The World Health Organization (WHO) and the Food and Agriculture Organisation set up an international network of researchers with the mission to study acrylamide in food, and the HEATOX (Heat-Generated Food Toxicants, Identification, Characterisation and Risk Minimisation) project was started. The project allowed researchers and consumers to have a much better understanding of how acrylamide is formed, its effect on health, and importantly, how different methods of preparing and cooking food affect the levels of acrylamide in the final product.

When discussing the risks associated with acrylamide, it is important to bear in mind the fact that people have been heating starchy foods for centuries, and the food group where acrylamide presents higher levels forms an important part of a healthy diet.

On 3 May 2007 the Commission adopted a Recommendation on the monitoring of acrylamide levels in food. This recommendation has been extended by Commission Recommendation 2010/307/EU of 2 June 2010.

On 10 January 2011 the Commission adopted a Recommendation on investigations into the levels of acrylamide in food. Member States are recommended to carry out investigations in cases where the levels of acrylamide in a foodstuff, tested in the monitoring exercise, exceeds certain acrylamide indicative values. The Member States were

recommended to report the results back to the Commission who assessed the situation by December 2012. Acrylamide data collected from EFSA (European Food Safety Agency) and published a report in 2014 with the results of four years of monitoring (2007 to 2010). In this report, EFSA's Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) assessed the toxicity of acrylamide for humans and updated its estimate of consumer exposure through the diet (EFSA, 2013).

The food and drink industry (FoodDrinkEurope, FDE), the national authorities and the European Commission, developed a "toolbox" with instructions to lower the levels of acrylamide in food for industry and home-cooking. The toolbox was divided into specific brochures designed to help food business operators to implement the recommendations that are relevant for their sector. These brochures are available in 22 Community languages.

1.2- Organization and objectives of the dissertation

The present dissertation is organized into 6 Chapters. In Chapter 1 a review of the state of art about acrylamide is presented including properties, toxicity, mechanisms of formation, mitigation strategies and analytical methodologies.

In Chapter 2 are presented the analytical methodologies developed to quantify acrylamide in food. Chapter 3 presents the results related to acrylamide in coffee and the influence of variety and processing on acrylamide formation. Chapter 4 is dedicated to mitigation strategies to reduce acrylamide formation during home made cooking. In Chapter 5 is discussed the exposureto acrylamide of the population of Porto. And finally in Chapter 6 are presented the main conclusions of the work.

CHAPTER 1

State of the Art Regarding Acrylamide Toxicity, Formation, Mitigation and Analysis

2. Acrylamide properties, toxicity and exposure

2.1. Chemical Properties of Acrylamide

Acrylamide is an industrial chemical, synthesized for the first time in 1893 and commercially available in 1954. Acrylamide monomer is used mainly in the production of polyacrylamides with widely different physical and chemical properties, which are used in water and waste-water treatment, crude oil production processes, paper and pulp processing, mineral processing, concrete processing, cosmetic additives, soil and sand treatment, coating applications, textile processing and other miscellaneous (Smith and Oehme, 1991).

Acrylamide, whose official chemical name is 2-propenamide, with the molecular formula C_3H_5NO and molecular weight of 71.08, is a white solid, odorless, crystalline at room temperature. Polymerizes rapidly and exothermically at temperatures above its melting point (81.0 to 84.5 ° C), in concentrated solutions or in an acid medium is exposed to ultraviolet (Shirshin and Kazantsev 2004). It is highly soluble in water and polar organic solvents such as methanol, ethanol, dimethyl ether and acetone, insoluble in benzene and heptane. Slowly sublimes at room temperature (Girma et al., 2005).Approximately 90% of the produced acrylamide is used in polyacrylamide synthesis of 9% is used as a chemical intermediate (for example in the formation of N-butoxiacrilamida and N-metoxiacrilamida) and 1% for various uses. Polyacrylamide, unlike acrylamide, is not toxic. However, in each of these uses, the final product always remains a residual amount of the unpolymerized acrylamide which may constitute a risk, especially with regard to drinking water.Acrylamide can complex with transition metals which can be used in the production of water-soluble polymers and copolymers with many commercial and scientific applications (Girma et al., 2005).

2.2. Exposure Assessment of Consumers to Acrylamide

Acrylamide may be released to the environment during production and use of polyacrylamides in water treatment. Residual monomer in the polyacrylamide is the main source of drinking water contamination by acrylamide. Acrylamide is rarely found in the atmosphere due to its low vapor pressure and high water solubility, and is not expected to be an air contaminant (WHO 2003). Acrylamide is not considered strongly persistent in the

environment and is expected to be highly mobile in soil and water due to its high susceptibility to biodegradation in both media. It is not expected to significantly bioconcentrate in aquatic organisms due to its great water solubility and its ability to be degraded by microorganisms (Buranasilp and Charoenpanich, 2011; EPA 2006; WHO 2003). Enzyme-catalyzed hydrolysis is the dominant mechanism for removal of acrylamide from soils (ASTDR, 2012; WHO 2003).

General population exposure to acrylamide can usually occur via inhalation of tobacco smoke, including passive smoke, due to the presence of the compound in the smoke (Schumacher et al. 1977, Hagmar et al., 2005, Schettgen et al. 2004b). Exposure can also result from ingestion of drinking water containing residual monomer used in water treatment (EPA 2006; WHO 2003; Sorgel et al. 2002, van Dijk-Looijaard and van Genderen 2000).

Since 2002, after the discovery of acrylamide in foodstuff, it is known that food contributes significantly to the overall exposure of the general population to acrylamide. Short-term exposure was estimated at 1 μg/kg bw day to average adult consumers and up to 4 μg/kg bw day for consumers with high dietary exposure (98th percentile) (JECFA, 2010). Long-term intake estimates ranged from 0.3 to 0.8 μg/kgbw/ day for the adult population (WHO, 2003). Tareke et al. (2002) concluded that acrylamide levels found in heated foods could result in a daily intake of a few tens of micrograms. Exposure in children is expected to be 2– 3 times that of adults, on a body weight basis (WHO, 2003). The median acrylamide exposure in children was determined to be 0.54 μg/kg bw/day, mainly resulting from dietary sources (Heudorf et al. 2009). Children can also inhale acrylamide via passive smoke (Hagmar et al., 2005). After exposure, acrylamide is widely dispersed by body fluids, and can also cross the placental barrier, exposing the fetus to high levels of acrylamide (Sorgel et al. 2002, WHO 2003). According to Sörgel et al. (2002), acrylamide can also be transferred via breast milk when mothers consume foods with high acrylamide concentrations. The same authors calculated that the ingestion of 500 mL of breast milk per day can result in up to 10 μg of acrylamide transferred to the baby. Women who eat food with lower amounts of the compound may still transfer 2 μg of acrylamide to the baby. For these two assumptions, the doses for a 3 kg child would be 3.3 and 0.66 μg/kg, respectively (Sorgel et al. 2002).

Acrylamide is found in many common staple foods derived from potatoes and cereals (Tareke et al., 2002; Friedman et al., 2008; EFSA, 2013). In Figure 2.1 is presented acrylamide levels found in different foods across the European Union (EFSA, 2010).

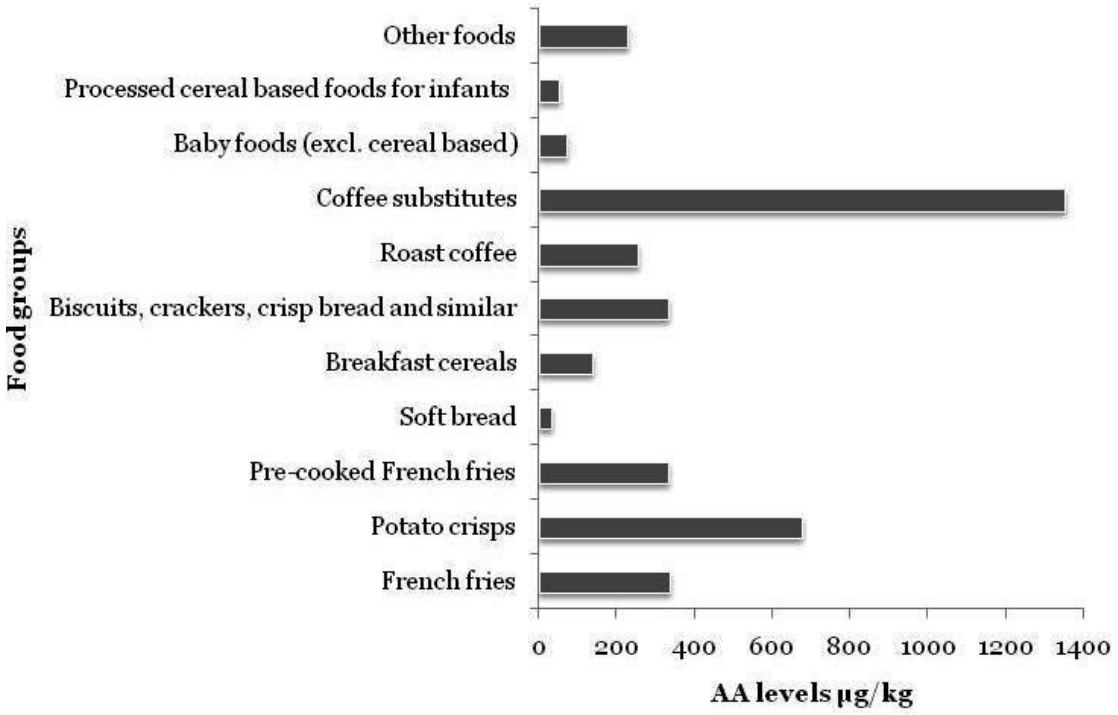

Figure 2.1- Acrylamide levels in selected food groups from Europe (Adapted from EFSA, 2010)

Acrylamide sources vary according to the country that is being considered, depending on local consumption habits and used processing methods (Dybing et al., 2005; Freisling et al., 2013; EFSA, 2013).

In Figure 2.2 the food groups responsible for the highest acrylamide contribution are presented for the general population in 27 centres in 10 European countries that participated in the EPIC (European Prospective Investigation into Cancer) study (Freisling et al., 2013).

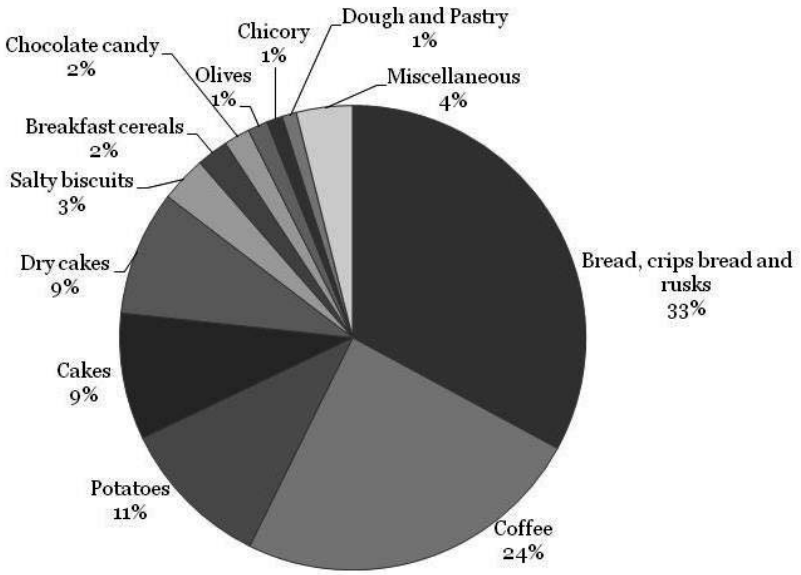

Figure 2. 2- Contribution of different food groups to acrylamide intake by the general population enrolled in the EPIC study. Miscellaneous include: nuts and seeds, fish, confectionary (non chocolate), meat, fruit, dairy products, flour flakes, condiments, soups and other source with a contribution percentage inferior to 1 %. (Adapted from: Freisling et al., 2013)

According to this study, bread and coffee are the most important sources of acrylamide intake, followed by potato products and cakes.

Recently EFSA report about acrylamide in food (2013), was disclosed for public consultation and the contribution to acrylamide dietary exposure was assessed for ten food groups and 6 food subgroups for different food surveys and age groups. The acrylamide exposure patterns were presented for infants, toddlers, children and adolescents and adults, elderly and very elderly according to European surveys collected and analyzed by EFSA Consulting Panel. In table 1 the maximum percentage intakes per food group and per age presented in the different surveys analysed by EFSA (2013) are summarized.

Taking into account these results it is clear that children and adults have different exposure patterns. For adults, coffee and fried potatoes are important contributors to acrylamide intake with values up to 33% and 49%, respectively. For children and adolescents, cereal-based and potato-based products are the main sources of the compound with values up to 51 and 60%, respectively. Although bread presents low amounts of acrylamide (Figure 2.1) its contribution to acrylamide intake is around 22% for adults due to its high consumption in a typical diet.

Table 2.1. Maximum contribution in % to acrylamide intake per food group and per age reported by EFSA (2013)

Food groups and subgroups	Infants	Toddlers	Other children and addolescents	Adults, elderly and very elderly
Potato fried products	< 5	5	51	49
Potato crisps and snacks	< 5	< 5	11	< 6.6
Soft bread	7.2	> 25	> 25	22
Breakfast cereals	17	21	< 10	11
Biscuits, crackers, crisp bread	20	> 25	> 25	19
Coffee		< 10	< 10	33
Baby foods, other than processed cereal-base	60	< 10	< 10	< 5
Processed cereal-based baby foods	31	14	< 5	< 5
Porridge	< 5	< 10	< 5	11
Cake and pastry	< 5	< 10	15	13
Savoury snacks other than potato-based	< 5	< 10	< 10	< 5
Other products based on cereals	29	> 25	> 25	21
Other products based on potatoes	50	> 25	> 25	31
Other products based on cocoa	< 5	< 10	< 10	< 5
Other products not based on cereals, potatoes and cocoa	< 5	< 10	< 10	< 6.6

Similar results were also reported by other studies (Figure 2.3). Friedman and Levin (2008) compared the percentage that different food groups contributed to acrylamide intake in USA and several European countries. Clearly, fried potato products contribute the most to the compound uptake in all age groups, followed by coffee and bread for adults and cereal-based products for children.

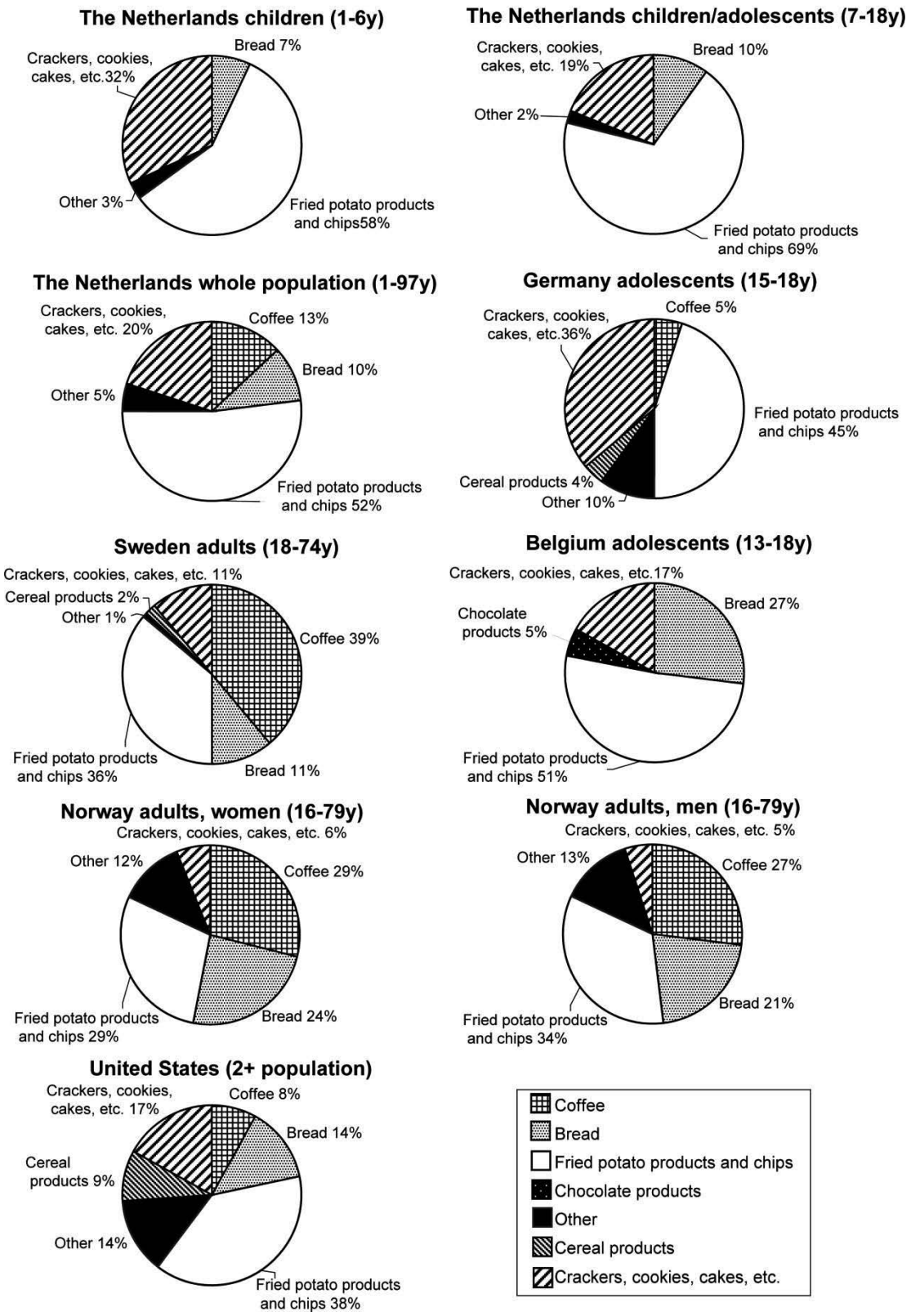

Figure 2.3- Contribution of different food groups to acrylamide intake. Foods were added into larger groups to permit comparisons between different cultures. Crackers, spice biscuits, biscuits, Dutch spiced cakes, cookies/biscuits/wafers, prepared toast, crisp bread, and crisp bread/thin unleavened bread were all combined into just one food category.Other categories include other snacks, special snacks, roasted nuts, and popcorn. Reprinted from Friedman and Levin, 2008.

Several international groups have estimated the dietary exposure to acrylamide being the highest reported by JECFA, which concluded in 2005 that acrylamide mean dietary exposure estimates were 1 μg/kg b.w. per day, and 4 μg/kg b.w. per day for a consumer at a high percentile of the distribution (JECFA, 2010). These estimates also included children. Recently, exposure assessments also revealed that infants, toddlers and other children were the most exposed groups with mean ranges from 0.6 to 1.9 μg/kg b.w. per day, with the 95th percentile from 1.4 to 3.4 μg/kg b.w. per day depending on the survey and age group. Adolescents, adults, elderly and very elderly had mean exposure estimates ranging from 0.3 to 0.9 μg/kg b.w. per day, and the 95th percentile estimates from 0.5 to 2.0 μg/kg b.w. per day depending also on the survey and age group (EFSA, 2013).

2.3. Metabolic Pathways and Toxicity of Acrylamide in Humans

Acrylamide is a probable carcinogen in humans, with the potential to cause nervous system damage, weakness, and incoordination of the legs following short-term exposure to high levels. Long-term effects of acrylamide include nervous system damage, paralysis, and cancer (Arisetto et al. 2007; EPA 2006, ASTDR, 2012).

Recently, EFSA delivered a scientific opinion on acrylamide (AA) in food. In this latest report, 43 419 analytical results obtained from food commodities collected and analysed since 2010 were presented. The highest levels of AA were found in coffee and coffee substitutes, followed by potato crisps and snacks and Potato fried products. Mean and 95th percentile dietary AA exposures crosswise surveys and age groups were estimated to be 0.3 to 1.9 μg/kg body weight (bw) per day and 0.6 to 3.4 μg/kg bw per day, respectively. Home-cooking preparations can have a substantial impact on human dietary AA exposure. Taking into account the toxic effects reported to animals, the consulting Panel concluded that the current levels of dietary exposure to AA are not of concern with respect to non-neoplastic effects. However, although the human studies have not demonstrated AA to be a human carcinogen, the margins of exposure (MOEs) across dietary surveys and age groups indicate a concern with respect to neoplastic effects (EFSA, 2013).

The estimation of dietary AA intake is difficult to compare across populations because of the different methodologies used to calculate intake values. Intake levels depend heavily on the thermal processing of specific types of foods and preparation methods vary between populations and regional habits. By using open-ended 24-h dietary recalls (24-HDR) it is possible to collect sufficient descriptive details about the food sources of AA and compare measurements in multi-center studies because procedures such as administration

and structure of interviews, food description and quantification, or quality controls can be standardized across centers. In the European Prospective Investigation into Cancer and Nutrition (EPIC) calibration study, almost 37 000 participants randomly selected in 27 regions of 10 European countries participated in standardized 24-HDR. Besides, a harmonized database of AA levels in foods was prepared, which allows a reliable comparison and interpretation of AA intake between these regions or countries. Although the studied populations of the EPIC study are not nationally representative of the European general population, the results from this study permits to identify important differences in AA intake across Europe and relate it to lifestyle characteristics like alcohol consumption, smoking status, physical activity, body mass index (BMI), and education that are relevant for dietary policy making (Freisling et al., 2013).

Recently a critical review on methods for assessing dietary AA exposure and its effects on health in humans was published (Riboldi et al., 2014). The authors highlight the necessity to conduct wide-scale epidemiological studies, preferably with prospective cohorts, but more importantly is to developed high-quality methods for accurate evaluation of dietary AA exposure and that wide databases are constructed and validated. Cancer risk due to acrylamide intake is still unclear, but improvements in measurement methods can optimize consumption estimates (Riboldi et al., 2014).

2.4. Distribution

Exposure to acrylamide can occur via ingestion, dermal contact, inhalation, and intraperitoneal routes (Sörgel et al. 2002). Once in the body, acrylamide is widely dispersed by body fluids, even crossing the placental barrier (WHO 2003).

Studies about distribution of acrylamide in human tissues is scarce, but those existent on several animal indicate that, following absorption, radioactivity from radiolabeled acrylamide is widely distributed in the body, with no specific accumulation in any tissues other than red blood cells (Barber et al. 2001). Exposure of pregnant animals to acrylamide indicates that acrylamide and/or its metabolites readily cross the placenta and are distributed within the developing fetus in a manner similar to that of the pregnant mother (Ferguson et al. 2010). Recently, a study designed to assess hemoglobin adduct levels of acrylamide in blood samples of pregnant women and umbilical cord blood of newborns, found that the concentration in umbilical cord blood was approximately 50% of that found in the blood of the mother, indicating that acrylamide readily passes from mother to developing fetus (Schettgen et al. 2004a).

2.5. Metabolism

The metabolic pathways for acrylamide in a living organism is widely studied in rats and mice, and several studies indicate that acrylamide is rapidly metabolized and excreted predominantly in the urine as metabolites (Sumner et al. 1992, 1999, 2003; Twaddle et al. 2004). Metabolism is assumed to take place primarily in the liver.

Figure 2.4 represents a metabolic scheme for acrylamide (adapted from ATSDR (2012) and based on reports from Calleman (1996), IARC (1994), and Sumner et al. (1992, 1999). According to the metabolic scheme, acrylamide reacts with glutathione to form a glutathione conjugate, which is further metabolized to N-acetyl-S-(3-amino-3-oxopropyl)cysteine or S-(3-amino- 3-oxopropyl)cysteine. Another initial step, catalyzed by the enzyme CYP2E1, involves oxidation of acrylamide to its epoxide derivative, glycidamide. Glycidamide can also react with glutathione to form conjugates that are further metabolized to N-acetyl-S-(3-amino-2-hydroxy-3-oxopropyl)cysteine or N-acetyl-S-(1-carbamoyl-2-hydroxyethyl)cysteine. Glycidamide may also undergo hydrolysis, perhaps catalyzed by epoxide hydrolases, leading to the formation of 2,3-dihydroxypropionamide and 2,3 dihydroxypropionic acid. Both acrylamide and glycidamide react with nucleophilic sites in macromolecules (including hemoglobin and DNA) in Michael-type additions (Bergmark et al. 1991, 1993).

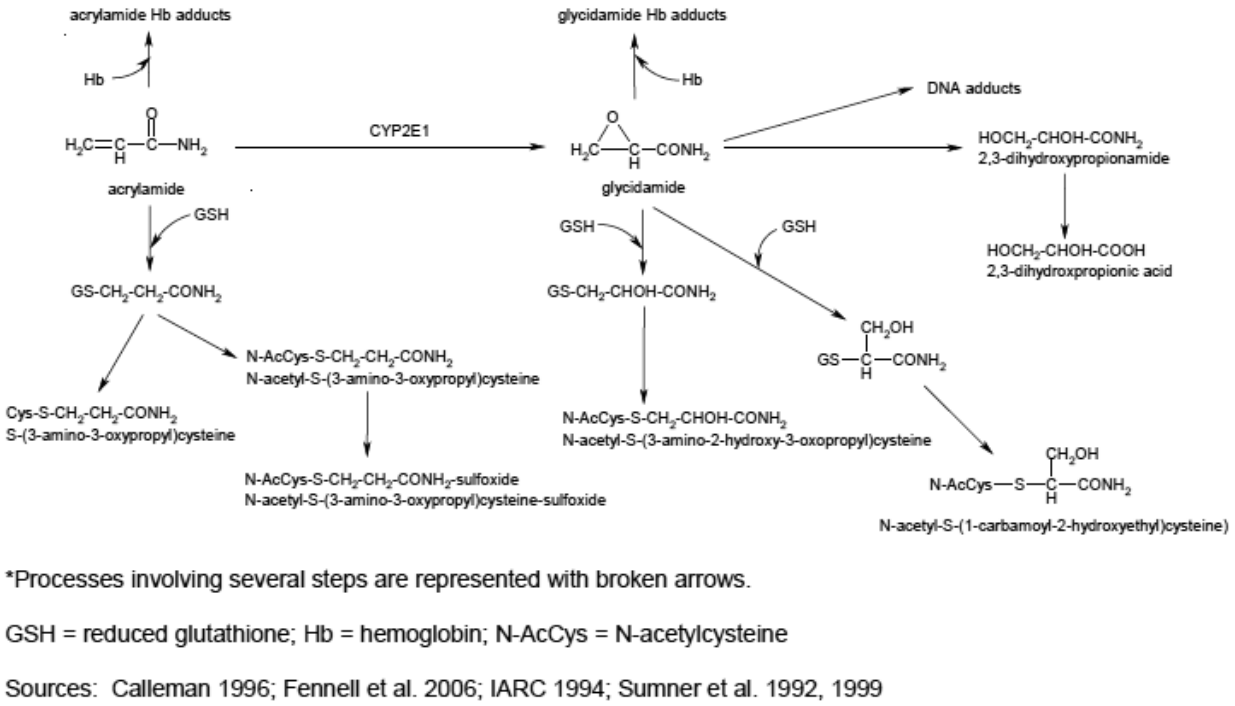

Figure 2.4. Metabolic scheme for acrylamide and its metabolite glicidamide in rats and mice. Adapted from ATSDR (2012).

Acrylamide metabolism in the human body has also been investigated in controlled studies (Boettcher et al. 2006a; Fennell et al. 2005, 2006; Fuhr et al. 2006). Fennell et al. evaluated metabolism, hemoglobin adduct formation (Fennell et al. 2005) and kinetics of elimination of urinary metabolites (Fennell et al. 2006) following oral and dermal administration of [1,2,3-$^{13}C_3$)-acrylamide and/or [2,3-^{14}C]-acrylamide to 24 adult male volunteers. All volunteers were aspermic (i.e., clinically sterile because of the potentially adverse effects of acrylamide on sperm), and had not used tobacco for the prior 6 months. The volunteers health was continually controlled. Acrylamide was administered in aqueous solutions (single dose of 0.5, 1, or 3 mg/kg) to the volunteers. Metabolism of the administered acrylamide was investigated by ^{13}C nuclear magnetic resonance (NMR) spectroscopy (Fennell et al. 2005) and by liquid chromatography-tandem mass spectroscopy (Fennell et al. 2006). Urinary metabolites constituted approximately 40% after a 3 mg/kg oral dose of acrylamide and 4.5% of a 3 mg/kg/day dermal dose for 3 consecutive days (Fennell et al. 2006). These findings demonstrate that orally-administered acrylamide is rapidly and readily absorbed by the gastrointestinal tract. Approximately 86% of the recovered urinary metabolites were derived from glutathione conjugation and excreted as N-acetyl-S-(3-amino-3-oxopropyl)cysteine and its S-oxide. Glycidamide, glyceramide (2,3-dihydroxypropionamide), and low levels of N-acetyl-S-(3-amino-2-

hydroxy-3-oxopropyl)cysteine were also detected in urine (Fennell et al. 2005). It seems that the main pathway of metabolism in humans is via direct glutathione conjugation, forming N-acetyl-S-(3-amino-3-oxopropyl)cysteine and its S-oxide (Fennell et al. 2006). Epoxidation to glycidamide was the other important pathway, with glyceramide formed as a major metabolite in humans. Glycidamide however, was detected in low amounts. The glutathione conjugation of glycidamide, which is a major pathway in rodents, appeared to occur at very low levels in humans. Metabolism via glycidamide in humans was approximately 12% of the total urinary metabolites. This is considerably lower than the amount of glycidamide-derived metabolites reported for oral administration of acrylamide in rats (28% at 50 mg/kg, [Sumner et al. 2003]) and in mice (59% at 50 mg/kg [Sumner et al. 1992]). Fennell et al. (2005) study also provided data on the amount of hemoglobin adducts derived from acrylamide and glycidamide following administration of a defined dose of acrylamide to the volunteers. Fennell et al. (2005) calculated the expected amount of adduct that would accumulate in men from continuous exposure, based on the amount of adduct formed/day of exposure, and from the lifespan of the erythrocyte. Oral intake of 1 μg/kg acrylamide/day for the lifespan of the erythrocyte (120 days) was estimated to result in the accumulation of adducts to 63 fmol/mg globin. Daily dermal exposure to 1 μg/kg acrylamide for the lifespan of the erythrocyte (120 days) would result in the accumulation of adducts to 10.8 fmol/mg globin.

Boettcher et al. (2006a) investigated human metabolism to mercapturic acid (MA) metabolites of acrylamide (AA) AAMA and its oxidative metabolite glycidamide (GA) GAMA in a healthy male volunteer who received a single dose of about 1 mg deuterium-labeled (d(3)) acrylamide, representing 13 μg/kg body weight, in drinking water. Urine samples before exposure and within the next 46 hours were analyzed for d(3)-AAMA and d(3)-GAMA using liquid chromatography-mass spectrometry (LC-MS). Total recovery in urine after 24 hours were about 51%, as the sum of AAMA and GAMA, which provides additional demonstration of the rapid and extensive absorption of ingested acrylamide and was similar to recoveries in rats (53–66%) given a feeding dose of 0.1 mg/kg (Doerge et al. 2007). After 2 days, AAMA accounted for 52% of the total acrylamide dose, and was the major metabolite of acrylamide in humans. GAMA accounted for 5%, and appeared as a minor metabolite of acrylamide. A urinary ratio of 0.1 was observed for GAMA/AAMA compared to previously reported values of 0.2 for rats and 0.5 for mice (Doerge et al. 2005b, 2005c). The authors concluded that the metabolic fate of acrylamide in humans was more similar to that in rats than in mice.

Fuhr et al. (2006) evaluated the urinary levels of acrylamide, AAMA, glycidamide, and GAMA (using LC-MS) in six young healthy volunteers after the consumption of a meal

containing 0.94 mg of acrylamide. Urine was collected up to 72 hours from the exposure. No glycidamide was found in urine. The authors identified the presence of unchanged acrylamide, AAMA, and GAMA that accounted for urinary excretion of approximately 4.5, 50, and 6% of the dose, respectively. Overall, approximately 60% of the dose was recovered in the urine. These results indicate that most of ingested acrylamide with food is absorbed in humans. Conjugation with glutathione exceeded the formation of the reactive metabolite glycidamide.

Hemoglobin adducts of acrylamide and glycidamide and urinary metabolites have been used as biomarkers of exposure to acrylamide (Bergmark 1997; Bergmark et al. 1993; Boettcher et al. 2005; Calleman et al. 1994).

Boettcher et al. (2006b) also tested the influence of acrylamide rich diet on the excretion of urinary mercapturic acid metabolites derived from acrylamide in three healthy volunteers who fasted for 48 hours. Urinary acrylamide mercapturic acid metabolites were considerably reduced after 48 hours of fasting, with levels even lower than the median level in nonsmokers. These results indicate that acrylamide in the diet is the main source of environmental acrylamide exposure in humans, apart from smoking.

Bjellaas et al. (2007a) reported urinary mercapturic acid derivatives of acrylamide in a clinical study comprising 53 subjects. Median intakes (range) of acrylamide were estimated based on 24-hour dietary recall as 21 (13–178) μg for nonsmokers and 26 (12–67) μg for smokers. The average dietary exposure to acrylamide was estimated to be 0.47 (range 0.17–1.16) μg/kg body weight per day. The median (range) total excretion of acrylamide in urine during 24 hours was 16 (7–47) μg for nonsmokers and 74 (38–106) μg for smokers. The authors found a correlation between urinary excretion of acrylamide metabolites and intake of aspartic acid, protein, starch, and coffee from the diet.

Heudorf et al. (2009) reported median levels of 36 μg AAMA/L and 13 μg GAMA/L in the urine of 110 children (63 boys and 47 girls; 5–6 years of age). Children who consumed higher quantities of French fries regularly had significantly higher urinary levels of acrylamide metabolites. Based on the urinary levels of AAMA and GAMA, mean estimated acrylamide dietary intakes were 1.13 μg/kg/day and 0.81 μg/kg/day for children. The ratio GAMA/AAMA was approximately 0.4, which is 2-times higher than 0.1 ratio observed by Boettcher et al. (2006a) in adults. Based on this finding, Heudorf et al. (2009) suggest that acrylamide may undergo oxidative metabolism to a greater extent in children than adults.

2.6. Elimination and Excretion

The studies presented previously suggests that in human and animals, urinary excretion of acrylamide metabolites is the primary route of elimination of acrylamide absorbed after exposure (Barber et al. 2001a; Boettcher et al. 2006a; Doerge et al. 2007; Fennell et al. 2005, 2006; Fuhr et al. 2006; Sumner et al. 1992, 1999, 2003).

2.7. Biomarkers Used to Characterize Effects Caused by Acrylamide

Glycidamide-derived DNA adduct formation has been quantified in rats and mice exposed to acrylamide (Doerge et al. 2005a; Gamboa da Costa et al. 2003). There are no other known biomarkers of effect that are considered to be specific to acrylamide exposure. It should be noted however, that hemoglobin adducts of N-methylolacrylamide are indistinguishable from hemoglobin adducts of acrylamide (Fennell et al. 2003; Paulsson et al. 2002).

Results of epidemiological studies support the use of hemoglobin adducts of acrylamide and/or glycidamide as biomarkers of exposure to acrylamide (Bergmark et al. 1993; Boettcher et al. 2005; Calleman et al. 1994; Fennell et al. 2005, 2006; Hagmar et al. 2001; Olesen et al. 2008). As discussed in previous paragraphs reported results of animal studies indicate similar biomarkers of exposure to acrylamide. Metabolites can be measured for assessment of recent exposure. Hemoglobin adducts provide a biomarker of exposure for longer periods.

2.8. Toxicity of acrylamide

Acrylamide and its main epoxide metabolite, glycidamide, react with various biologically significant molecules. The chemical basis for these interactions is strongly associated with the degree of electrophilicity (electron deficiency) with nucleophilic centers (unshared electrons). Due to its α,β-unsaturated structure and its capacity to undergo Michael-type additions, acrylamide react readily with nucleophiles such as the thiol groups of proteins or glutathione. Glycidamide, however, has a relatively high positive charge density, and is more capable of reacting with centers of high electronegativity, such as the purine and pyrimidine bases in DNA (Dearfield et al. 1995; Lopachin and DeCaprio 2005). Hemoglobin adduct levels provide a direct measure of the total amount of acrylamide and glycidamide, in the blood over a given period of time. Acrylamide and glycidamide readily bind to hemoglobin, but do not accumulate appreciably in any tissues. Acrylamide

metabolism is relatively rapid; both for acrylamide and glycidamide, and appears to be involved in the observable toxicity (ATSDR, 2012).

2.8.1. Neurotoxic Effects

Since the 1950s, neurotoxicity from acrylamide exposure is known and assessed through numerous animal studies. Major signs and observable symptoms include twitching, loss of balance, tremors, lethargy, and general weakness and more subtle indicators of functional deficits such as decreased rotarod performance and increased limb or foot splay. Evidence of degenerative lesions in peripheral nerve fibers, as observed by light and electron microscopy, have been detected at oral doses lower than those provoking clinical signs (ATSDR, 2012).

Some of the neurological effects observed in animals can be derived by administration of acrylamide or glycidamide. Observations of neurological symptoms in acrylamide-exposed humans include muscle weakness and other signs of functional deterioration (Calleman et al. 1994; Hagmar et al. 2001; He et al. 1989).

Specific mechanisms of acrylamide neurotoxicity are not yet elucidated. However, there are currently two major hypotheses; first, acrylamide-induced disruption of fast axonal transport (Sickles et al. 2002) and second, acrylamide-induced disruption of nitric oxide signaling at nerve terminals (LoPachin and Barber 2006; LoPachin et al. 2008). More recently, the work by Zhu et al. (2008) provided some support to a hypothetical mode of action whereby acrylamide exposure results in reactive oxygen species increase, with consequent damage to cellular macromolecules, and subsequent degeneration of neural tissues.

Most evidence of neurological effects in humans derives from occupational exposures,that predominantly involved inhalation and dermal routes. Available information regarding the neurological effects of oral exposure in humans is limited to two case reports: first, the persistent peripheral neuropathy in a subject who intentionally ingested 18 g of acrylamide crystals (ATSDR, 2012) and second, signs of central and peripheral neurological deficits in an entire family exposed (likely via oral and dermal routes) to acrylamide in well water at a concentration of 400 ppm (Igisu and Matsuoka 2002). Epidemiologic studies designed to evaluate noncancer health effects in groups of orally-exposed subjects have not been conducted.

2.8.2.Reproductive Effects.

Mechanisms of acrylamide-induced reproductive toxicity are poorly understood. There is some indication that mutagenic effects on male germ cells may play a significant role. Available data provide suggestions that acrylamide-induced male dominant lethal mutations may involve clastogenic events from binding of acrylamide and/or glycidamide to spermatid protamines or spindle fiber proteins and/or direct alkylation of DNA by glycidamide (Adler et al. 2000; Perrault 2003; Tyl and Friedman 2003; Tyl et al. , 2000a, 2000b). Tyl and Friedman (2003) also suggested that adverse effects on mounting, sperm motility, and intromission could be related to distal axonopathy resulting from binding of acrylamide to motor proteins. No known studies regarding acrylamide-induced reproductive effects in humans exist.

2.8.3. Developmental Effects

No studies were found regarding acrylamide-induced developmental effects in humans. Several reports are available in which developmental toxicity was assessed in the offspring of rat or mouse exposed to acrylamide via daily feed during gestation and/or lactation (Field et al. 1990; Friedman et al. 1999; Takahashi et al. 2009; Wise et al. 1995). Body weight decreases and decreased auditory startle response were noted in offspring of female rats exposed to 5 and 15 mg/kg-day, respectively, on gestation days 6–10 (Wise et al. 1995). No exposure-related fetal malformations or variations (gross, visceral, or skeletal) were found in offspring of rats and mice exposed to high doses of acrylamide (Field et al. 1990).

2.8.4. Carcinogenic Effects

Specific mechanisms whereby acrylamide induces tumors in laboratory animals are not understood at present. However, the weight of evidence supports a mutagenic mode of action (Besaratinia and Pfeiffer 2004, 2007; Dearfield et al. 1995). Evidence includes findings that: acrylamide is metabolized by CYP2E1 to DNA-reactive glycidamide; acrylamide and glycidamide induce mutations in lymphocyte HPRT and liver cII cells; DNA adducts of glycidamide have been detected in tissues of all relevant tumor targets of acrylamide-and glycidamide-exposed male and female rats and mice; glycidamide is

mutagenic to bacteria and male mouse germ cells and male and female mouse somatic cells *in vivo*; acrylamide induces heritable translocations and specific locus mutations in germ cells of exposed male mice; acrylamide induces clastogenic effects in mouse lymphoma assays; and dominant lethal effects in rodents occur at subchronic oral exposure levels comparable to those associated with carcinogenic effects in chronically-exposed rats (ATSDR, 2012). These findings support a proposed mode of action whereby acrylamide is metabolized to the relatively long-lived epoxide, glycidamide, which reacts with proteins and DNA, causing mutations that persist in viable somatic cells, resulting in tumor formation. Ghanayem et al. (2005) observed significant dose-related increases in micronucleated erythrocytes and DNA damage in somatic cells (leukocytes, liver, lung) of acrylamide-treated wild-type mice, but not in CYP2E1-null mice, indicating that genetic damage in somatic cells is dependent on metabolism of acrylamide by CYP2E1.

Another hypothetical mode of action involves disruption of hormone levels or hormone signaling for acrylamide-induced tumors in hormonally-sensitive tissues including mammary gland and thyroid or tissues adjacent to hormonally sensitive tissue, such as tunica vaginalis mesothelium (Dourson et al. 2008; Haber et al. 2009; Klaunig 2008; Shipp et al. 2006).

Available epidemiology studies on increased risk of cancer from acrylamide in food include case-control studies (Lin et al. 2010; Michels et al. 2006; Mucci et al. 2003, 2004, 2005; Pelucchi et al. 2006, 2007, 2011a; Wilson et al. 2009a) and prospective cohort studies (Hogervorst et al. 2007, 2008a, 2008b, 2009a, 2009b; Larsson et al. 2009a, 2009b, 2009c, 2009d, 2009e; Mucci et al. 2006; Schouten et al. 2009; Wilson et al. 2009b, 2010). These studies provide miscellaneous results regarding possible associations between dietary acrylamide intake and selected cancer types.

2.9. Case-control Studies

No significant associations were found between self-reported consumption of foods with high (300–1,200 μg/kg) or moderate (30–299 μg/kg) acrylamide concentrations and increased risk of large bowel, kidney, or bladder cancer in a population-based case control study (692 controls and 875, 391, and 186 large bowel, kidney, and bladder cancer cases, respectively) (Augustsson et al. 1999; Mucci et al. 2003). Information on intake of various foods and nutrients was assessed by questionnaire. Mucci et al. (2005) assessed acrylamide intake of >43,000 women, including 667 breast cancer cases, who participated in the Swedish Women's Lifestyle and Health Cohort. The estimated average daily acrylamide

intake among the participants was 25.9 μg/day and was based on results of food frequency questionnaires (FFQs) and the Swedish National Food Administration database of information on acrylamide content of selected food items. After ranking the women into quintiles of estimated acrylamide intake, there was no significant increased risk of breast cancer in the higher quintiles compared to the lowest quintile. A Swedish nationwide, population-based case-control study reported a significant association between dietary acrylamide intake and risk of esophageal cancer (Lin et al. 2010). The study included 189 cases of exophageal adenocarcinoma, 262 cases of gastroexophageal junctional adenocarcinoma, 167 cases of esophageal squamous cell carcinoma, and 820 control participants. Dietary acrylamide intake was assessed by questionnaire and categorized into quartiles. For the highest quartile, the adjusted risk of all esophageal tumors combined was increased and was higher among overweight or obese patients. The association appeared to be strongest for esophageal squamous cell carcinoma, particularly among nonsmokers in the highest quartile of acrylamide exposure.

Within an integrated network of Italian and Swiss hospital-based case-control studies to investigate the relation between dietary acrylamide intake and cancers at several sites, no significant associations were found between estimated dietary acrylamide intake and cancers of the oral cavity and pharynx, esophagus, large bowel, larynx, breast, ovary, or prostate (Pelucchi et al. 2006). Dietary acrylamide intake was estimated based on results of FFQs and average content of acrylamide in foods from resources of the World Health Organization and Swiss Federal Office of Public Health. In a case-control study that included four areas of Italy, no significant association was found between total dietary acrylamide intake and renal cell cancer (Pelucchi et al. 2007). In this study, some correlation was noted for weekly white bread consumption and cancer risk but the authors indicated that the relationship between white bread consumption and renal cell cancer might be explained by a high glycemic content and consequent effect on levels of insulin-like growth factors. Another case-control study performed by Pelucchi et al. (2011) found no significant association between dietary acrylamide and pancreatic cancer.

Wilson et al. (2009a) conducted a case-control study to assess possible associations between acrylamide and prostate cancer risk using two measures of acrylamide exposure: intake from FFQs and acrylamide hemoglobin adduct levels in blood samples. Dietary data were available for 1,499 prostate cancer cases and 1,118 controls from a Cancer of the Prostate in Sweden (CAPS) population-based case-control study. Acrylamide-hemoglobin adduct levels were measured in blood samples from a subset of 170 prostate cancer cases and 161 controls. No significant association was found between acrylamide exposure (as measured by FFQ or acrylamide-hemoglobin adduct levels) and risk of prostate cancer.

Michels et al. (2006) conducted a case-control study to evaluate whether diet during preschool age affected a woman's risk of breast cancer later in life. Cases and controls were selected from participants in two prospective cohort studies, the Nurses' Health Study and the Nurses' Health Study II. Information concerning childhood diet of the nurses at ages 3–5 years was obtained from FFQs filled out by the mothers of the participants. The median year of birth of the mothers was 1914 for case mothers and 1913 for control mothers. The results indicated an increased risk of breast cancer among woman who had frequently consumed French fries at preschool age. For one additional serving of French fries per week, the risk for breast cancer adjusted for adult life breast cancer risk factors was 1.27. Consumption of whole milk was associated with a slightly decreased risk of breast cancer. Intake of none of the nutrients calculated was related to the breast cancer risk in this study. The authors noted that they did not observe a similar association of breast cancer with frequent consumption of hot dogs or ground beef, suggesting that French fry consumption was not a marker of fast food habits. The study results suggest a possible association between diet before puberty and the subsequent risk of breast cancer, but the conclusions and the study are of limited use. No information is available on cooking methods or acrylamide content in the foods being evaluated, and the ability of mothers to accurately recall preschool diets of their daughters is questionable. No significant associations were found between acrylamide-hemoglobin or glycidamide-hemoglobin adduct levels and total breast cancer in a Danish nested case-control study that examined breast cancer and acrylamide exposure using acrylamide-hemoglobin and glycidamide-hemoglobin adduct levels in red blood cells as presumed biomarkers for oral exposure to acrylamide (Olesen et al. 2008). After adjusting for confounding factors including smoking behavior, the study authors noted that a 10-fold increase in acrylamide-hemoglobin adduct levels was associated with a 1.9 times higher risk of breast cancer and a 5-fold increase (which corresponds to the range in acrylamide-hemoglobin adduct levels among nonsmokers) was associated with a 1.6 times higher risk. A significant positive association was observed between acrylamide-hemoglobin adduct level and ER+ breast cancer; a 10-fold increase in adduct level was associated with a 4.9 times increased risk in smokers and 2.7 times increased risk after adjustment for smoking. However, this study is limited by the relatively small number of subjects (374 cases and 374 controls) and uncertainty regarding extrapolation of acrylamide exposure as assessed by a few months of acrylamide-hemoglobin adduct measurements to a lifetime of exposure. Mucci et al. (2004) analyzed data from a large population-based Swedish case-control study of renal cell cancer. FFQs were used to collect information on intake of 11 food items with elevated acrylamide levels as ascertained through extensive food databases in Sweden and the United States, and quartiles of daily food and acrylamide intake were created. This study found no evidence

that food items with elevated acrylamide were associated with a higher risk of renal cell cancer risk.

2.10. Prospective Cohort Studies

Recent and ongoing prospective studies designed to evaluate possible associations between acrylamide in food and risk of cancers at various countries include cohorts from Sweden (Larsson et al. 2009a, 2009b, 2009c, 2009d; Mucci et al. 2006), the United States (Wilson et al. 2009b, 2010), and the Netherlands (Hogervorst et al. 2007, 2008a, 2008b). Most studies found no statistically significant associations between acrylamide in food and risks of cancers of the oro-hypopharynx, larynx, or thyroid gland (Schouten et al. 2009); esophagus, stomach, or pancreas (Hirvonen et al. 2010; Hogervorst et al. 2008b); colon or rectum (Hirvonen et al. 2010; Hogervorst et al. 2008b; Larsen et al. 2009c; Mucci et al. 2006); bladder or prostate (Hirvonen et al. 2010; Hogervorst et al. 2008a; Larsson et al. 2009e); lung (Hogervorst et al. 2009b); brain (Hogervorst et al. 2009a); breast (Hogervorst et al. 2007; Larsson et al. 2009d; Wilson et al. 2009b, 2010); endometrium (Hogervorst et al. 2007; Larsson et al. 2009a); ovarian epithelium (Larsson et al. 2009b), or lymphomas (Hirvonen et al. 2010). However, Wilson et al. (2010) reported increased risk for endometrial cancer for "high" acrylamide consumers among women in the Nurses' Health Study. Wilson et al. (2010) also reported a slightly increased risk for ovarian serous tumors. Hirvonen et al. (2010) reported increased risk for lung cancer in the highest quintile (compared to the lowest quintile) of dietary acrylamide intake within a cohort of 27,111 male smokers identified through the Finnish Cancer Registry without history of cancer prior to a 10.2-year follow-up period. Two prospective studies of a Dutch population reported increased risks of postmenopausal endometrial and ovarian cancer (Hogervorst et al. 2007) and renal cell cancer (Hogervorst et al. 2008a) with increasing dietary acrylamide in prospective studies of a Dutch population, but in these studies, estimations of dietary acrylamide levels in foods on the market in 1986 were based on food samples analyzed since 2001 and questionnaires did not include details regarding specifics of food preparation. Some of the tumor sites observed in animal studies (thyroid, testis, central nervous system) have not been evaluated in humans, and there are limitations in some of the study methods and cohort sizes in the prospective studies. Pelucchi et al. (2011) performed meta-analyses for various cancer end points from a number of prospective cohort and case-control studies that assessed dietary intake of acrylamide. The meta-analyses results indicated a lack of increased risk for cancers of the esophagus, colorectum, colon, rectum, breast, endometrium, ovary, prostate, bladder, and kidney. The relative risk of 1.12 was considered

to indicate that a possible association between dietary acrylamide intake and kidney cancer should not be excluded.

2.11. Final considerations

The majority of the reviewed epidemiologic studies estimates punctual exposures from the baseline FFQs assuming that the dietary acrylamide content as well as the individual exposures over time remained constant. It is necessary to take into account the number of new food items that are introduced in the market each year or new cooking practices. Factors such as seasonality, prices, sales, as well as social factors such as holidays resulting in potential changes in dietary acrylamide exposure must be taken into consideration also. Future studies about dietary acrylamide exposure assessment should be improved, including HbAA adducts analysis every 3 months, along with improved tools of dietary assessment. Until an enhanced exposure assessment method is incorporated, epidemiologic studies assessing relationship between dietary acrylamide and cancer will not have any meaningful interpretations (Virk-Baker et al., 2014). These authors also point the fact that in the reported epidemiologic studies, dietary acrylamide exposure assessment has been inadequate, potentially leading to misclassification. Besides, case-control studies have reported nearly the same values for dietary acrylamide exposures among both cancer cases and controls. For disease endpoint such as cancer, exposure assessment methods that could capture long-term exposures are highly recommended.

3. Acrylamide formation pathways

Acrylamide presence in a broad range of foods has lead to a huge amount of studies that intended to determine the formation pathway of the compound. The most accepted route of formation is the Maillard reaction that occurs between reducing sugars and amino acids (Mottram et al., 2002; Stadler et al., 2002). All the studies agree that in order to detect the presence of acrylamide, foods must be heated at temperatures higher than 120ºC, for moderately long periods of time under limited water presence. The cooking process, in particular frying or roasting at high temperatures, induces a higher degree of acrylamide formation.

Many studies reported the details in many aspects of acrylamide formation specially trying to identify intermediaries and other by-products of the reaction. As the studies proceeds, some important and direct precursors contributing to the formation of acrylamide were demonstrated as 3-aminopropionamide (Granvogl et al., 2004), decarboxylated Schiff base (Zyzak et al, 2003), decarboxylated Amadori product (Yaylayan et al., 2003) acrylic acid (Becalski et al., 2003; Stadler et al., 2004) and acrolein (Yasuhara et al.,2003).

On the other hand, other reaction routes for the formation of acrylamide in foods was being discussed in order to explain the presence of the compound matrices with low amounts of asparagine and/or reducing sugars.

In the following points is presented a non exhaustive review about the possible pathways for acrylamide formation.

3.1. The Maillard Reaction: The Asparagine Route

Mottram et al. (2002) and Stadler et al. (2002) were the first to describe that acrylamide could result from a Maillard thermal mechanism between asparagine (an acidic amino acid) and reducing sugars (glucose and fructose). Those authors conducted a series of experiments in which equimolar solutions of glucose with several amino acids (asparagine, glycine, cysteine, methionine, and glutamine) were subject to heat treatment. The authors found that in the experiments at 165 ° C and pH 5.5 only asparagine resulted in rather high amounts of acrylamide. Evidence that the carbon skeleton and the amide group from acrylamide was derived from asparagine were obtained using an isotope-labelled amino acid (^{13}C and ^{15}N), realizing that the new molecule contained more than 98% of these labelled atoms (Mottram et al., 2002; Stadler et. al, 2002;. Zyzak et al, 2003). The

asparagine route that leads to acrylamide is complex and may involve many intermediates. Mottram et. al. (2002) proposed the involvement of a chemical process known as "Strecker degradation." The schematic representation of this mechanism is shown in Figure 3.1.

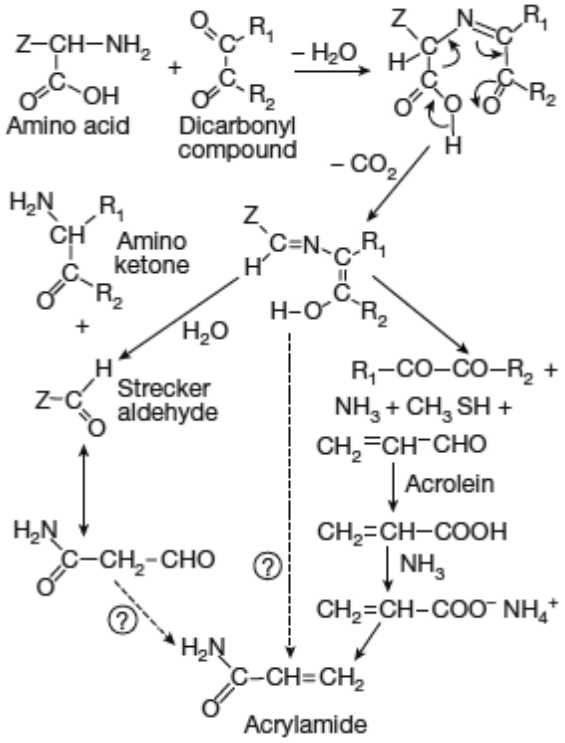

Figure 3.1. Pathway for the formation of acrylamide after the Strecker degradation of asparagine in the presence of dicarbonyl products. Reprinted from Mottram et al., 2002.

Yaylayanet. al. (2003) andZyzaket. al. (2003) presented evidence foran alternative routethat involvesdecarboxylationin the early stagesof the N-glycoside asparagineviaformation ofa Schiff base. A schemeof this mechanism isshown in Figure3.2.

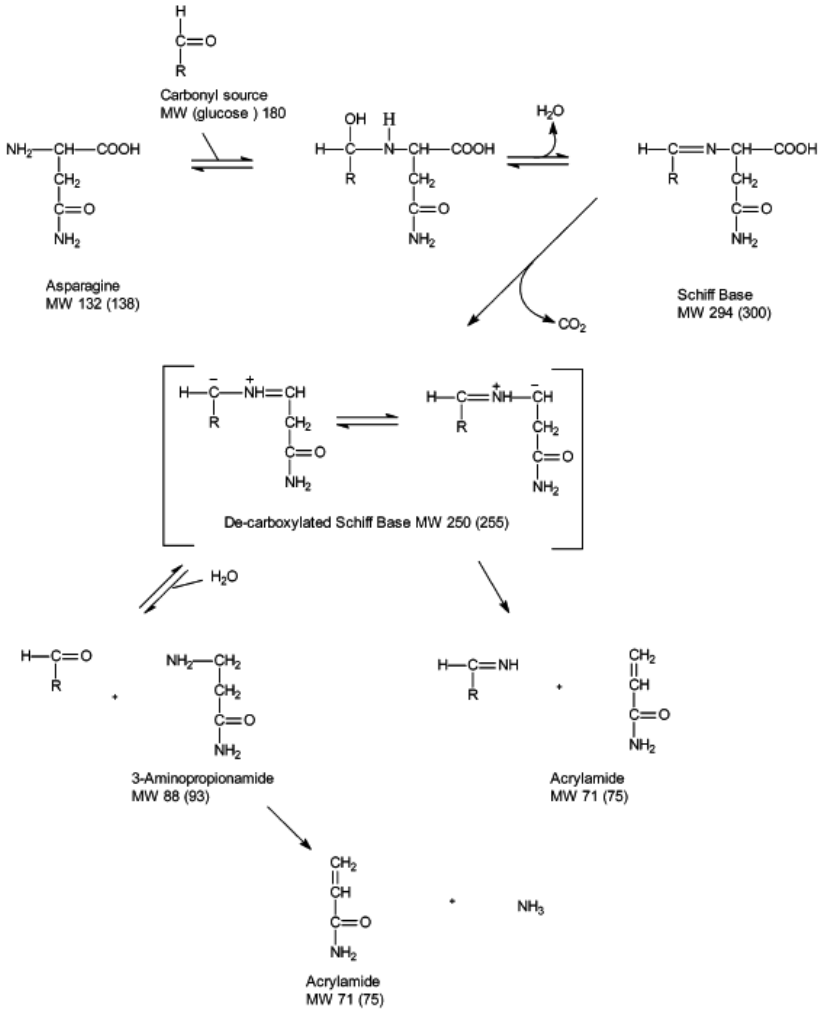

Figure 3.2. Mechanism of formation of acrylamide through decarboxylation of asparagine. (Reprinted from Zyzak et al., 2003)

The asparagine route can explain the formation of acrylamide in foods that have high amounts of asparagine and reducing sugars like potatoes and cereals and positive correlations were found between acrylamide and its precursosrs (Zhu et al., 2010).

3.2. Formation of acrylamide via the 3-Aminopropionamide pathway

Besides being a possible intermediate during the Maillard reaction (Figure 2), 3-APA can also be formed in raw foodstuffs by enzymatic decarboxylation of asparagine. It was described, that in the presence of 3-APA in food matrix, acrylamide can be formed easily, even under aqueous conditions during heating. In this pathway, the Maillard reaction is not taking place and acrylamide can be formed even if there are no reducing sugars in the system

via direct deamination of 3-aminopropionamide (Figure 3.3). This can explain the fact why acrylamide is found in raw materials containing low amounts of asparagine (Granvogl et al., 2004).

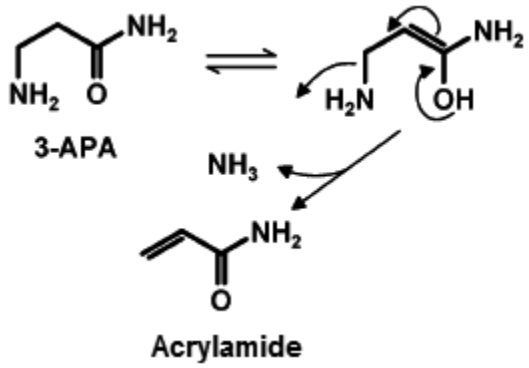

Figure 3.3. Formation of acrylamide through direct deamination of 3-APA

Zamora et al. (2009) made some more studies regarding the effect of 3-aminopropionamide and 3-(alkylamino)propionamides (aminopropionamides that can be naturally present in food) on acrylamide formation. They concluded that, there are possibly diverse pathways by which 3-aminopropionamide and 3-(alkylamino)propionamides are converted into acrylamide depending on water activity and presence of other carbonyl compounds.

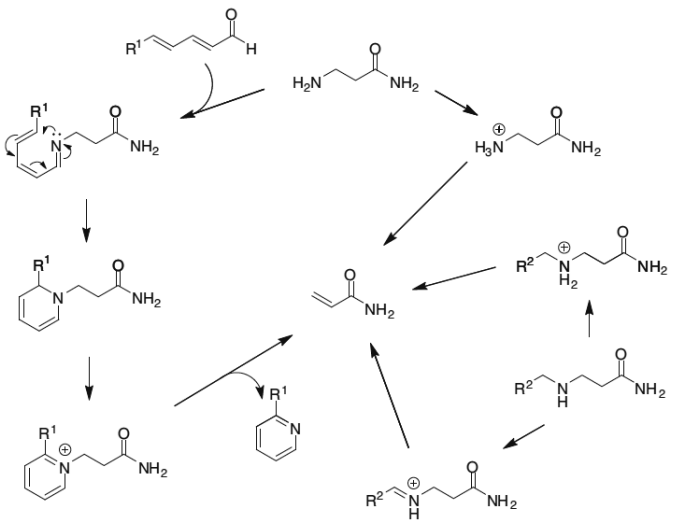

Figure 3.4. Possible pathways for the degradation of 3-aminopropionamide and 3-(benzylamino)propionamide. Reprinted from Zamora et al. 2009.

The authors proposed pathways presented on Figure 3.4, suggest the existence of diverse competitive pathways by which 3-aminopropionamide and 3-(alkylamino) propionamides are converted into acrylamide. The authors concluded that at low water activity and high temperatures, both types of amino compounds are converted rapidly into acrylamide to high extent (40–50%) and carbonyl compounds apparently do not play a significant role, at least in relation to the amount of acrylamide produced. When water activity increases, the conversion of 3-(alkylamino) propionamides into acrylamide does not seem to change significantly. However, the amount of acrylamide produced from 3-aminopropionamide decreases and carbonyl compounds have a positive effect in the amount of acrylamide formed. Concluding, the type of precursor involved is likely to play a major role in the amount of acrylamide produced (Zamora et al, 2009).

3.3. Acrolein Pathway

Acrolein is an unsaturated aldehyde and can be produced from lipids (triglycerides) by strong heat treatment. Small amounts of acrolein can be found in some foods, such as fried foods, cooking oils and roasted coffee (ATSDR, 2007).

Yasuhara et al. (2003) have shown that acrylamide can be formed from acrolein and these authors proposed two pathways (Figure 3.5). In the first pathway, acrylic acid produced from acrolein reacts with ammonia to produce acrylamide. The second pathway involves an acrylic radical formed from hemolytic fusion of acrolein that absorbs an amine radical formed from an amino acid to yield acrylamide (Yasuhara et al., 2003). The reaction of acrolein via acrylic acid mechanism was also proposed by other research groups (Gertz and Klostermann 2002; Stadler et al. 2004; Becalski et al. 2003).

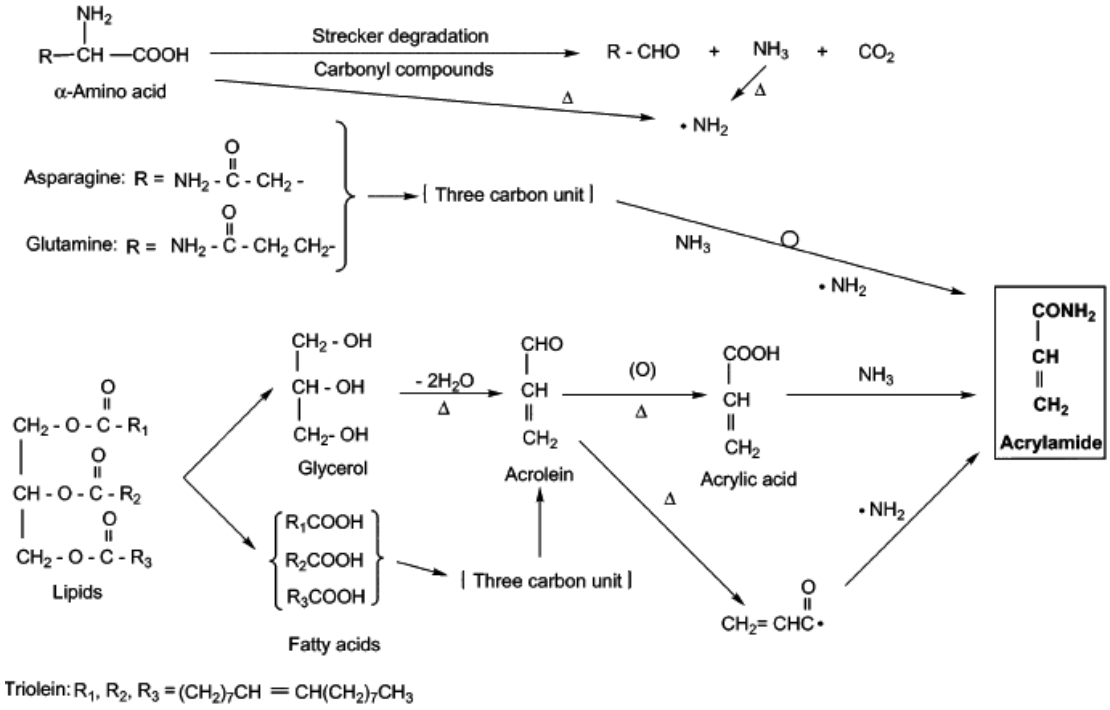

Figure 3.5. Formation of acrylamide via the acrolein pathway. (reprinted from Yasuhara et al.,2003)

3.4. Acrylic acid pathway: The contribution from different amino acids

Acrylic acid, which plays a role in the formation of acrylamide from acrolein (Yasuhara et al., 2003), can be formed by thermal decomposition of aspartic acid, carnosine and β-alanine (Stadler et al., 2004; Yaylayan et al., 2004; Yaylayan and Stadler, 2005). Some amino acids can generate acrylic acid directly during their thermal decomposition. Such amino acids require the presence of ammonia to convert acrylic acid into acrylamide. The necessary ammonia can be easily obtained in foods from is the free amino acids. Sohn and Ho (1995) have identified asparagine, glutamine, cysteine and aspartic acid as the most efficient ammonia generating amino acids under thermal treatment. However, the efficiency of the conversion of acrylic acid into acrylamide is limited by the availability of free ammonia in the vicinity of its production in the food matrix, in addition, this limitation is further compounded by the extreme volatility of ammonia at temperatures that are conducive to acrylamide formation.

The mechanism of decomposition of β-alanine to generate both reactants required for the formation of acrylamide, ammonia and acrylic acid, is shown in Figure 3.6. Pyrolysis of β-alanine alone generates mainly acrylic acid and acrylamide, indicating deamination as

a major pathway of thermal decomposition of β-alanine. The resulting acid can then interact with the available ammonia to form acrylamide.

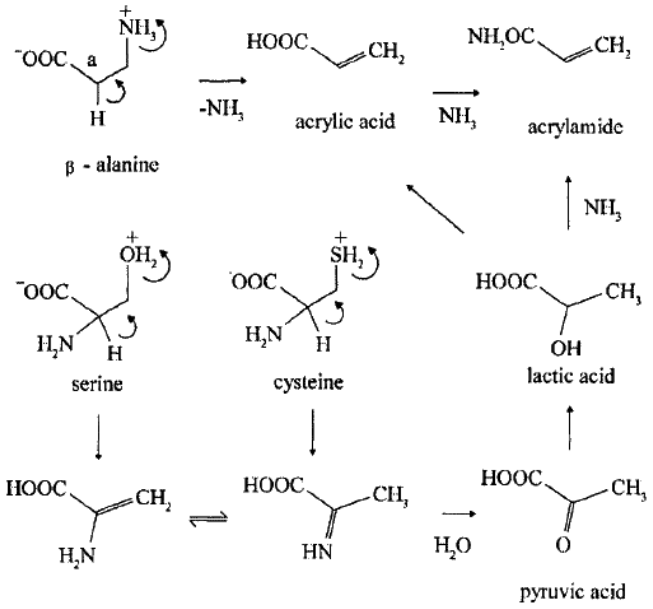

Figure 3. 6. Conversion of aminoacids β-alanine, serine and cysteine to acrylamide. (Reprinted from Yaylayan et al., 2005)

Aspartic acid, on the other hand, can also form acrylic acid and subsequently acrylamide (Stadler et al., 2003; Yaylayan et al., 2004; Becalski et al, 2003). This pathway can occur via a concerted mechanism where decarboxylation occurs simultaneously with deamination as shown in Figure 3.7.

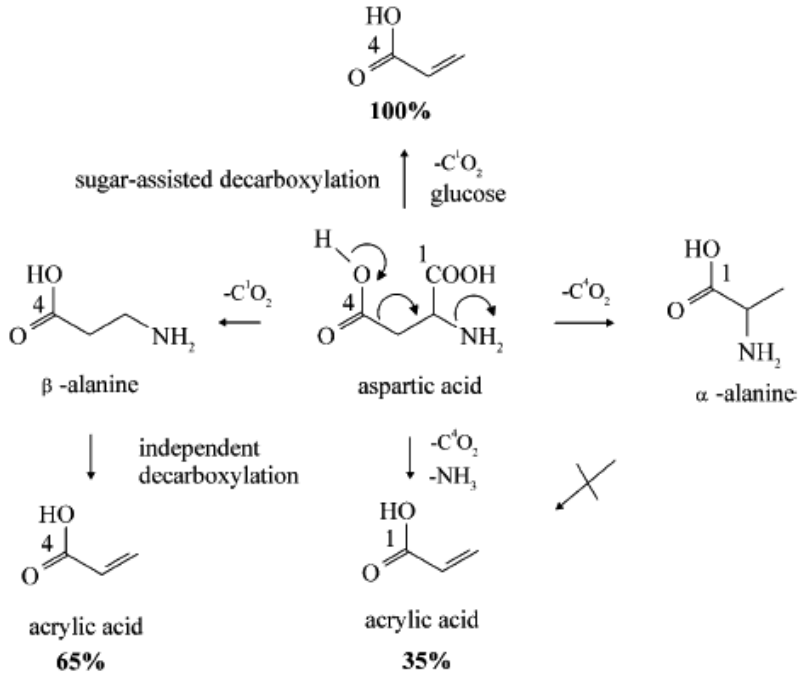

Figure 3.7. Decarboxylation pathways of aspartic acid. Reprinted from Yaylayan et al., 2005.

3.5. Formation of acrylamide from carnosine and creatine

The carnosine content in beef (33 mmol/g of fresh beef, Koutsidis et al., 2008) is comparable with that of asparagine in potatoes (15 *m*mol/g of fresh potato, Friedman, 2003), it is reasonable to assume that acrylamide should be formed in cooked meat as abundantly as in potatoes. According to Yaylayan et al. (2005) the dipeptide carnosine (N-β-alanyl-L-histidine) when pyrolyzed alone produced acrylic acid and acrylamide in amounts higher than asparagine/glucose model system. However, in the presence of glucose the amounts became comparable due to the interaction of carnosine with reducing sugars (Chen and Ho, 2002). Figure 3.8 depicts two possible pathways of formation of acrylamide from carnosine, one through hydrolysis of the peptide bond and release of β-alanine and its subsequent deamination, the second through release of 3-aminopropanamide and its deamination.

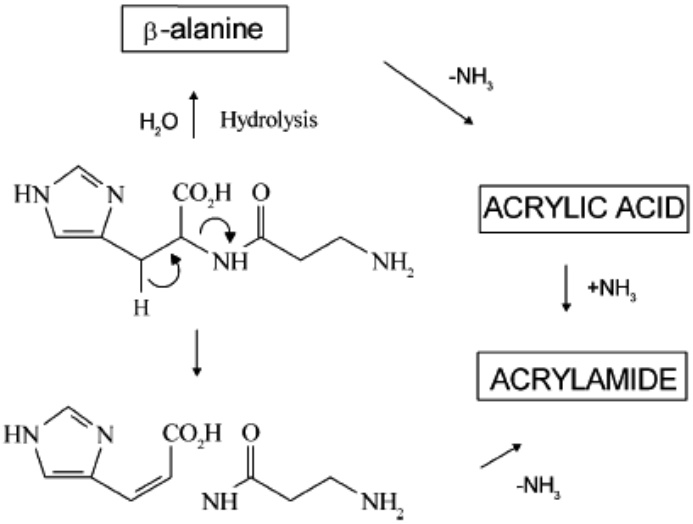

Figure 3.8. Different pathways of acrylamide formation from carnosine. Reprinted from Yaylayan et al., 2005.

However, the absence of high levels of acrylamide in meat products at the scale expected to that of potatoes (Friedman, 2003) has lead Yaylayn et al. (2005) to investigate its possible fate in meat products using carnosine containing model systems. Carnosine was reacted in the presence of lysine (a reactive amino acid) and creatine (a major constituent of meat: 40.5 mmol/kg fresh beef, Koutsidis et al., 2008) and their effect on the amounts of acrylamide and its precursor acrylic acid was calculated. Lysine did not exert any significant effect on the formation efficiencies of acrylamide and acrylic acid. Creatine on the other hand, not only significantly reduced the acrylic acid content but also gave rise to two new potentially toxic acrylamide derivatives; N-methylacrylamide and N,N-dimethylacrylamide. The decrease in acrylic acid formation can be explained by its accelerated conversion into acrylamide derivatives due to the efficient generation of ammonia and methylamines from added creatine (Yaylayan et al., 2004).

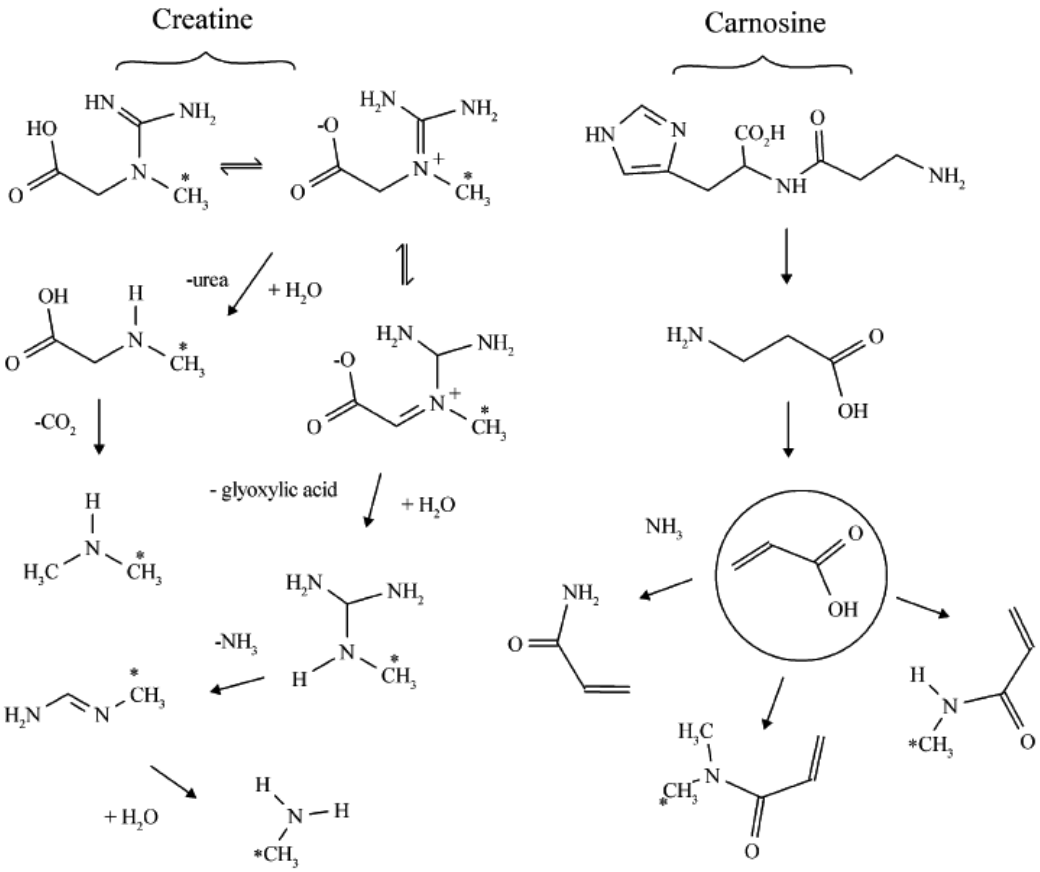

Figure 3.9. Mechanism of generation of methyl and dimethylamines from creatine and formation of N-methylated acrylamides. Reprinted from Yaylayan et al., 2004

In conclusion, considering the relatively high detection limit of the method employed in measuring acrylamide derivatives in meat samples, the results obtained have provided enough evidence to speculate that levels of N-methylacrylamide in cooked meat could be as high as acrylamide levels in potato products. Further studies are needed to quantify the levels of N-methylacrylamide in different meat-related consumer products to assess the risk factors associated with its consumption (Yaylayan et al., 2004, 2005).

3.6. Lipid oxidation contribution to Acrylamide formation

Recent studies have pointed to some oxidized lipids as potential inducers in the conversion of asparagine into its vinylogous derivative (Zamora and Hidalgo, 2008). Among them, alkadienals exhibited the highest reactivity for this reaction. The mechanism for this reaction seems to take place in two main steps, namely, the decarboxylation of the amino acid and the later deamination of the produced 3-aminopropionamide (Figure 3.10).

However, the role of alkadienals in the decarboxylation reaction of asparagine has not been yet analyzed. In an attempt to understand the reaction pathways by which carbonyl compounds are able to convert asparagine into acrylamide, this study analyzes the decarboxylation reaction of asparagine in the presence of alkanals, alkenals, and alkadienals, among other lipid derivatives (Hidalgo et al., 2010).Capuano et al. (2010) reported that lipid oxidation had a positive influence in the formation of acrylamide, especially in sugar-free systems where lipids became the main sources of carbonyls. In systems containing higher amount of water, acrylamide formation was delayed due to evaporative cooling.

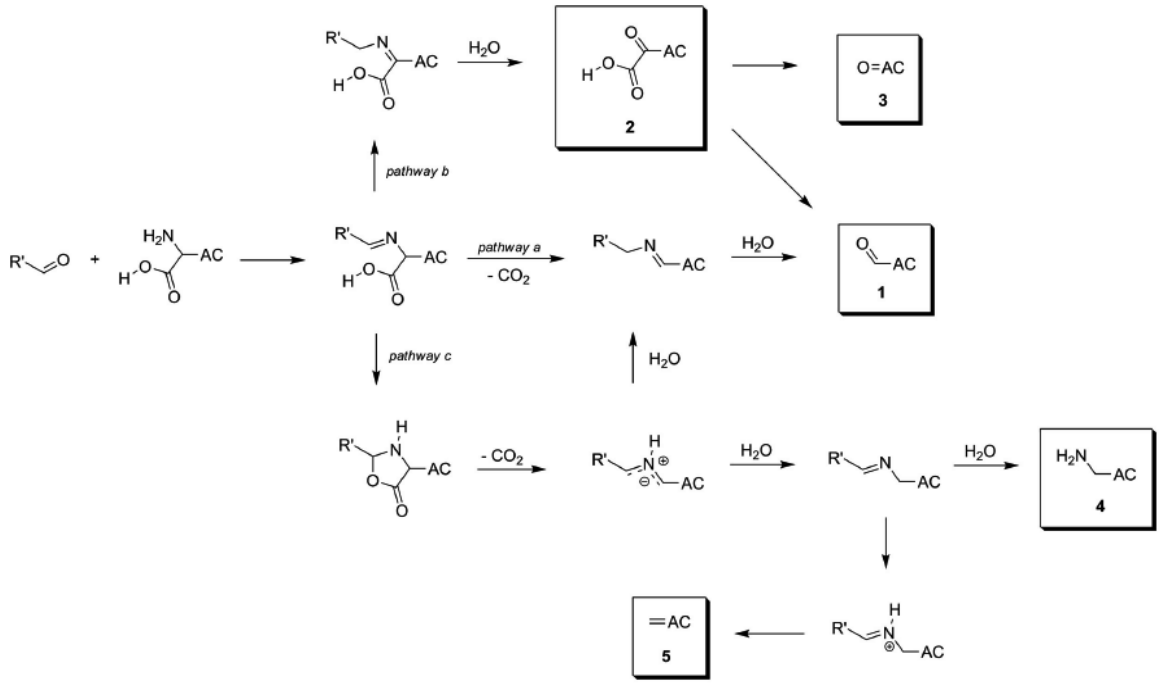

Figure 3.10. Amino acid degradation in the presence of lipid carbonyls. 1- α-keto acids; 2- other amino acid-derived aldehydes; 3- biogenic amines; 4- vinylogous derivatives of the amino acids; 5- AC amino acid chain. Reprinted from Zamora and Hidalgo, 2011.

3.7. Depolymerization ofpolyacrylamide

Ahn and Castle (2003) investigated the possibility of the presenceof acrylamide in foods resulting from thermal depolymerization of polyacrylamide used in agricultural processes. They concluded that even if the polyacrylamide contaminate crops and foods derived therefrom (which in itself is unlikely), there is no evidence that the

depolymerisation process occurs in significant amounts during heating of the food in order to free acrylamide.

3.8. Factors affecting acrylamide formation in foods

3.8.1. Presence of precursors and processing conditions

The formation of acrylamide is affected by multiple factors. At the early stage of studies, researchers focused on the parameters, such as heating time, heating temperature, the ratio of amino acid and reducing sugar, etc. Initial studies were almost performed in model systems with pure chemicals, such as the asparagine-glucose model system. Compared to model systems, the formation of acrylamide in actual food matrices produced via various heat processing methods, such as frying, baking, and roasting, is more complex. Meanwhile, the control of acrylamide content and maintenance of original food quality need to be simultaneously considered during heat processing (Zhang et al., 2009). Fiselier et al. (2006) summarized the effect of frying temperature on the formation of acrylamide and demonstrated that the temperature during the second half of the process is the most important since acrylamide is formed toward the end of frying. Romani et al. (2008) indicated that the increase of frying time becomes a key factor in terms of the quantity of acrylamide and its formation rate when the temperatures of the potato surface and the oil bath reach 120 and 140 ^{0}C, respectively, after around 4 min of frying. Besides, other factors related to the formation of acrylamide include precursor levels and water content in raw materials, pretreatment, pH, etc. The contents of precursors, including asparagine, glucose, and fructose, play an important role in the generation of acrylamide. Since the asparagine content is approved as a prerequisite for the heat-induced formation of acrylamide, control of free asparagine could turn out to be a useful approach to mitigate acrylamide formation. A direct relationship between the acrylamide contents and asparagine levels was demonstrated in baked/toasted wheat and rye breads (Granby et al., 2008). The reducing sugars glucose and fructose have also been reported to serve as important contributors (Vivanti et al., 2006). Recently, a function of different glucose/fructose ratios in raw potato products using several color measurement methods was optimized to investigate the relationship between acrylamide content and Maillard browning in French fries (Mestdagh et al., 2008). Based on current findings, acrylamide is formed in different amounts with several mono- or disaccharides. Even nonreducing sugars, such as sucrose, are efficient reactants, leading to the release of reducing sugars that are then available to react with the

$R\text{-}NH_2$ group of asparagine via the Maillard pathway after thermally induced hydrolysis (Zhang et al., 2009). Some other factors such as water content and pretreatments also need to be indicated. Crust temperature in combination with water content has a significant effect on acrylamide formation during the baking of white bread. Acrylamide concentration was observed to decrease at very high temperatures and lower water contents (Ahrné et al., 2007).

The choice for raw material is usually related with the amounts of aprecusrsors present. The agronomic factors that affect raw material composition mainly include fertilization methods, harvest season, and climatic conditions. A reverse correlation between the amount of fertilizer applied in potato cultivation and the acrylamide content in the edible products has been revealed, since reducing sugar contents are elevated while crude protein and free amino acids decrease when less nitrogen-fertilizer is given(De Wilde et al., 2006). When wheat was grown under conditions of severe sulfate depletion or sulfur deficiency during fertilization, dramatic increases in the concentration of free asparagine were found and subsequently enhancement of acrylamide content during baking was observed (Granvogl et al., 2007). Independent of fertilization, harvest year and climatic conditions turn out to be other factors influencing acrylamide formation. Favorable light and temperature conditions during the cultivation period enhance amino acid and protein contents, thus promote the formation of acrylamide during baking (Amrein et al., 2004).

Besides climatic conditions affecting precursors composition, the variety of the raw material also influences the amount of asparagine and reducing sugars, particularly in potatoes (Vicklund et al., 2008).Other factor is the storage condition, sine many researchers (Grob et al., 2003) demonstrated that the acrylamide level in potato chips made from tubers stored at low temperature is much higher than that from those stored at high temperature. It seems that storage of raw materials at low temperatures should be avoided for the control of precursors. However, storage at too high a temperature can reduce the preservation period and sensory attributes of raw materials. Overall, the effect of variety and storage conditions of raw materials is ascribed to the variation of amino acids and reducing sugars.

To effectively control the formation of acrylamide, some tips regarding heat processing methods were recommended such as low temperature vacuum frying, short time heating, and avoiding the use of palm olein as for modification of processing (Zhang et al., 2009).

Color not only is visually considered one of the most important parameters in the definition of quality of fried products but also is the result of the Maillard reaction, which depends on the content of reducing sugars and amino acids or proteins at the surface, and the temperature and time of frying (Pedreschi et al., 2007). A direct correlation between the

acrylamide generation and color development of products was approved in many studies (Lukac et al., 2007; Pedreschi et al., 2007; Gokmen and Senyuva, 2006). Compared to previous studies since 2002, the goal of formation studies in 2006-2008 is to find new factors and improve acknowledged factors contributing to the formation of acrylamide (Zhang et al., 2009).

3.9- Acrylamide presence in selected food matrices

The concentration of acrylamide in banana fritter of both banana varieties was enhanced by the increase in the concentration of the reducing sugars, glucose and fructose with the increase of the maturity stages of banana. This finding demonstrated that the acrylamide concentration is strongly dependent on the concentration of reducing sugars and the reducing sugars are the limiting factor for the formation of acrylamide in banana fritters (daniali et al., 2013).

Coffee is usually roasted at temperatures in the range of 220-250 ºC. Coffee beans are processed at relatively higher temperatures than those for other foods, and therefore the reaction and formation of acrylamide during roasting are more complex than those for other matrices. Acrylamide in coffee is formed at the very beginning of the roasting step, reaching more than 7 mg/kg and then declining sharply toward the end of the roasting cycle due to high rates of elimination (Stadler and Scholtz, 2004). Deep roasting as a potential choice to mitigate acrylamide could generate other undesirable compounds and negatively impact the sensory properties of the product. In view of all possible changes of acrylamide content, its formation and elimination during roasting, storage, and brewing need to be sequentially considered. Lantz et al. 2006) systematically showed that the main factors affecting the level of acrylamide in roasted coffee appear to be the Arabica/Robusta ratio (with Robusta giving higher levels), time and degree of roast (with both shorter and lighter roasting at the edges of the normal roasting range giving higher levels), and storage conditions and time (with clear reduction at ambient storage).

Almonds also contain both acrylamide precursors in appreciable levels. The content of free asparagine was reported in the range of 2000-3000 mg/kg while glucose and fructose levels were analyzed to be 500-1300 mg/kg, and sucrose contents ranged from 2500 to 5300 mg/kg (Zhang et al., 2009). As a result, the detection of acrylamide in roasted almonds in concentrations from 260 to 1530 μg/kg42a was not surprising. Interestingly, acrylamide was found to decrease in roasted almonds during storage at room temperature (Amrein et al., 2003). The formation of acrylamide in almonds starts only when the kernel

temperature exceeds approximately 130 ºC. The color as measured by the degree of brightness correlates well with the acrylamide content, as acrylamide content increases with increasing darkness. At constant roasting conditions, almonds with higher initial moisture content contain less acrylamide after roasting, which is probably due to the influence of moisture on the development product temperature during roasting (Lukac et al., 2007; Zhang et al., 2011; Amrein et al., 2005).

Black ripe olives are one of the most important classes of table olive commercialized in the world. Acrylamide levels in black ripe olives are affected by variations in processing conditions at the different manufacturers, as well as in different forms of olive presentation (sliced, pitted, or chopped) by the same manufacturer. The effects of darkening method and olive cultivar on the acrylamide content were pronounced as the key factors. Acrylamide contents do not significantly differ after 6 months of storage. The small amounts of free amino acids and reducing sugars found in olives before sterilization do not significantly correlate with the acrylamide formed (Casado and Montano, 2008). Recently, Casado et al. (2013) ruled out glucose and other carbonyl compounds as acrylamide precursors in sterilized olives in contrast with other heated foods such as fried potatoes, bread, roasted coffee and almonds. Suggesting that peptides and/or proteins are the precursors of acrylamide formation in sterilized olives.

The presence of acrylamide in roasted tea samples was reported by Mizukami et al. (2008) that optimized the roasted green tea (Houjicha) processing by applying roasting treatments to reach AA reduction without affecting the quality. Analysis of 82 tea samples revealed that the Asn content in tea leaves was a more important factor that the reducing sugars contents as regards the acrylamide formation in roasted products (Mizukami et al., 2006). Apparently acrylamide is formed in tea samples via the Maillard reaction.

3.10. Final considerations

Acrylamide formation is foodstuff is still not fully understood since mechanisms of formation are not completely elucidated. The presence of this compound in foods were the Maillard reaction is no expected to be the main pathway opens the field to further investigation. The parameters concerning food processing are usually also responsible for the organoleptic features of food and it could be difficult to change them without affecting consumer acceptability of a food item. In the next Chapter acrylamide mitigations possibilities are going to be discussed in more detail.

4. Mitigation Strategies to Reduce the Formation of Acrylamide

4.1. Introduction

Since the discovery of acrylamide in a variety of heated foods, in 2002, considerable efforts have been undertaken at a cross-scale –envolving scientists, food manufacturers, and legislators – to devise strategies to minimize the acrylamide content of the diet. In addition to attempts to act gradually in terms of changing eating habits, the large majority of efforts have focused in the reduction of acrylamide levels in the main foods of concern: potato products, cereal products (biscuits, breakfast cereals, bread, crisp bread, crackers, etc.), and coffee and coffee substitutes. The real challenge is to reduce acrylamide levels in foods as much as possible without adversely affecting the nutritional quality, safety, and sensory attributes, including colour and flavour, while maintaining consumer acceptance. Research performed to date demonstrates the necessity of a farm-to-fork approach in order to reduce acrylamide in fried potato products.

Once known the definitive importance of the reaction between free asparagine and reducing sugars in the formation pathway of acrylamide, it is not surprising that most of the methods proposed to mitigate acrylamide rely on actions to reduce its precursors in raw materials or, alternatively, changing some processing parameters in order to decrease the extent of the reaction. On the other hand, post-processing techniques which remove or trap acrylamide after it is formed in foods are also being tested. Overall, it was the combination of several technologies that mitigate acrylamide that has been found to be the most effective approach, since the organoleptic quality of foods is not affected (Mariotti et al., 2011). It should be noted that most of the mitigation measures proposed so far were only tested at laboratory or at pilot scale. Therefore, for those mitigation measures it is not clearly known whether the percentage of reduction in acrylamide claimed at laboratory scale could ever be achievable in food processed at an industrial scale. When several mitigation strategies are applied to the same food product, the overall percentage of reduction in acrylamide level is not merely the sum of the percentages achievable when each single measure is applied. The interactions among different measures are not clearly known as well and should be taken into account (Capuano and Fogliano, 2011). Lab scale studies in acrylamide mitigation research should always be interpreted with utmost care.

Friedman and Levin (2008) pointed-out 30 different parameters that have been evaluated for mitigation purposes, which can be grouped as follows: (a) selecting potato, cereal, and other plant varieties for dietary use that contain low levels of the acrylamide

precursors, namely, asparagine and glucose; (b) removing precursors before processing; (c) using the enzyme asparaginase to hydrolyse asparagine to aspartic acid; (d) selecting processing conditions (pH, temperature, time, processing and storage atmosphere) that minimize acrylamide formation; (e) adding food ingredients (acidulants, amino acids, antioxidants, nonreducing carbohydrates, chitosan, garlic compounds, protein hydrolysates, proteins, metal salts) that have been reported to prevent acrylamide formation; (f) removing/trapping acrylamide after it is formed with the aid of chromatography, evaporation, polymerization, or reaction with other food ingredients.

The more successful mitigation options that have been proposed and tested so far have been collected by the Food and Drink Europe (FDE, formed termed Confederation of the Food and Drink Industries of European Union (CIAA)) in a guidance document, the so-called "toolbox" for acrylamide. The latest edition (updated at 2011) includes information from food and beverage manufacturers in the USA, provided through the Grocery Manufacturers Association (GMA). Most of the approaches defined in the "toolbox" have been summarized by a Codex document, named as *Code of Practice for the reduction of acrylamide in foods* (Codex 2009).Suitable or even applicable mitigation strategies for products such as coffee are still a pending issue (Capuano et al., 2011).

In the next sections some of the most relevant acrylamide mitigation technologies tested in potato, cereal-derived bakery products, and coffee are discussed.

4.2. Potato products

As highlighted before, potato products are strongly susceptible to acrylamide formation because they contains the acrylamide precursors (asparagine and reducing sugars) and, on the other hand, the traditional applied baking conditions such as frying and roasting favor the occurrence of the Maillard reaction. Thus, all potential strategies to prevent acrylamide formation may be resumed in two major approaches, removal of the acrylamide precursors or interference with the Maillard reaction. Research performed to date demonstrates the necessity of a farm-to-fork approach in order to reduce acrylamide in fried potato products (Vinci et al, 2012).

Contrary to what happens with most of cereals, asparagine levels in potatoes are in excess compared with reducing sugars contents, so is the sugar level that constitute the limiting factor in acrylamide formation as firstly reported by Amrein et al. (2003), and further confirmed by several other authors. De Wilde et al. (2006), e.g., reported that acrylamide levels in fried potatoes derived from 16 different varieties correlated to reducing

sugar content of the potatoes (R^2 = 0.82, n = 96). The only exception to this consensus comes from Matsuura-Endo et al. (2006) who found that when the relationship fructose / asparagine was greater than 2, the limiting factor becomes asparagine, but this finding was not subsequently confirmed by other groups.

Hence, careful control of reducing sugar levels can reduce acrylamide formation in finished potato products such as french fries, potato chips, and roasted potatoes. The attainment of raw material with reduced levels of reducing sugars encompasses different kind of actions; a) selection of potato variety, b) adherence to agronomy best practices, (c) paying attention to the maturity of tubers at harvest, (d) selection of lots based on sugars or colour assessment, (e) controlled storage conditions for tubers from farm to factory (>6 ºC), and (f) reconditioning the potatoes when appropriate (Lineback et al., 2012).

In what concerns potato variety, the ones with lower contents of reducing sugars should be chosen, which is not a easy task because they may vary by region and by season. Some varieties are more suitable than others for French fries production, as fully demonstrated by earlier works. Granda et al. (2004) obtained acrylamide levels of 5021 μg/kg, 646 μg/kg, and 466 μg/kg, from 3 different cultivars prepared and fried under exactly the same conditions: 165 ºC, 4 minutes. Acrylamide contents ranging from 50 to 1800 μg/kg were obtained from 66 different potato samples fried at 180 ºC for 3,5 minutes (Becalski et al., 2004).Cultivars with large, long, oval tubers containing moderately high dry matter and low reducing sugar contentsare the most appropriate (Medeiros Vinci et al., 2012).The US Snack Foods Association recommends the use of Russet variety for chipping (FDE, 2011). Potato maturity at the time of harvest should be optimized, in order to avoid immature potato tubers, which have higher reducing sugar contents.

Environmental factors, such as climatic conditions, growing location, and fertilization regimes, are not to be neglected, as these may have an impact on the sugar concentration of the tubers (Haase, 2006). The mineral content of the soil has also some importance as observed by Whittaker at al. (2010) which found a negative correlation between potassium and calcium levels and reducing sugar content and a positive correlation with zinc and copper contents. High nitrogen fertilization has been reported in the literature to have a positive impact in asparagine content and a negative impact in the sugar content, so moderate nitrogen fertilization combined with a good provision of potassium may result in lower levels of reducing sugars in potato tubers (Heuser et al, 2005). The climatological conditions also have an impact on sugar content, as observed by De Meulenaer et al., 2008, which found that exceptionally warm summers resulted in lower reducing sugar contents.

A key aspect in controlling the amount of reducing sugars of raw potatoes is the storage temperature. Certain storage conditions can cause potatoes to accumulate

unacceptable quantities of sugars, even though levels were acceptable at harvest. Briefly, there are two different processes leading to a rise in the free sugar content of tubers: senescent sweetening and cold temperature (Vinci et al. 2012). The former results from an enzymatic process which occurs more rapidly at higher storage temperatures (>8 ºC) being related to the start of sprout growth (Amrein et al., 2004). The increase in the amount of free sugars is not always too marked because sugars and starch remain in balance, with the free sugars either transforming into starch or being used up in other reactions (Friedman and Levin, 2008). To avoid tubers sprouting is usual to store potatoes at cold temperatures (<8 ºC). This alternative however, has a major impact on reducing sugar accumulation and subsequently in acrylamide formation in fried potato products, as stressed by several authors, and as can be illustrated in Figure 4.1, from De Wilde et al. (2005). Cold induced sweetening is presumably a defence mechanism of tubers to protect themselves from frost, and therefore start mobilizing sugars from starch at temperatures <8 ºC (Blenkinsop et al., 2002). Biederman et al. (2013) showed that to achieve lower acrylamide levels, potatoes used for roasting and frying should contain less than 1 g/kg of reducing sugars, which can be achieved by avoiding storage of fresh potatoes at 4ºC.

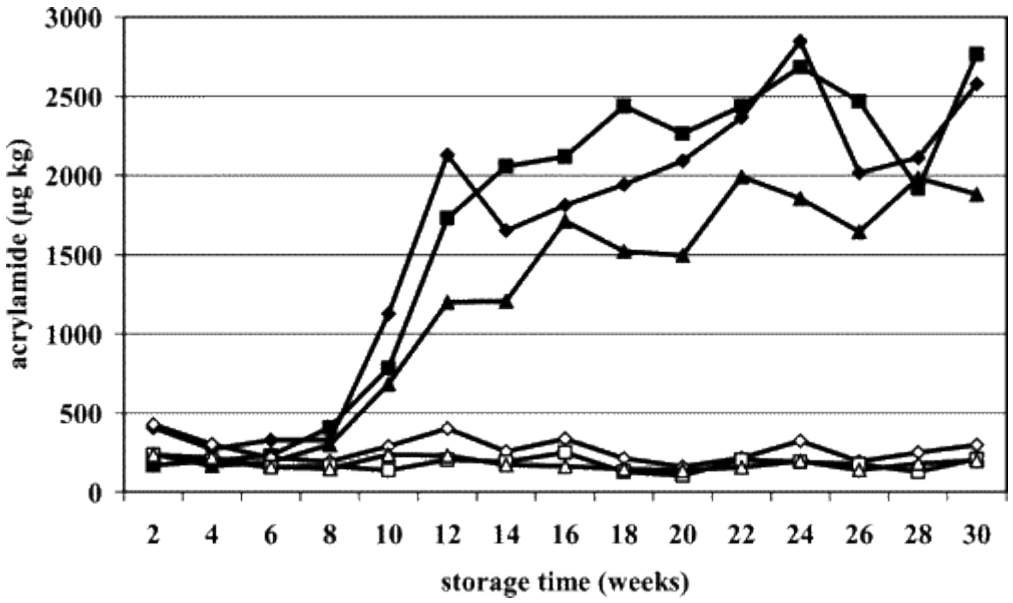

Figure 4.1. Influence of storage time and temperature on acrylamide formation during frying of three varieties (Bintje, Ramos, Saturna) stored at 4 °C and 8 °C over 24 weeks, expressed in µg kg-1. (◆ = Bintje, 4 °C; ▪ = Ramos, 4 °C; ▲ = Saturna, 4 °C; ◇ = Bintje, 8 °C; □ = Ramos, 8 °C; Δ = Saturna, 8 °C.). [Reprinted from De Wilde et al. (2005)].

Taking into account that reducing sugar content is not significantly influenced when potatoes are stored at 8 ºC, potato tubers should be ideally stored at intermediate temperature of approximately 8 ºC. When cold conditions are unavoidable during winter

periods the reconditioning of the potatoes should be done, usually by submitting them at 15 ºC for a period of 3 weeks which may result in a reversible reduction of the reducing sugar levels (Biedermann et al., 2003; Blenkinsop et al., 2002; De Wilde et al., 2005).

Potato processing is executed in multiple steps. Throughout the earlier stages of the process prior to frying and roasting operations, there are also opportunities to lower the content of free sugars and also asparagine and thus obtain final products with a lower content of acrylamide.

Blanching is a well-established procedure in the French fries production. During this step enzymes are inactivated and a layer of gelatinized starch is formed which limits oil absorption and improves texture (also contributing for a uniform colourof the product after final frying. During this step, acrylamide precursors are leached out, resulting in the reduction of acrylamide content in the final product. Several authors have tried the optimization of the blanching conditions, namely temperature, time, and replacement frequency of the used water in order to maximize reducing sugar and asparagine extraction while maintaining the final product specifications constant (*Medeiros Vinci et al., 2010*, Mestdagh et al., 2008, Pedreschi et al., 2004, Pedreschi et al., 2007, *Pedreschi et al., 2009*; *Viklund et al., 2010*). Usually an increase in the intensity of blanching conditions is required when higher reducing sugar levels are present. However, extreme blanching conditions result in textural and nutrient loss issues and therefore blanching can only be adapted within certain limitations. Several authors have concluded that the application of low temperature and long blanching time (~70 ºC, 15 min) may be considered an effective acrylamide mitigation technique for fried potatoes, since under these pre-treatment conditions not only is acrylamide reduction achieved but also some desirable quality attributes such as firmness and low oil uptake are maintained (Pedreschi et al., 2007; Pedreschi et al., 2004; Mestdagh et al., 2008). Besides time and temperature, the concentration of soluble components extracted from the potato cuts during this continuous process will also influence the efficiency of sugar extractability and therefore affecting acrylamide formation in potato products. The continuous replacement of the blanching water with fresh water is however not feasible, both from environmental and economical point of view (Medeiros Vinci et al., 2011).

Asparaginase pre-treatments have been suggested as a promising technological intervention for acrylamide mitigation. Asparaginase, an enzyme that hydrolyses asparagine to aspartic acid and ammonia is claimed to significantly reduce acrylamide levels by converting asparagine into aspartic acid, maintaining intact the sensorial attributes of the final product (Ciesarova et al., 2006; Pedreschi et al., 2008; Pedreschi et al., 2011).Commercial enzymes, mainly from the companies Novozymes and DSM became

available around 2008, being widely applied in potatoes and other many different foods as judged by the entries in the FDE Toolbox and communication on mitigation tools by the different food sectors (Lineback et al., 2012). However, incorporation of the enzyme into potato products which consist of solid cut pieces seems to be the challenge in terms of acrylamide mitigation efficiency (Anese et al., 2011) because a finest contact between enzyme and substrate is required (*Hendriksen et al., 2009*). *Pedreschi et al. (2011)* have performed lab-scale studies on the effect of blanching of potato slices and asparaginase treatment on acrylamide formation, claiming up to 90% reduction when combining the two treatments. Blanching makes the tissues more permeable and consequently the enzyme is more accessible to the substrate. For that reason, a blanching step prior to enzyme application is required for better asparaginase-asparagine contact (*Pedreschi et al., 2008*). Trials on the application of asparaginase in chilled french fries have shown some promising results (*Medeiros Vinci et al. 2011*). In this study, longer enzyme-substrate contact times resulted in a major reduction of asparagine in the enzyme-treated fries after four days of cold storage. As expected, acrylamide contents in these fries were significantly reduced by approximately 90% with no effects on the sensorial properties of the product upon final frying. However, according to the authors, introduction of this measure implies major line modification to ensure better temperature control and results must be confirmed by production at factory scale in order to deliver a final product of comparable quality and shelf-life stability.

An alternative to removing reducing sugars in potatoes is through fermentation. Lactic acid bacteria metabolize simple sugars rapidly, producing lactic acid, which lowers pH and reduces the Maillard reaction. This method has been applied to french fries prior to the prefrying step, with a reduction of up to 90%. However, this has not yet been applied in commercial products, possibly because of the impact on quality/sensorial properties of the finished products (*Blom et al., 2009*).

Besides the reduction of sugar content of raw material, most of the mitigation strategies to reduce acrylamide levels of fried/roasted potatoes are directed to decrease the extent of the Maillard reaction by changing some processing parameters, such as pH, time and temperature, and additives used. However, since the Maillard reaction is essential for the desired and characteristic flavor and colourformation of potato products, this constitutes a major challenge for food scientists on how to reduce acrylamide formation without affecting final product specifications and quality.

Taking into account that lower pH lessens the extent of the Maillard reaction, one of the proposed alterations to the cooking recipes was the previous acidulation of raw material. The internal pH of potatoes as well as other foods can play a major role in governing

acrylamide formation. Working with a potato matrix in a model asparagine/glucose system, and a frying temperature of 160 ºC, De Vleeschouwer et al. (2006) found a 10-fold decrease in acrylamide formation with decreasing pH from 8 to 4. Rydberg et al. (2003) studied the effect of pH on acrylamide formation, concluding that it exhibited a maximum around at a pH value of 8. Lower pH values enhanced acrylamide elimination thus decelerating its formation rate, e.g. a reduction of the pH from 5,72 to 2,96 resulted in a decrease of 70% in the acrylamide formed. The beneficial effect of low pH results primarily from protonation of the reactive free -NH_2 group of asparagine to the non-reactive R-$NH3^+$ form (Jung et al., 2003), but also from partial acid-catalyzed hydrolysis of asparagine to aspartic acid and of acrylamide to acrylic acid (Friedman and Levin, 2008). Acrylamide reductions of 90% and 50%, respectively, were obtained at laboratorial scale in potato crisps by immersing them in acetic acid solution or soaking or blanching in citric acid solution (Kita et al., 2005). Is worth noting that under normal processing conditions, potato pH level is ≈4.7 due to addition of sodium acid pyrophosphate with the aid to reduce the darkening of the blanched potato cuts, caused by the ferri-dichlorogenic acid complex formation during potato cooking and air exposure (Vinci et al., 2012). Lower pH may adversely affect the sensorial properties of the fries according to the organic acid concentration (Mestdagh et al., 2005). Even though the significant acrylamide reductions in preliminary laboratory experiments, these treatments did not translate in consistent acrylamide reductions in the industrial production of par-fried French fries (Vinci et al., 2011). This authors showed that pH reduction treatments were only effective in reducing acrylamide formation (up to 39%) when applied at extremely exaggerated low pH values (e.g. pH 3 and 2 *vs* pH 4.7 of the standard production process) therefore are not realistic and feasible in terms of acrylamide mitigation in the industrial production of French fries.

Frying conditions - temperature and time - dramatically affect acrylamide levels of the products as they are eaten, since it is during this last step that acrylamide is actually formed. It is well established that the rate of acrylamide formation is function of temperature and longer cooking times increase acrylamide levels, as can be seen in Figure X, retired from Taeymans et al. (2004). From the same figure we can see that acrylamide formation is particularly intense at the end of the frying process, which can be explained by a fall of the moisture content to very low values, usually below 3 %.

Taking into account that acrylamide formation is simultaneously with other key events that take place during frying (colour, texture, and flavour development) given that both are linked to the Maillard reaction, the applied frying conditions (time and temperature) affect both in similar manner, so any alteration in order to mitigate acrylamide will have adverse consequences on other important features of the final product.

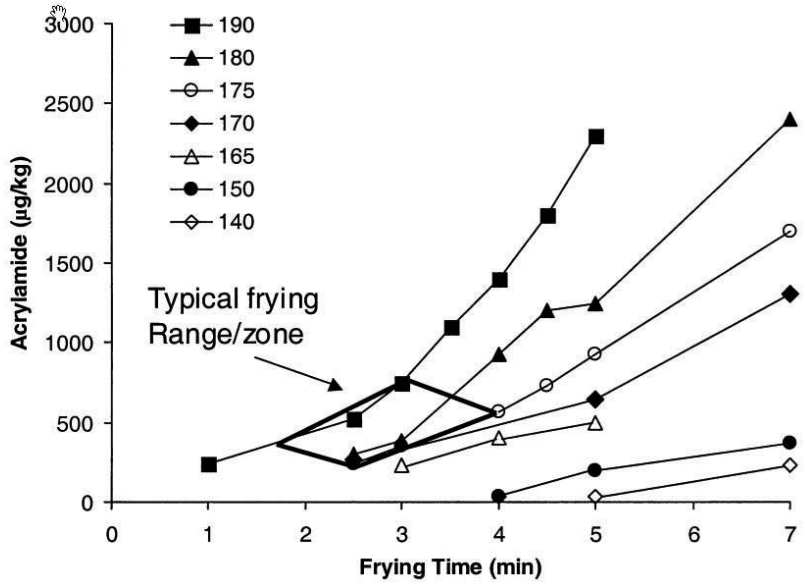

Figure 4.2. Influence of reconstitution temperature and frying time on acrylamide formation in lab processed French fries. [Reprinted from Taeymans et al. (2004)].

From the Figure 4.2 we can also concluded that under typical temperature frying conditions (170 ºC - 190 ºC) a heating time of 3-4 minutes should not be exceeded in order to avoid the attainment of a final product with very high levels acrylamide. Last guidelines provided by the regulatory authorities (FDE, 2011) advise the use of frying temperatures no higher than 175 ºC, targeting higher moisture endpoints (1,3-1,5 %), while providing acceptable sensorial properties. Frying at lower temperatures may cause higher fat uptake and affect crispness, whereas moisture endpoint levels greater than 1,5 % may affect flavour texture, and shelf life. The product/oil ratio may influence a drop of initial frying temperature and therefore longer frying periods would be needed resulting in higher acrylamide contents.

Flash frying (frying at high temperatures for short periods of time) with rapid cooling and vacuum frying(frying under reduced atmospheric pressure to allow use of a lower oil temperature) may be useful approaches for reducing acrylamide levels for some manufacturers (FDE, 2011). Vacuum frying may reduce acrylamide formation significantly without significant changes in organoleptic properties. Using a frying temperature of 118 º C and a vacuum pressure of 10 Torr, Granda et al (2004) obtained a decrease of 94 % in acrylamide content of potato chips while maintaining desirable yellow golden colourand texture attributes compared with those fried in the traditional way. However, the process is difficult to industrial application because it have limited throughput capacity (FDE, 2011).

It also may be helpful to use a multi-stage cooking process, in which higher temperatures are applied initially, followed by lower temperatures near the end of the cooking process when moisture levels are lower and the products are more susceptible to acrylamide formation. Par-frying coupled with dry steam cooking, used to produce low-fat potato chips, also may produce lower acrylamide levels (Foot et al., 2007).

Addition of non-asparagine amino acids reduced acrylamide formation markedly, probably either by promoting competitive reactions and /or by covalently binding acrylamide which is formed through Michael type addition reactions (*Anese et al., 2009*, Claeys et al., 2005, *Kim et al., 2005*, Lindsay et al., 2005, *Low et al., 2006*, Mestdagh et al., 2008, *Ou et al., 2008*). First studies by Rydberg et al. (2003) showed that glycine, lysine, and 4-aminobutyric acid are the amino acids more effective in that reduction. The great impact (reduction levels between 30% and 70% compared to controls) of glycine on decreasing acrylamide levels in fried potatoes, as well as in cereal based products, was later confirmed by other authors (Brathen et al., 2005; Kita et al., 2005). However, this 'glycine methodology' still needs some improvements due to its negative impact on the flavor profile of the final products (Low et al., 2006). The addition of glycine to potato-based model systems heated at 180 ◦C for 5–10 min has been shown to significantly alter the distribution pattern of alkylpyrazines (Low et al., 2006, Low et al., 2007) which are important flavor volatiles in baked potatoes. It could be explained since the Strecker degradation of glycine leads to formaldehyde, which may react directly with dihydropyrazine intermediates to give alkylpyrazines with an additional methyl substituent derived from glycine (Low et al., 2007).

Mono and divalent cations, e.g. Na^{+} and Ca^{2+} and also Fe^{3+} were indicated to efficiently reduce acrylamide formation in model solutions. These ions could interact with asparagine suppressing early-stage Maillard reactions, namely by inhibiting the formation of the intermediate Schiff base that leads to acrylamide formation (*Gökmen and Senyuva, 2007*, *Lindsay and Jang, 2005*, *Mestdagh et al., 2008a*, *Mestdagh et al., 2008c*, *Ou et al., 2008*, *Park et al., 2005*, *Pedreschi et al., 2010* and *Pedreschi et al., 2007b*). The addition of calcium ions has also been related to a pH decrease of the food matrices and accordingly associated to acrylamide reductions (*Levine and Ryan, 2009* and *Mestdagh et al., 2008c*). The effective lowering of the acrylamide content was observed at only 1% NaCl addition, which makes it applicable to real technological procedures of food processing (Kolek et al., 2006). The mitigating action of salts seems to be more effective when added during the blanching procedure (Lindsay et al., 2005; Pedreschi et al., 2007; Pedreschi et al., 2009). The latter authors observed acrylamide reductions of 62% in potato slices blanched in NaCl solutions; however, only about half of this percentage (~27%) could be attributed to the

effect of NaCl and 35% to the effect of the slight heat treatment during the salt immersion step (25 ◦C for 5 min). These authors suggested that the addition of salt in the water solutions changes the osmotic potential and, in order to establish equilibrium, the liquid is transported out of the potato and a simultaneous washing out of reducing sugars occurs. In several works from the group of Friedman and collaborators, the authors found that changes of the ionic strength induced by positively charged Na+ ions affect the rate of addition reactions of amino groups of amino acids to the double bonds of conjugated vinyl compounds such as acrylamide (Friedman and Levin, 2008). NaCl has also been proposed to accelerate acrylamide elimination via polymerization in a model food matrix (Kolek et al., 2006). Regarding calcium salts, these have been utilized successfully by Lindsay et al., (2005), which obtained a reduction of about 95% in acrylamide levels. However, Ca^{2+} should be used only in low concentrations to avoid off-tastes. Moreover Ca^{2+}tends to influence pectin binding (possibly cross-links) in the cell walls, and harder and more brittle crisps are obtained.

Some authors have been proposed the mitigation of acrylamide by addition of antioxidants in consequence of their known influence on Maillard reaction (Becalski et al., 2013).Acrylamide reductions were reported in potatoes cuts when treated with extract of bamboo leaves (*Zhang et al., 2007*) and oregano phenolic extract (Kotsiou et al., 2010). Although the mechanism of antioxidants on acrylamide formation is however not yet fully understood,(Kotsiou et al., 2010) concluded that phenolic compounds without aldehydic groups in their structures are more effective in acrylamide reduction.In contrast, other studies either described positive correlations between acrylamide and antioxidants or no correlations were found (Bassama et al., 2010, Becalski et al., 2010, Ehling and Shibamoto, 2005, Rydberg et al., 2003 and Serpen and Gokmen, 2009). Supplementary factors associated to the addition of antioxidants, such as pH decrease or the presence of amino acids present in the extracts could influence acrylamide contents and therefore hinder the comparison of results between different studies (Mestdagh, et al., 2009).

4.3. Cereal based foods

Cereal-based products include foods such as bread, breakfast cereals, crackers, cookies, and cakes that are cooked from cereal crops such as wheat and maize. Bakery products are a major dietary source of acrylamide, contributing around 30% -40% of the total human exposition. As well as above described for potatoes, the potential strategies to prevent acrylamide formation may be resumed in two major approaches,

removal/reduction of the acrylamide precursors present in raw materials or interference with the Maillard reaction by changes in processing and/or used ingredients. Some of the mitigation approaches are similar to that used in potatoes (e.g. use of asparaginase treatment, calcium supplementation, lowering thermal input through modifying baking times and temperatures throughout the processing) while others are specifically designed for cereal-based foods.

Unlike what happens with potatoes, asparagine, rather than reducing sugars, is the main determinant of acrylamide formation in products made from cereal grains. Asparagine levels are highly variable, depending on cereal grain type, grain variety, and growing conditions and this variability can significantly impact acrylamide levels in bakery products ((Friedman and Levin, 2008; Konings et al., 2007). Besides other aspects, it was found that asparagine content of wheat is highly dependent of the amounts of sulphur in the soil. A severe sulphate depletion can lead to asparagine levels 30 times greater than normal ones (Muttucumaru et al., 2006). An excessive nitrogen fertilization may also increase asparagine levels in cereal crops. On the other hand it is known that whole grains are richer in asparagine than refined grains, producing more acrylamide in finished products.

Taking into account the above described picture, viable mitigation measures by acting on cereal raw materials are i) the use of cereal varieties poorer in asparagine, ii) use of wheat grown with adequate soil sulphate and without excessive nitrogen fertilization. Reducing whole grain content may also reduce acrylamide, but authorities does not recommend this approach given their benefits.

The majority of the tools proposed to reduce acrylamide formation in bakery products are focused on the processing stage, i.e., recipes/ingredients and thermal input. Several authors have showed that almost all the acrylamide formed during baking is found in the crust of the finished foods, that acrylamide levels of the crusts were related to temperature and baking time, and finally that surface colourof the crust is strongly correlated with acrylamide content (Surdyk et al., 2004; Ahrné et al., 2007). Depending of the type of products with which we are dealing, lower acrylamide levels can usually be obtained by baking at lower temperatures for longer periods of time (Claus et al., 2008; Konings et al., 2007; Jensen et al., 2008). Another approach is to increase temperatures earlier in the baking process when higher moisture levels should prevent acrylamide formation and use lower temperatures during the final stages of cooking when the moisture levels are low and acrylamide formation is more likely (Konings et al., 2007). Potential disadvantages of temperature reductions include slower production lines, lighter color, less crispness, and shorter shelf life of higher moisture products (FDE, 2011). Alternative baking technologies, such as steam baking, baking with lidded pans, and infrared radiation also can

reduced acrylamide levels. The colourof many baked goods (e.g. bread) can be a useful indicator to monitor acrylamide levels, although the correlation between colourand acrylamide may have to be determined on a product by product basis, because in a few products, such as breakfast cereals, darker colourmay be associated with less acrylamide (CIAA, 2007).

In what concerning recipes, significant reductions could be achieved in baked products, by replacing the chemical leavening agent ammonium bicarbonate with the corresponding sodium salt. The promoting effect of the ammonium salt appears to be due to its ability to enhance the formation of reactiveR-dicarbonyl intermediates, which then combine with NH3, forming sugar imines that are then transformed to acrylamide (Amrein et al., 2006). Besides sodium bicarbonate alternative leavening agents could be used, e.g., disodium diphosphate, potassium bicarbonate with potassium bitartrate and some organic acids. According to industrial organizations replacement of ammonium bicarbonate is one of the most effective mitigation techniques in cookies, due to reasonable costs and limited impact of quality (FDA, 2013). However, the use of sodium bicarbonate have raise concerns about increased sodium intake, being important to consider whether less sodium can be added elsewhere in the baking process (FDE, 2011). Moreover, alternative leavening agents may have unwanted effects on taste and texture, and cause decreased browning, more limited leavening, and slower gas generation in baked goods (Sadd et al., 2008).

As well as in the potatoes products, asparaginase treatment is one of the most effective mitigation practices adopted by manufacturers for cereal goods. Acrylamide reductions of approximately 35 to 90 percent have been reported for asparaginase treatment of various cereal-dough-based products in commercial or trial use, such as tortilla chips, corn chips, pretzels, crackers, crispbread, cakes, cookies (including gingerbread), honey cakes, and hydrolysed ready-to-eat cereal products (CIAA, 2009; Hendriksen et al., 2009). Kukurova et al. (2009) achieved a 60–90% reduction (from an initial acrylamide content of 1400 μg/kg) for some fried pastry products. A major advantage of asparaginase treatment is the limited effect on product characteristics or organoleptic properties, as might occur with recipe or process changes.

The primary factors affecting successful use of asparaginase in cereal-based products are dose, dough contact time, and dough water content. The enzyme can be added in a granular form to dry ingredients or as a liquid. While some modifications to manufacturing lines may be necessary, e.g., to increase holding time in the presence of asparaginase, dry addition typically requires only minimal process changes (Hendriksen et al., 2009). Asparaginase activity is greater in high moisture doughs compared with low moisture doughs, such as ginger cookies. Also, asparaginase is not effective for breakfast

cereals that have low moisture content or that are based on coarsely ground flours because of limited penetration of asparaginase into the product (FDE, 2011). Working with biscuit formulations, Anese et al. (2011)observed 48% and 58% acrylamide reduction when 10% and 20%of water added, respectively. On the contrary, the presence of fat significantly reduced acrylamide development and enzyme activity as compared with the fat free formulation. The authors hypothesized that the presence of fat would hamper the interaction between the precursors in the aqueous phase, leading to lower amounts of acrylamide.Chlorine content may reduce asparaginase activity in cereal-dough-based products, while the influence of pH depends on enzyme origin (FDE, 2011).

According Friedman and Levin (2008), beneficial findings with asparaginase suggest that other enzymes may be effective in modifying acrylamide precursors, including commercially available glucose oxidase that catalyzes the oxidation of glucose. A complementary approach would be to use so-called acrylamidinases to hydrolyse acrylamide in food to acrylic acid (CH2=CH-COOH) and ammonia or to otherwise metabolize the molecule to nonreactive and safe products. According the same authors, although such enzymes are not currently available, they could be produced by recombinant DNA techniques. It would also have to be shown that such enzymes are safe to consume.

Replacement of reducing sugars in the recipe (glucose, inverted sugar, corn syrup, fructose, honey) by nonreducing sugars such as sucrose or trehalose has proved to be a successful strategy for acrylamide mitigation in baked goods, particularly in the cases where browning is not crucial. Amrein et al (2004) observed in a 20-fold decrease in acrylamide formation in gingerbread by replacing inverted sugar and honey with the sucrose, while Oku et al. (2005) observed similar effectswhen adding the nonreducing disaccharide trehalose (currently used in many commercial food applications) to glucose/asparagine or ascorbic acid/asparagine mixtures. However, when sucrose is used, a slightly lower pH causes an increment of acrylamide levels,presumably due to hydrolysis of sucrose to the reducing sugars glucose and fructose. For recipes that require reducing sugars, fructose is preferable to glucose. This kind of replacements may cause a lighter colored product or interfere with flavor formation for some products (e.g., gingerbread), but not have a significant effect or unacceptable effect on other products (Amrein et al., 2004; CIAA, 2009). Reducing sugars applied as coatings after cooking do not influence acrylamide levels (FDE, 2011).

Yeast fermentation of wheat and rye doughs result in a significant reduction on acrylamide content in baked cereal goods such as crisp breads. Fredriksson et al. (2004) achieved a reduction of 87 % and 77 %, respectively, when comparing prolonged fermentation of the whole dough with a short fermentation time, for wheat and rye bread. Similar results were observed by Mustafa et al. (2005), who achieved whole-grain rye crisp

breads with 10-30 μg/kg of acrylamide, compared to amounts up to 1900 μg/kg in commercial samples. These finding can be explained by the fact that yeast use asparagine during growth, and it is likely that most of the original asparagine transforms to other products during fermentation. Longer fermentation time may be hence a useful strategy to mitigate acrylamide formation in breads, crispbreads, and crackers (Claus et al., 2008; Koenings et al., 2007; Sadd et al., 2008), although extended fermentation also may have unwanted effects, such as weakened gluten and flatter breads (Claus et al., 2008). Yeast activity can be affected by other mitigation techniques that alter dough properties, e.g. excess sodium chloride. The use of yeast strains with increased rates of asparagine consumption may also prove useful in the future (Chhun and Husnik, 2011).

Other minor ingredients, such as calcium, magnesium, amino acids, antioxidants (rosemary extract, tea polyphenols), phytic acid, and organic acids, have been tested either at laboratory, pilot, or factory scale (Sadd et al., 2008*;*Konings et al., 2007*;*Capuano et al. 2009).

Calcium fortification of bread (0,3-1 % of calcium carbonate or calcium chloride) reduces acrylamide by approximately 30% while magnesium fortification at equivalent levels reduced acrylamide formation at a lesser degree (CIAA, 2009). Fortification of wheat flour with calcium is already a common practice in most countries. Taking into account that acrylamide forms primarily in bread crusts, a useful approach could be to apply calcium salts directly to dough surfaces (*Sadd et al., 2008*; CIAA, 2009). Calcium propionate should be avoided as it caused an increase of greater than 90 % in acrylamide levels in bread (FDE, 2011). For crackers, cookies, calcium and magnesium supplementation also showed potential in reducing acrylamide in laboratory trials but product quality has been poor (Sadd et al., 2008). Breakfast cereals are in most cases already fortified with calcium. Manufacturers should consider calcium addition when non-fortified cereals are used (FDE, 2011).

Addition of glycine and cysteine has been shown in laboratory and pilot plant trials to reduce acrylamide formation in various types of bread (Claus et al., 2009; Sadd et al., 2008). However, glycine may reduce fermentation ad cysteine can negatively affect bread structure and flavour. The addition of glycine in the recipes of other baked goods such as flake cereals, cookies, and crackers, always result in reduction of acrylamide levels, but poor product colourand taste are obtained. According to FDE (2011) researchers have not been able up to now to mitigate glycine´s effects oc colourand taste, while meeting requirements for moisture, texture and shelf life. When methods of acrylamide mitigation that reduce browning are employed, such as glycine addition and cooking at lower temperature, one possible way to be explored in the future will be the addition of lysine to flours and doughs,

which may significantly increase the extent of browning (Friedman and Levin, 2008). Furthermore, high nutritional quality products will be obtained because cereals are usually lacking of sufficient amounts of lysine.

Acidulants such as citric acid reduce acrylamide levels in cereal base products through their interference with the Maillard reaction (Amrein et al., 2007; Sadd et al., 2008). There are claims of a reduction up to 90 % in gingerbread and up to 60 % in breakfast cereals in industrial cereals (FDE, 2011). However, some authors report significant impacts in baked goods including sour taste, less browning, and also the formation of the undesirable chemical by-product 3-monochloropropanediol (Konings et al., 2008; CIAA, 2009). Spraying dough surfaces with acidulants, rather than incorporating them in dough, may avoid some side effects (Konings et al., 2008).

4.4. Coffees

Coffee is one of the major sources to the total acrylamide content of the diet, depending of the dietary habits of the populations. It is known that acrylamide formation starts rapidly at the beginning of the roasting process and it decreases shortly after reaching a maximum level, probably due to physical and chemical losses (Bagdonaiteet al., 2008; Guenther et al., 2007; Lantz et al., 2006; Senyuva and Gökmen, 2005; Taeymans et al., 2004). Therefore, the degree of roasting will be a key factor in acrylamide content, with light roasted coffee attaining significantly higher amounts when compared with dark roasted coffees (Bagdonaite et al., 2008; Guenther et al., 2007; Lantz et al., 2006; Senyuva and Gökmen, 2005; Taeymans et al., 2004). When comparing the two coffee species of higher economic importance, namely Coffea arabica and Coffea canephora (also known as arabica and robusta coffees, respectively) increased levels of acrylamide are described for the latter (Bagdonaite et al., 2008; Guenther et al., 2007; Lantz et al., 2006; de la Calle, et al., 2007). One interesting feature of coffee products is the decrease of acrylamide content observed during the storage, which can reach values as high as 65 % in opened ground coffee stored at room temperature over 6 months (Andrzejewski et al., 2004). This reduction may be the result of reactions of acrylamide with SH- and NH_2-containing amino acids, peptides, and proteins in the solid state (Hoenicke and Gatermann, 2005). Other possibilities include hydrolysis, degradation, and polymerization of acrylamide during storage (Friedman and Levin, 2008).

There are currently no viable strategies for minimizing the acrylamide content in coffee without adversely affecting the organoleptic qualities of the finished product. Generally acrylamide mitigation strategies are based on the restriction of the Maillard

reaction. Maillard reactions also yield aroma and flavor development in the product, hence in aromatic products such as coffee, the use of these strategies are disadvantageous. Because acrylamide and melanoidins are both Maillard reaction products formed during the roasting of coffee, typically conducted at temperatures between 220 and 250 °C, any attempt to inhibit the Maillard reaction as a possible measure to minimize the formation of acrylamide also lead to a reduction of the antioxidant capacity of coffee. Notwithstanding, some proposals could be viable in the future.

Asparaginase pretreatment of green beans may represent a viable way to reduce acrylamide in roasted coffee as described above for potatoes and cereal based products (Capuano and Fogliano, 2011). Studies with whole grain cereals show that a major bottleneck in asparaginase treatment is the accessibility to the substrate (i.e., enzyme penetration through the cell wall). The magnitude of reduction depends on the blend and roasting/conditions, could reaching 45% (Coffee: Emerging Health Effects and Disease Prevention, 2012). However, testing of acrylamide levels over time shows that after 3 months of storage (i.e., the average age of coffee in the market), the difference between treated and untreated beans was reduced to 20%-30% at most, because the acrylamide content of roast and ground coffees decrease during storage. Moreover, the sensory properties of the enzyme-treated beans were clearly altered, leading to off-flavours that depreciate the final product. The company Novozymes claimed that their asparaginase can reduce acrylamide levels in bean coffees from Arabica and Robusta varieties by up to 70%, and that this approach is being applied commercially (*http://www.novozymes.com/en/news /news-archive/Pages/new-solution-to-reduce-acrylamide-levels-in-coffee.aspx*; accessin January 2015). However, the company also stated that the taste is less bitter in the case of lower quality beans.

Removing or trapping acrylamide after it is formed with the aid of chromatography, evaporation, and polymerization or reactions with other food ingredients has also been applied to coffees (Friedman et al., 2008). Similarly, the removal of acrylamide from coffee through supercritical CO_2 extraction has been investigated. Supercritical treatment may reduce acrylamide content by up to 79% (with the addition of ethanol as supercritical fluid) without affecting the caffeine content of the coffee. However, to consider this method as a mitigation technology it is necessary to test its influence over the sensorial quality of the final product (Banchero et al., 2013). Pastoriza et al. (2012) suggested that addition of soluble melanoidine could modulate the content of acrylamide in the final coffee brew.

A recent study by Akillioglu and Gökmen (2014) showed that yeast fermentation with *Saccharomyces cerevisiae* (1-2 %) is promising for the mitigation of acrylamide in instant coffees. Acrylamide contents were reduced exponentially at varying rates,

depending upon fermentation medium, namely the addition of sucrose important for promoting yeast activity, and time. After 48 h, acrylamide concentration was decreased by about 70%.Authors claim thatthe method developed by the integration of the fermentation step into the regular production of instant coffee would not cause aroma losses can be easily adapted by the industrybecause is easy to adapt into the regular instant coffee production line. Furthermore *Saccharomyces cerevisiae* yeast is easily accessible and harmless to humans.

Finally, the use of bacterial enzymes to control acrylamide levels in coffee has been studied. Cell-free extracts containing acrylamide-degrading enzymes, which hydrolyse acrylamide to acrylic acid, were directly applied to coffee, to remove acrylamide. Although acrylamide was not totally degraded at higher concentrations of coffee in water, it totally disappeared in a 10 mg coffee per mlsolution in water, which is a coffee concentration slightly more diluted than what people commonly drink (Cha et al., 2013).

4.5. Final notes

It is worth noting that some of these acrylamide mitigation strategies are not only associated with a loss of quality attributes but also with an increase in the formation of other toxic compounds. For instance, the use of $Ca^{2+}$and glycine is reported along with a concomitant effect on hydroxymethylfurfural (HMF) concentration. In the first case, this may be explained by cations preventing the formation of the Schiff base, changing the reaction path towards the dehydration of glucose and leading to HMF (Capuano et al., 2009). On the other hand, when glycine is added, overall Maillard reaction is accelerated, improving HMF generation (Capuano et al., 2009).Hence, manufacturers must individually assess whether any sensorial deviations are acceptable to their products, as the final choice is made by the consumer. Moreover, any measures identified to work in an industrial line should not violate health targets, e.g., by increasing sodium or reducing the portion of whole grain in the products (FDE 2011).

5. Analytical Methods for Acrylamide Determination in foods

In general, food is a complex heterogeneous mixture of a wide range of chemical components. Measuring its minor individual components, present at residual levels, is often a difficult task, usually involving sophisticated analytical techniques. Among these, chromatographic techniques such as high liquid-performance chromatography (HPLC) and gas-chromatography (GC) assume particular relevance. The use of chromatographic methods, however, demands several previous steps, generically called sample preparation. The main objective of this procedure is to transfer the analyte(s) from the matrix to an extract, usually of an organic dolvent, as free as possible from interfering compounds, and compatible with the chromatographic system and detection method.

As expected, acrylamide quantification in foods is a difficult task, not only due the complexity of the matrices involved, as mentioned, but mostly due to acrylamide properties: low molecular weight, high polarity, absence of any specific wavelength absorption maxima and absence of native fluorescence. For all these reasons, acrylamide analysis in food usually involves a complex sample preparation process, which may include several steps: a) homogenization, b) swelling, c) extraction of acrylamide, d) purification and concentration of the extracts, including fat elimination and protein precipitation and, e) derivatization. The number and particularities of each step are related with the food matrix specificities and the chromatographic technique used.

5.1. Sample preparation

5.1.2. Homogenization

Sample homogenization is the initial step of any analytical process and aims to obtain a proper mixing of all its components. In solid samples is usually necessary to reduce the particles size, which is achieved by cutting, breaking and spraying the matrix (Lincoln, 1996). In acrylamide analysis, special care should be taken in this step, since formation of acrylamide takes place mainly at the surface of the food not inside. It is at the food surface where the most suitable temperature and humidity conditions for Maillard reactions occur.

Thus, is necessary to ensure that the aliquot taken for analysis includes all parts of the food, in correct proportions, to ensure its representativeness. Petersson et al. (2006)

demonstrated the importance of using use small particles (<1000 microns) in order to obtain homogenous sampling and satisfactory extraction yields.

Different procedures have been published to homogenizing samples, according to the matrix. Household blender and grinding have been used for potato chips and cereal based products (Bermudo et al., 2006b; Petersson et al., 2006). The use of a mortar and pestle for manual disruption of potato chips and wafers samples was also proposed (Fernandes and Soares, 2007; Kim et al. 2007) or an electric mill for meat and coffee samples (Hoenicke et al., 2004). Ultra-turrax homogenization for 1 to 2 minutes of potato chips or bread immersed in the extraction solvent also showed good results (Delatour et al., 2004; Gökmen et al., 2005; Pittet et al., 2004).

5.1.2. Extraction

Water is generally considered the first choice as extractive solvent for safety, cost, environmental and efficiency reasons. Even when organic solvents are used, the pre-addition of water (swelling) to the matrix is usually performed to provide better access to potentially adsorbed or enclosed acrylamide. Usually, homogenized samples are mixed with water, followed by an adequate amount of a specific internal standard solution, and kept at a pre-specified temperature for 10 to 30 min. Depending on the matrix, swelling yields an increase in analyte recovery of up to 100-fold (Biedermann et al. 2002a). When using water, extraction is usually carried out at room temperature (Tareke et al., 2002; Pittet et al., 2004), although some authors advise hot water (60-80°C), since acrylamide is relatively stable at this temperature and the yield can be reasonably growth (Dunovská et al., 2004; and Şenyuva Gökmen, 2006). Increased recovery rates could also be expected by treating the samples in an ultrasonic baths (30 min at 60°C) (Schaller 2003).

Although liquid-base extraction using water provides satisfactory results, the co-extracted components, such as sugars, proteins, amino acids, organic acids, etc., might affect the chromatographic performance. Thus, some authors suggest the use of organic solvents instead, as methanol (Şenyuva and Gökmen 2006), ethanol (Ciesarová et al. 2004), isopropanol (Pabst et al., 2005), acetonitrile/water (Jezussek and Schieberle, 2003) and methanol/water (Petersson et al, 2006), in order to reduce the co-extraction of these major components.

The effects of other factors, such as salt addition and pH, on acrylamide extraction have also been studied. Jiao et al. (2006) suggests the extraction with concentrated sodium chloride solution in order to prevent the formation of emulsions during purification,

increasing the extraction yield. Some authors also suggest the use of acidic aqueous solutions to increase yield extraction. Gertz and Klostermann (2002) used aqueous acetic acid at 37 °C and Taubert et al. (2004) used aqueous perchloric acid solution. On the other hand, higher amounts of acrylamide (three or four times) were observed in food samples by increasing to pH> 12. On reason might be that during normal water extraction polyacrylamide sterically hinders all of the acrylamide from getting into the solutions (Keramat et al., 2011). Alkaline pH can also change the structure of the matrix and facilitate the free acrylamide to get into the solution. Also, increasing pH releases chemically bound acrylamide (bound with protein and carbohydrate) to become available for analysis with this extraction technique (Keramat et al., 2011).

Generally, liquid-based extraction at room temperature and pressure remains the most common approach to extract acrylamide from foods, typically with a 10:1 liquid solvent-to sample ratio aided by a bleeding device for better extraction. For solid samples, sonication, mixing with a blender, extended shaking or mixing on a vortex with water mixed or not with organic are commonly employed. Also, extraction of acrylamide with methanol by means of a Soxhlet devise, far exceeded liquid-base extraction (<90%) (Keramat et al., 2011). However, one drawback is the long extraction time mentioned by the authors, of 10 days, while no information is available on the potential acrylamide formation during the extraction itself (Wenzl at al. 2003).

To overcome these problems, instrumental technique that control pressure and/or temperature of the solvent during extraction have been developed and employed. Pressurized liquid extraction (PLE) technique known commercially as ASE - Accelerated Solvent Extraction (Dionex, 2003) has been used in acrylamide extraction. This technique consists in extracting solid samples or semi-solid with conventional liquid solvents at elevated temperatures and pressures to increase the process efficiency. This technique reduces labour, solvent consumption and variability through automation. Yusá et al. (2006) applied this technique to cereals and potatoes chips samples using acetonitrile as extractive solvent. According to the authors, the technique allows a fast and automated extraction of acrylamide in various food samples.

5.1.3. Purification

As above mentioned, an inherent difficulty in acrylamide extraction with water is the co-extraction of major components of the matrix. The presence of these interferents may cause a multitude of problems, including the formation of emulsions, incorrect

chromatographic separation and detector contamination, with decreasing of the overall method performance.

Different clean-up procedures have been used in acrylamide analysis. The most common are: a) selective precipitation of proteins and carbohydrates, b) additional liquid-liquid extraction for fat elimination and c) solid-phase extraction.

5.1.3.1. Selective precipitation of proteins, carbohydrates and polysaccharides

Selective precipitation can be used to separate proteins, carbohydrates and polysaccharides from food matrices by using different processes: isoelectric point precipitation, precipitation with organic solvents, addition of non-ionic hydrophilic polymers, and flocculation by polyelectrolytes (Linden and Lorient, 2000).

Proteins can be removed with addition of potassium hexacyanoferrate trihydrate solution, K_4 [Fe (CN) $_6$].$3H_2O$ (Carrez I) followed by a heptahydrate zinc sulfate solution, $ZnSO_4.7H_2O$ (Carrez II). In this process, proteins are precipitated by molecular occlusion during the formation of a white precipitate (K_2Zn_3 [Fe $(CN)_6$]2), eliminating about 85% protein sample (Sun et al, 2003). Several authors applied this procedure in potato chips and cereal products (Ciesarová et al., 2004; Hamlet et al., 2004; Gökmen et al, 2005; Şenyuva and Gökmen, 2005a and 2006). Other authors proposed the use of organic solvents such as methanol (Jezussek and Schieberle, 2003), 1-propanol (Dunovská et al., 2004) and acetonitrile (Hoenicke et. al., 2004) to deproteination.

Polysaccharides are mainly responsible by the formation and stability of coffees foam, but they can preclude the correct quantification of their acrylamide content. The use of ethanol to reduce foam formation and precipitate proteins combined with isolute SPE extraction showed be efficient in acrylamide extraction of coffee and soluble coffee (Soares et al. 2006).

5.1.3.2. Liquid-liquid extraction

Liquid-liquid extraction with hexane (Dunovská et al., 2004; Ciesarová et al., 2004; Wenzl et al., 2004; Bermudo et al., 2006a), petroleum ether (Jiao et al, 2005; Zhang et al, 2005), cyclohexane (Petersson et al., 2006), dichloromethane (Delatour et al., 2004) or 1,2-

dichloroethane (Garcia-Molina et al. 2014), can help in the fat elimination from water and/or acetonitrile solutions. Fat can be also removed by simple filtration after acylglicerol crystallization at low temperatures (below 0°C). Other methods include phase separation by centrifugation followed by removal of the water fraction by azeotropic distillation (Biedermann et al. 2002a, 2002b)

5.1.3.3. Solid-phase extraction

Solid phase extraction has been widely used as a purification technique alone or in combination with additional purification steps already presented. In acrylamide analysis, SPE involves usually the combination of various sorbents (Florisil, alumina, silica gel) to ensure multi-interactions and extend the range of eliminating interfering compounds. As a rule, this separation is carried out in aqueous extracts immediately before the chromatographic process, or prior to derivatization when it takes place. In some cases, the organic extraction is applied after the derivatization and extraction steps, on the formed derivative.

Some research groups suggested the use of non-polar sorbents such as C18 (Şenyuva and Gökmen, 2005; Gökmen et al., 2005; Jiao et al., 2005; Kim et al. 2007) or graphite (Tareke et al., 2002) to purify aqueous extracts of potato chips, breakfast cereals and bread samples. Other authors proposed sorbents mixtures (polar, anion exchange and cation exchange) for purification of potato chips and cereal extracts (Nielsen et al., 2006) and aqueous samples of coffee, powdered chocolate and cocoa (Delatour et al., 2004). Jezussek and Schieberle (2003) used diatomaceous earth columns to purify aqueous potato chips extracts before GC-MS analysis. Other groups suggest a sequential combination of two or more different sorbents. Govaert et al. (2006) and Petersson et al. (2006) proposed non-polar sorbents followed by mixed-mode sorbents to purify extracts of potato chips, coffee, chocolate, bread and cereal. Riediker and Stadler (2003) used a combination of a mixed-mode and cation exchange sorbents for purification of cereal based before analysis by LC-MS/MS. In all these works water is used as the elution solvent.

More recently, Wenzl et al. (2009) purified aqueous extract of coffee by passage through two SPE columns: an Isolute Multimode followed by an Isolute Env$^+$. Isolute Multimode contains a silica-based C-18 group as well as anion and cation exchangers. Acrylamide is not retained in the column, but many matrix components (nonpolar and ionic compounds) that could exert interference are retained. At the second solid-phase extraction step, an Isolute ENV$^+$ cartridge that contains a polymer-based phase with high capacity to

bind acrylamide. The analysis of performed by HPLC after elution of acrylamide with 60% methanol in water.

Generally, SPE techniques are applied to aqueous extract prior derivatization or directly before LC-MS/MS or GC-MS analysis. However, SPE can be also applied to derivatize organic extracts. Some authors have suggested Florisil to perform the purification of brominated extracts of potato chips, and cereals samples (Pittet et al., 2004).

In general, after extraction and purification procedures, it is further necessary to remove the solvent excess. In most of works described previously, vacuum-vaporation is carried out between 50 and 60°C. However, this step is also critical or the effectiveness of the global methods, because most acrylamide is loss when the aqueous extracts are evaporated to dryness (Biedermann et al., 2002a). Furthermore, when samples are excessively concentrated by evaporation, gelatinous solutions could be formed which decrease recovery (Ahn et al., 2002). To overcome these problems Yamazaki et al. (2012) proposed the addition of diethylene glycol solution to the aqueous extract before concentrating it on a rotatory evaporator.

5.2. Acrylamide quantification

The quantification of acrylamide is usually achieved using chromatographic techniques, both HPLC and GC. The major difference between the two is that the first easily allow direct acrylamide analysis while GC usually requires a prior derivatization with consequent time-consumption and labor. Common to both techniques is the use of mass spectrometry (MS) detector, justified due the complexity of the matrices analyzed and the high sensitivity levels required.

5.2.1. Internal standards

The use of structural analogs was initially proposed by several authors in particular N, N-dimethylacrylamide (Tareke et al., 2000 and 2002), methacrylamide (Taubert et al., 2004), propionamide (Biedermann et al. 2002a, 2002b; Ciesarová et al., 2004) and butyramide (Biedermann et al., 2002a).

However, the use of isotopic analogs of acrylamide as internal standard has enormous advantages over other substances, since they have identical chemical and

physical properties. Its use in chromatographic methods, however, is confined MS detectors, because they allow mass separation of the analyte and their isotopic analog.

Three stable isotopes are used to internal standards-deuterium (2H), carbon-13 (13C), and nitrogen-15 (15N). The number of isotope and their position in the molecule are important to ensure the correct separation of the analyte and their isotope; ideally three atoms are incorporated in positions that not allow structural isotope exchange. Among the isotope commercially available $^{13}C3$-acrylamide (Tareke et al., 2002; Riediker and Stadler, 2003; Pittet et al., 2004), $^{13}C1$ (Nemoto et al., 2002) and $^{2}H3$-acrylamide (Ono et al., 2003; Peng, 2003) have been commonly applied in acrylamide analysis.

5.3. Acrylamide analysis by GC-MS

5.3.1. GC-MS with derivatization of acrylamide

Although acrylamide can be analysed without derivatization, the molecule is generally brominated to form 2,3-dibromopropionamide that presents the best chromatographic properties when using GC-MS .

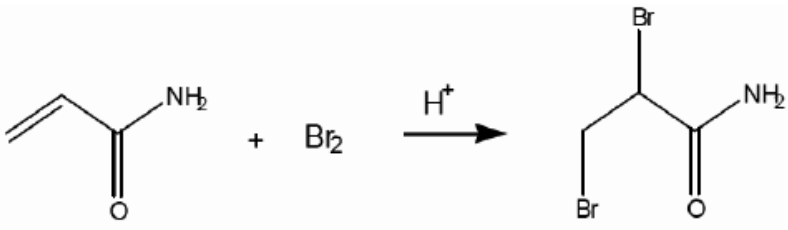

Figure 5.1. Derivatization of acrylamide to 2,3-dibromopropionamide

The main advantages of acrylamide derivatization of acrylamide is to form a relatively volatile compound with less polarity and higher molecular weight, thus more easily detectable by mass spectrometry (due to the greater mass of the fragments formed). Additionally, the derivative formed can be extracted with an organic solvent which allows obtaining final extracts free of polar compounds usually present in large amounts in the aqueous extract: sugars, proteins, amino acids, etc. The brominated derivative is identified by its retention time and ratio mass of characteristic ions.

In practice, derivatization process involves the addition of potassium bromide (KBr), hydrobromic acid (HBr) and bromine (Br2) saturated solution to the aqueous extract allowing, to react for at least one hour at 0° C in an oil or ice bath (Hardas et al., 1999). Excess bromine is removed by the addition of a sodium thiosulfate solution until the solution becomes colourless (Ono et al. 2003; Ciesarová et al., 2004; Hamlet et al. 2004; Pittet et al., 2004; Williams, 2005). The brominated derivative formed is less polar compared with the original module, and is therefore easily extracted with ethyl acetate (Ono et al. 2003; Jezussek and Schieberle, 2003; Hamlet et al., 2004) or a mixture of ethyl acetate and n-hexane in a ratio of four to one (4:1) (Tareke et al., 2000 and 2002; Pittet et al., 2004). However, Andrawes et al. (1987) have shown that, under certain conditions, the 2,3-dibromopropionamide fromed can be converted into a more stable derivative 2-bromopropenamide on GC inlet or directly on the capillary column. Because of this decomposition a decrease in reproducibility and accuracy can occur, being preferable to deliberately convert 2,3-2-dibromopropionamide to 2-bromopropenamide prior to GC analysis, which can be achieved by the addition of 10% triethylamine to the final extract before injection into the chromatograph (Hamlet et al., 2004; Pittet et al., 2004). This conversion is virtually instantaneous at room temperature and it is quantitative and reproducible (Andrawes et al., 1987).

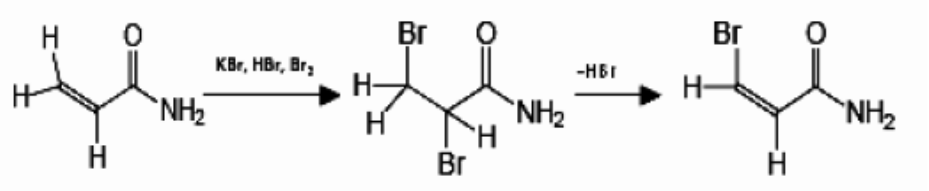

Figure 5.2. Conversion of acrylamide to 2,3-dibromopropionamide and then to 2-bromopropenamide. (Adapted from Rothweiller et al., 2003).

Robarge et al. (2003) converted 2,3-dibromopropionamide to 2-bromoproprenamide in *situ* by thermal decomposition in the injector, using ammonia as reagent gas. However, some care must be taken to quantify this acrylamide derived, since it affords the fragment *m/z* 149 which is subject to interference from phthalates present in food packing materials (Rothweiler et al., 2003).

Other bromination technique suggested by Nemoto et al. (2002), is based on the production of the bromine molecule be produced through an oxidation-reduction reaction between potassium bromate (KBrO3) and potassium bromide (KBr), eliminating the hazard of

handling elemental bromine. Despite not having assessed accurately its yield, this reaction it is undoubtedly a safer way to perform this derivatization.

In GC-MS analysis, the ions monitored for 2,3-dibromopropionamide are: $[C_2H_3{}^{79}Br]^+$= 106, $[C_2H_3{}^{81}Br]^+$= 108, $[C_3H_5{}^{79}BrNO]^+$= 150 and $[C_3H_5{}^{81}BrNO]^+$= 152 being the fragment m/z 150 used for quantification (Figure 5.3). For the internal standard 2,3-dibromo($^{13}C_3$)propionamide the ions are monitored included- $[^{13}C_2H_4{}^{81}Br]^+$= 110, $[^{13}C_3H_5{}^{79}BrNO]^+$= 153 and $[^{13}C_3H_5{}^{81}BrNO]^+$= 155 (Tareke et al., 2002). In the case of using a deuterated standard ions to be monitored are $[C_{32}H_3{}^{79}Br]^+$= 109, $[C_{22}H_3{}^{81}Br]^+$=111, $[C_3H_{22}H_3{}^{79}BrNO]^+$=153 and $[C_3H_{22}H_3{}^{81}BrNO]^+$= 155. In both cases the fragment *m/z* 155 is used for quantification (Ono et al. 2003; Ciesarová et al., 2004).

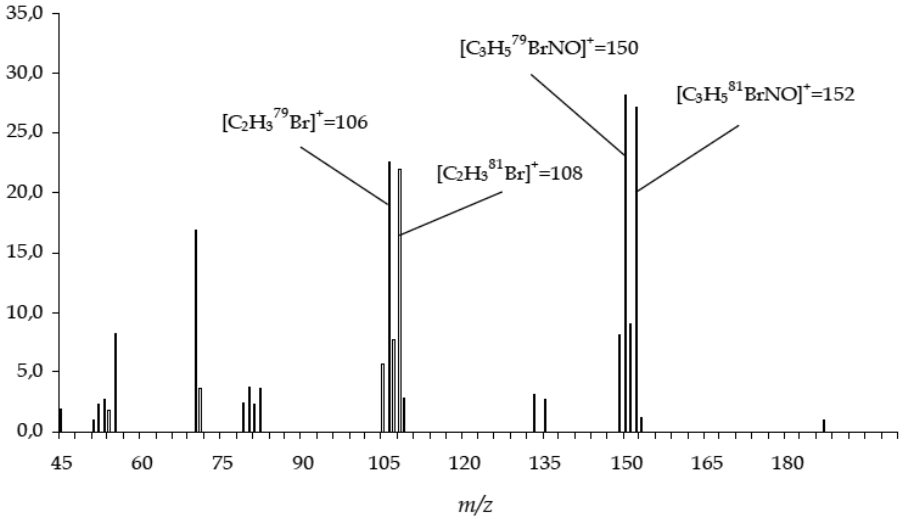

Figure 5.3. Mass spectrum of the brominated derivative of acrylamide: 2,3-dibromopropionamide.

In the case of conversion to 2-bromopropenamide, the ions to be monitored are $[C_3H_4NO]^+$= 70, the $[C_3H_4{}^{79}BrNO]^+$= 149 and $[C_3H_4{}^{81}BrNO]^+$= 151, using the fragment (*m/z*) 149 for quantification. Ions monitored to identify the 2-bromo($^{13}C_3$) propenamide internal standard are $[^{13}C_2H_3{}^{81}Br]^+$= 110, and $[^{13}C_3H_4{}^{81}BrNO]^+$= 154, using *m/z* 154 for the quantification. In the internal standard methacrylamide the monitored ions are *m/z* 120 and 122 (Ahn et al., 2002).

With the deuterated internal standard is possible to occur exchange between deuterium and hydrogen during derivatization reaction, which decreases the analysis accuracy. Thus, it is advisable to use acrylamide marked with ^{13}C.

Bromination results in an increased selectivity, which compensates for the hard and time consuming derivatization process. Generally, the detection limits of the methods that include bromination range from 2 to 25 mg/kg, depending on the sample and the purification procedures employed.

As an alternative to bromination, Lagalante and Felter (2004) proposed the silylation of acrylamide with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) to give the volatile compound N,O-bis(trimethylsilyl)acrylamide (BTMSA). The derivative was easily extracted by headspace solid phase microextraction (SPME) using a poly (dimethylsiloxane) fiber.

Recently, some studies have been published on a novel acrylamide derivatization technique, using xanthydrol as reagent (Figure 5.4), followed by GC-MS determination. This method was applied to diverse foodstuffs and water, and it is claimed to be more environmentally friendly, requiring mild reaction conditions at low temperature, and proceeds in aqueous solution (Tsukakoshi et al. 2012; Yamazaki et al. 2012 Lim and Shin 2013). Therefore, a series of drawbacks that occur with bromination derivatization are avoided when xanthydrol is used. For instance, obtaining unstable derivatives and inconsistent detection is usual in bromination procedure unlike with the stable xanthyl derivative obtained when xanthydrol is used (Yamazaki et al. 2012). Moreover, lower susceptibility to interference has been also attributed to the xanthyl-acrylamide due to its larger mass (m/z 251) compared with bromo derivative (m/z 150) (Mizukami et al. 2006). All these advantages together with its robustness and the possibility to be applied to a large variety of foods make this new and recent derivatization method an excellent alternative to the classical and tedious bromination (Garcia-Molina et al., 2014).

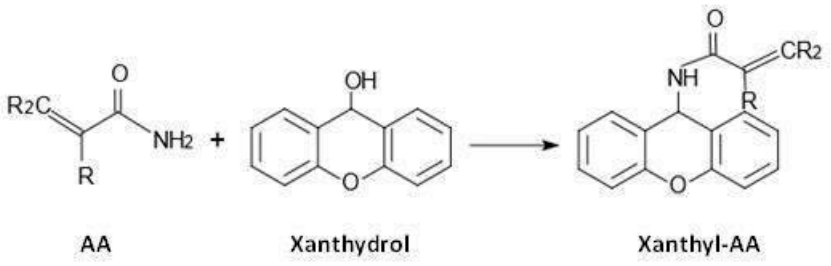

Figure 5.4. Derivatization reaction of acrylamide with xanthydrol.

The analysis of the acrylamide derivatives by GC/MS is always performed in capillary columns with midpolar to polar phases. Several GC capillary columns have been proposed, such as DB17 (50%-phenyl-dimethylpolysiloxane), ZB50 (50%-phenyl-dimethylpolysiloxane), Optima WAX (polyethyleneglycol 20 kDa), or RTX-200 Crossbond (trifluoropropylmethylpolysiloxane). Dimensions should be of 30 m of length and 0.25 mm of internal diameter with 0.25 μm of thickness. The injected volume varied between 1 and 2 μL of sample in splitless mode. The initial oven temperature ranges from 60-85 °C and in many cases, the heating ramp is 15°C per minute. The final oven temperature is typically less than 250°C.

5.3.2. GC-MS without derivatization acrylamide

Without derivatization, acrylamide analysis by GC-MS has been performed by researchers such as Ellenberg et al. (2004) Hoenicke et al. (2004) and Amrein et al. (2005). The major drawback of GC-MS analysis without derivatization was the lack of characteristic peaks in the mass spectra. The main ions are *m/z* 55 and 72, used also for quantification. Co-extracted substances such as maltol or heptanoic acid showed nearly the same fragmentation pattern and may therefore interfere (Biedermann et al., 2002a).In analysis of acrylamide without derivatization the detection limits range between 4 and 20 mg/kg.

5.4. Acrylamide analysis by LC-MS/MS

5.4.1. LC-MS/MS without derivatization

LC-MS/MS analysis of acrylamide features high sensitivity and makes it possible to avoid the derivatization step. For the chromatographic separation of acrylamide, the most authors have proposed reverse phase chromatography with C18 columns (Rufian-Henares and Morales, 2006). The main difficulty lies in the choice of the appropriate mobile phase to obtain adequate separation of the compound with a reasonable retention time due to the high polarity of acrylamide.

The LC-MS/MS technique has a high selectivity, particularly in MRM mode (Multiple Reaction Monitoring). In this way, the first quadrupole (Q1) selects and transmits a precursor ion with a specific m/z. This ion is then fragmented in the second quadrupole

(Q2 collision cell), and a specific product ion with a defined m/z is selected and transmitted in the third quadrupole (Q3).

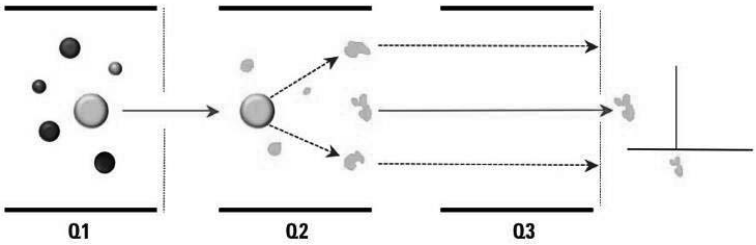

Figure 5.5. Multiple reactions occurring within the LC-MS/MS. (Adapted from: http: //www.chem.agilent.com)

The ions monitored for acrylamide analysis by LC-MS/MS are $[CH_2=CHC=O]^+$ *m/z* 55 and $[CH_2=CHC=NH]^+$ *m/z* 54, generated by collisions with argon in the second quadrupole from the fragment ($[CH_2=CHCONH_2]^+$) *m/z* 72 generated in the first quadrupole.

The transition 72> 55 is always selected for quantification because it has a high relative abundance (Ahn et al, 2002; Hoenicke et al., 2004; Murkovic, 2004). In some cases, other transitions have been used to confirm the presence of acrylamide, as 72 > 54, 72 > 72, and 44 > 27 (Leung et al., 2003; Delatour et al., 2004; Zhang et al, 2005b; Nielsen et al, 2006). For the detection of acrylamide isotopes used as internal standard, the transitions monitored are 75 > 58 in the case of $[^2H_3]$-acrylamide, and $[^{13}C_3]$-acrylamide (Ono et al., 2003; Kim et al., 2007), and 73 > 56 for $[^{13}C_1]$-acrylamide (Zhang et al., 2005a). Petersson et al. (2006) used a 55/58 ratio of ions to quantify the acrylamide.

Despite the high selectivity provided by tandem MS/MS, allowing good separation of analytes, the presence of interference in the extract of complex matrix is unavoidable (Becalski et al., 2003). These authors observed peaks with retention times identical to deuterated acrylamide and acrylamide. One promising approach is to re-extract the aqueous extract with a polar organic solvent as ethyl acetate in order to remove interfering constituents such as salts, sugars, starches, amino acids, etc. The obtained extract is usually concentrated before LC-MS/MS analysis (Hoenicke et al., 2004; Claus et al., 2005; Jiao et al., 2005; Zhang et al., 2005b). Becalski et al. (2003) reported the presence of an interfering compound when the transition 72 > 55 was used for detection of acrylamide. These authors

proposed the use of analytical columns with greater length (150 mm) and an additional purification step with mixed-phase sorbents to prevent this interference.

Recently, techniques based on ultra-performance liquid chromatography (UPLC) were developed, with improved resolution within a shorter retention time (Zhang et al., 2007). In this system the conventional LC with C18 columns, needed for separation of the polar compounds in order to avoid co-elution of acrylamide with other compounds, are replaced by sub-2 μm particle C18 columns which reduces run time and improves sensitivity. A comparative analyses of acrylamide in potato chip extracts, by two alternative LC–MS/MS systems the UPLC and the "classic" LC separation showed that (i) the values of height equivalent to the theoretical plate obtained in UPLC were mostly higher, however, their variability was also rather high, (ii) the analysis time in the system employing UPLC was reduced by more than 50% with similar analytical output, and (iii) UPLC provided significantly improved S/N followed by decreased limit of quantification (Zhang et al. 2007).

In general, methods based on LC-MS/MS are very sensitive and applicable to all types of samples showing lower detection limits of 10 mg/kg of acrylamide.

5.4.2. LC-MS/MS with derivatization

Jezussek and Schieberle (2003) developed a method for LC-MS/MS for quantification of acrylamide in cereal products based on derivatization with mercaptobenzoic acid. The detection limit obtained was of 6.6 mg/kg. The results were promising but the authors considered that it was still necessary to optimise the proposed methodology to complex samples as coffee and chocolate.

5.5. Other analytical techniques used to determine the acrylamide

Although, chromatography techniques are the most applied in acrylamide analysis several other methods have been used for such as capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MEKCC), time-of-flight mass spectrometry (TOF-MS), electrochemical detection, biosensors, and enzyme-linked immunosorbent assay (ELISA).

Capillary electrophoresis (CE) techniques was first proposed for the determination of acrylamide without derivatization, but this method may have a relatively high limit of detection value. An improved MEKCC method was developed for the separation and quantification of acrylamide and has been approved as a reliable method (limit of detection 0.1 μg/mL) (Zhou et al., 2007). On the basis of the above techniques, Bermudo et al. (2006a) presented a method in which the acrylamide was analysed by CZE after derivatization with mercaptobenzoic acid. In 2004, the same group had published a work in which acrylamide was analyzed by MEEKC. Both methods were applied to cereals and potatoes samples. The detection limits were 0.07 mg/mL and 0.7 mg/mL for CZE and MEEKC, respectively. To further improve the detection limits and to apply the method over a wide range of samples, field-amplified sample injection (FASI) was proposed (limit of detection 1 μg/L) (Bermudo et al., 2006a). Based on the FASI-CE technique, Bermudo et al. (2007) showed the applicability of CE coupled with MS/MS for the analysis of acrylamide in foods and obtained both good linearity and precision. In addition to the FASI-CE method, a relative field-amplified sample stacking (FASS) was also developed and regarded as a simple, rapid, and inexpensive choice (Tezcan and Erim, 2008).

With the rapid development of TOF–MS technique, the LC or GC method combined with a high resolution TOF–MS was used for the analysis of acrylamide and was validated by the Food Analysis Performance Assessment Scheme (FAPAS) (Dunovská et al., 2006). Compared to robust GC–MS or LC–MS/MS, the applicability of TOF–MS for the quantification of acrylamide still needs to be optimized andimproved. Electrochemical detection of DNA damage induced by acrylamide and its metabolites is an alternative new technique using the graphene-ionic liquid-Nafion modified pyrolytic graphite electrode (Qiu et al., 2011).

5.6. Official methods validated acrylamide analysis

There are several comprehensive reviews published on the numerous methods for acrylamide detection to date. However, an official method of acrylamide quantification in food has not yet been recommended. Efforts on standardization of methods used to detect and quantify acrylamide in foods have continued. A draft for an official method of acrylamide in foods has already been established by the European Committee of Normalization (Comité Europeén de Normalisation, CEN) under a specific European Commission mandate M/463 dated April 30, 2010. Regularly, Food Analysis Performance Assessment Scheme (FAPAS) organizes interlaboratory trials for quality assessment and validation of acrylamide methods in a proficiency testing scheme. Proficiency tests have shown that two main approaches are used for acrylamide: HPLC coupled to tandem mass

spectrometry (MS/MS) and GC of the mono- or dibromo derivative of acrylamide with electron ionization and mass spectrometry detection of the fragment ions (Morales and Mesias 2015).

6. References

Adler ID, Baumgartner A, Gonda H, Friedman MA, Skerhut M. 1-Aminobenzotriazole inhibits acrylamide-induced dominant lethal effects in spermatids of male mice. Mutagenesis 2000; 15 (2): 133-136.

Agency for Toxic Substances , Disease Registry (ATSDR), Toxicological Profile for Acrolein, U.S. Department of Health and Human Services, Atlanta 2007.

Agency for Toxic Substances and Disease Registry (ATSDR). 2012. Toxicological profile for Acrylamide.Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

Agroportal, 2015. Available at: http://www.agroportal.pt/a/2013/tpetulante.htm

Ahn JS, Castle L, Clarke DB, Lloyd AS, Philo MR, Speck DR. Verification of the findings of acrylamide in heated foods. Food Addit Contam 2002; 19 (12): 1116-1124.

Ahn JS, Castle L. Tests for the depolymerization of polyacrylamides as a potential source of acrylamide in heated foods. J Agr Food Chem 2003; 51 (23): 6715-6718.

Akıllıoglu HG, Gokmen V. Mitigation of acrylamide and hydroxymethyl furfural in instant coffee by yeast fermentation. Food Res Int 2014; 61: 252-256.

Amrein T. Systematic Studies on Process Optimization to Minimize Acrylamide Contents in Food". Diss. ETH, 16311, Zurique, 2005.

Amrein TM, Bachmann S, Noti A, Biedermann M, Barbosa MF, Biedermann-Brem S, et al. Potential of acrylamide formation, sugars, and free asparagine in potatoes: a comparison of cultivars and farming systems. J Agr Food Chem 2003; 51: 5556–5560.

Amrein TM, Lukac H, Andres L, Perren R, Escher F, Amadò R. Acrylamide in roasted almonds and hazelnuts. Agr Food Chem 2005; 53 (20): 7819-7825.

Amrein TM, Schonbachler B, Escher F, Amado R. Acrylamide in Gingerbread: Critical Factors for Formation and Possible Ways for Reduction. J Agric Food Chem 2004; 52 (13): 4282-4288.

Andersen ML, Outtrup H, Skibsted LH. Potential antioxidants in beer assessed by ESR spin trapping. J Agr Food Chem 2000; 48 (8): 3106-3111.

Andrawes F, Greenhouse S, Draney D. Chemistry of acrylamide bromination for trace analysis by Gas Chromatography and Gas Chromatography-Mass Spectrometry. J Chromatogr 1987; 399: 269-275.

Anese M, Bortolomeazzi R, Manzocco L, Manzano M, Giusto C, Nicoli MC. Effect of chemical and biological dipping on acrylamide formation and sensory properties in deep-fried potatoes. Food Res Int 2009; 47: 142–147.

Anese M, Quarta B, Frias J. Modelling the effect of asparaginase in reducing acrylamide formation in biscuits. Food Chem 2011; 126: 435–440.

Arisseto AP, Toledo MC, Govaert Y, Van Loco Y, Fraselle S, Weverbergh E, et al. Determination of acrylamide levels in selected foods in Brazil. Food Addit Contam 2007; 24 (3): 236-241.

Arvanitoyannis I S,Dionisopoulou N. Acrylamide: Formation, Occurrence in Food Products, Detection Methods, and Legislation. Crit Rev Food Sci 2014; 54 (6): 708-733.

Augustsson K, Skog K, Jagerstad M, Dickman PW, Steineck G. Dietary heterocyclic amines and cancer of the colon, rectum, bladder, and kidney: A population-based study. Lancet 1999; 353 (9154): 703-707.

Bagdonaite K, Derler K, Murkovic M. Determination of acrylamide during roasting of coffee. J Agr Food Chem 2008; 56: 6081–6086.

Banchero M, Pellegrino G, Manna L. Supercritical fluid extraction as a potential mitigation strategy for the reduction of acrylamide level in coffee. J Food Eng 2013; 115: 292–297.

Barber DS, Hunt JR, Ehrich MF, Lehning EJ, LoPachin RM. Metabolism, toxicokinetics and hemoglobin adduct formation in rats following subacute and subchronic acrylamide dosing. Neurotoxicology 2001; 22 (3): 341-353.

Bassama J, Brat P, Bohuon P, Boulanger R, Günata Z. Study of acrylamide mitigation in model system: Effect of pure phenolic compounds. Food Chem 2010; 123 (2): 558-562.

Becalski A, Benjamin P-YL, Lewis D, Stephen WS. Acrylamide in Foods: Occurrence, Sources, and Modeling. J Agr Food Chem 2003; 51: 802-808.

Becalski A, Lau B, Lewis D, Seaman S, Hayward S, Sahagian M, et al.. Acrylamide in French fries: influence of free amino acids and sugars. J Agr Food Chem 2004; 52 (12): 3801-3806.

Becalski A, Stadler R, Hayward S, Kotello S, Krakalovich T, Lau BP-Y, et al. Antioxidant capacity of potato chips and snapshot trends in acrylamide content in potato chips and cereals on the Canadian market. Food Addit Contam 2010; 27 (9): 1193-1198.

Bergmark E, Calleman CJ, Costa LG. Formation of hemoglobin adducts of acrylamide and its epoxide metabolite glycidamide in the rat. Toxicol Appl Pharmacol 1991; 111 (2): 352-363.

Bergmark E, Calleman CJ, He F, Costa LG. Determination of hemoglobin adducts in humans occupationally exposed to acrylamide. Toxicol Appl Pharmacol 1993; 120 (1): 45-54.

Bermudo E, Moyano E, Puignou L, Galceran MT. Determination of acrylamide in foodstuffs by liquid chromatography ion trap tandem mass-spectrometry using an improved purification procedure. Anal Chim Acta 2006b; 559: 207-214.

Bermudo E, Núñez O, Moyano E, Puignou L, Galceran MT. Field amplified sample injection-capillary electrophoresis-tandem mass spectrometry for the analysis of acrylamide in foodstuffs. J Chromatogr A 2007; 1159: 225–232.

Bermudo E, Núñez O, Puignou L, Galceran MT. Analysis of acrylamide in food samples by capillary zone electrophoresis. J Chromatogr A 2006a; 1120: 199-204.

Bermudo E, Ruiz-Calero V, Puignou L, Galceran MT. Microemulsion electrokinetic chromatography for the analysis of acrylamide in food. Electrophoresis 2004; 25: 3257-3262.

Besaratinia A, Pfeifer GP. A review of mechanisms of acrylamide carcinogenicity. Carcinogenesis 2007; 28 (3): 519-528.

Besaratinia A, Pfeifer GP. Genotoxicity of acrylamide in humans and mice: Promutagenic glycidamide-DNA adducts in the human p53 gene and the cII transgene. Abstracts: Proc Am Assoc Cancer Res 2004; 45: 452.

Biedermann M, Biedermann-Brem S, Noti A, Grob K, Egli P, Mandli H. Two GC-MS methods for the analysis of acrylamide in foods. Mitt Lebensm Hyg 2002b; 93: 638–652.

Biedermann-Brem S, Noti A, Grob K, Imhof D, Bazzocco D, Pfefferle A. How much reducing sugar may potatoes contain to avoid excessive acrylamide formation during roasting and baking?. Eur Food Res Technol 2003; 217 (5): 369-373.

Bjellaas T, Olesen PT, Frandsen H, Haugen M, Stolen LH, Paulsen JE, et al. Comparison of estimated dietary intake of acrylamide with hemoglobin adducts of acrylamide and glycidamide. Toxicol Sci 2007b; 98 (1): 110-117.

Bjellaas T, Stolen LH, Haugen M, Paulsen JE, Alexander J, Lundanes E, et al. Urinary acrylamide metabolites as biomarkers for shortterm dietary exposure to acrylamide. Food Chem Toxicol 2007a; 45 (6): 1020-1026.

Blenkinsop RW, Copp LJ, Yada RY, Marangoni AG. Changes in compositional parameters of tubers of potato (Solanum tuberosum) during low-temperature storage and their relationship to chip processing quality. J Agr Food Chem 2002; 50 (16): 4545-4553.

Blom H, Baardseth P, Sundt TW, Slinde E. Lactic acid fermentation reduces acrylamide formedduring production of fried potato products. Asp. Appl. Biol. 2009 http://zeracryl.files.wordpress.com/2010/10/blom-2009.pdf

Boettcher MI, Bolt HM, Angerer J. Acrylamide exposure via the diet: Influence of fasting on urinary mercapturic acid metabolite excretion in humans. Arch Toxicol 2006b; 80: 817-819.

Boettcher MI, Bolt HM, Drexler H, Angerer J. Excretion of mercapturic acids of acrylamide and glycidamide in human urine after single oral administration of deuterium-labelled acrylamide. Arch Toxicol 2006a; 80 (2): 55-61.

Boettcher MI, Schettgen T, Kutting B, Pischetsrieder M, Angerer J. Mercapturic acids of acrylamide and glycidamide as biomarkers of the internal exposure to acrylamide in the general population. Mutat Res 2005; 580 (1-2): 167-176.

Brathen E, Knutsen SH. Effect of temperature and time on the formation of acrylamide in starch-based and cereal model systems, flat breads and bread. Food Chem 2005; 92: 693–700.

Buranasilp K, Charoenpanich J. Biodegradation of acrylamide by Enterobacter aerogenes isolated from wastewater in Thailand. J Environ Sci 2011; 23 (3): 396–403.

Calleman CJ, Wu Y, He F, Tian G, Bergmark E, Zhang S, et al. Relationship between biomarkers of exposure and neurological effects in a group of workers exposed to acrylamide. Toxicol Appl Pharmacol 1994; 126 (2): 361-371.

Calleman CJ. The metabolism and pharmacokinetics of acrylamide: Implications for mechanisms of toxicity and human risk estimation. Drug Metab Rev 1996; 28 (4): 527-590.

Can NO, Arli G. Analysis of acrylamide in traditional and nontraditional foods in Turkey using HPLC-DAD with SPE cleanup. J Liq Chromatogr R T 2014; 37 (6): 850-863.

Capuano E, Ferrigno A, Acampa I, Serpen A, Acar OC, Gokmen V, et al. Effect of flour type on Maillard reaction and acrylamide formation during toasting of bread crisp model systems and mitigation strategies. Food Res Int 2009; 42: 1295–1302.

Capuano E, Fogliano V. Acrylamide and 5-hydroxymethylfurfural (HMF): a review on metabolism, toxicity, occurrence in food and mitigation strategies. LWT–Food Sci Technol 2011; 44: 793–810.

Capuano E, Oliviero T, Açar O, Gokmen V, FoglianoV. Lipid oxidation promotes acrylamide formation in fat-rich model systems. Food Res Int 2010; 43 (4): 1021-1026.

Cha M. Enzymatic control of the acrylamide level in coffee. Eur Food Res Technol 2013; 236: 567–571.

Chen F, Yuan Y, Liu J, Zhao G, Hu X, Survey of acrylamide levels in Chinese foods. Food Addit Contam B 2008; 1: 85–92.

Chen Y, Ho CT. Effects of carnosine on volatile generation from Maillard reaction of ribose and cysteine. J Agr Food Chem 2002; 50 (8): 2372-2376.

Chu YF. (Ed.). Coffee: emerging health effects and disease prevention (Vol. 59). John Wiley , Sons. 2012.

Chuda Y, Ono H, Yada H, Ohara-Takada A, Matsuura-Endo C, Mori M. Effects of physiological changes in potato tubers (Solanum tuberosum L.) after low temperature storage on the level of acrylamide formed in potato chips. Biosci Biotech Biochem 2003; 67 (5): 1188-1190.

CIAA (2011). Acrylamide toolbox 2011. http://www.fooddrinkeurope.eu/uploads/publications_documents/Toolboxfinal260911.pdf.

Ciesarová Z, Balasova V, Kiss E, Kolek E, Simko P, Kovac M. Comparison of Two Methods for Acrylamide Determination and Dietary Intake of Acrylamide from Potato Crisps in Slovakia. Czech J Food Sci 2004, 22: 251-254.

Ciesarova Z, Kiss E, Boegl P. Impact of L-asparaginase on acrylamide content in potato product. J Food Nutr Res 2006; 45: 141–146.

Claeys WL, De Vleeschouwer K, Hendrickx ME. Kinetics of acrylamide formation and elimination during heating of an asparagine–sugar model system. J Agr Food Chem 2005; 53: 9999–10005.

Claeys WL, De Vleeschouwer Kl, Hendrickx ME. Effect of amino acids on acrylamide formation and elimination kinetics. Biotechnol Progr2005; 21 (5): 1525-1530.

Claus A, Carle R, Schieber A. Acrylamide in cereal products: a review. J Cereal Sci 2008; 47: 118–133.

Codex. 2009. Code of Practice for the Reduction of Acrylamide in Foods (CAC/RCP 67–2009). http://wwwcodexalimentarius.net/download/standards/11258/CXP_067e.pdf

Commission Recommendation of 10.1.2011 on investigations into the levels of acrylamide in food. http://ec.europa.eu/food/food/chemicalsafety/contaminants/recommendation_10012011_acrylamide_food_pl.pdf. Accessed on 2013-08-15.

Commission Recommendation of 2 June 2010 on the monitoring of acrylamide levels in food (2010/307/EU). Off. J. Eur. Union L 137/4; 3.6.2010.

Commission Recommendation of 3 May 2007 on the monitoring of acrylamide levels in food (2007/331/EC). Off. J. Eur. Union L 123/33; 12.5.2007.

Cook DJ, Taylor AJ. On-line MS/MS monitoring of acrylamide generation in potato- and cereal-based systems. J Agr Food Chem 2005; 53: 8926–8933.

Daniali G, Jinap S, Hanifah NL, Hajeb P. The effect of maturity stages of banana on the formation of acrylamide in banana fritters. Food Control 2013; 32 (2): 386-391.

De Meulenaer B, De Wilde T, Mestdagh F, Govaert Y, Ooghe W, Fraselle S, et al. Comparison of potato varieties between seasons and their potential for acrylamide formation. J Sci Food Agr 2008; 88 (2): 313-318.

De Vleeschouwer K, Van der Plancken I, Van Loey A, Hendrickx ME Impact of pH on the kinetics of acrylamide formation/elimination reactions in model systems. J Agr Food Chem 2006; 54: 7847–7855.

De Wilde T, De Meulenaer B, Mestdagh F, Govaert Y, Ooghe W, Fraselle S, et al. Selection criteria for potato tubers to minimize acrylamide formation during frying. J Agr Food Chem 2006; 54 (6): 2199-2205.

De Wilde T, De Meulenaer B, Mestdagh F, Govaert Y, Vandeburie S, Ooghe W, et al. Influence of storage practices on acrylamide formation during potato frying. Journal of Agricultural and Food Chem 2005; 53 (16): 6550-6557.

Dearfield KL, Douglas GR, Ehling UH, Moore MM, Sega GA, Brusick DJ. Acrylamide: A review of its genotoxicity and an assessment of heritable genetic risk. Mutat Res 1995; 330 (1-2): 71-99.

Delatour T, Périsset A, Goldmann T, Riediker S, Stadler RH. Improved Sample Preparation to Determine Acrylamide in Difficult Matrixes Such as Chocolate Powder, Cocoa, and Coffee by Liquid Chromatography Tandem Mass Spectrometry. J Agr Food Chem 2004; 52: 4625-4631.

Doerge DR, Twaddle NC, Boettcher MI, MacDaniel P, Angerer J. Urinary excretion of acrylamide and metabolites in Fischer 344 rats and B6C3F1 mice administered a single dose of acrylamide. Toxicol Lett 2007; 169 (1): 34-42.

Doerge DR, Young JF, McDaniel LP, Twaddle NC, Churchwell MI. Toxicokinetics of acrylamide and glycidamide in B6C3F1 mice. Toxicol Appl Pharmacol 2005a; 202 (3): 258-267.

Doerge DR, Young JF, McDaniel LP, Twaddle NC, Churchwell MI. Toxicokinetics of acrylamide and glycidamide in Fischer 344 rats. Toxicol Appl Pharmacol 2005b; 208 (3): 199-209.

Dourson M, Hertzberg R, Allen B, Haber L, Parker A, Kroner O, et al. Evidence-based dose-response assessment for thyroid tumorigenesis from acrylamide. Regul Toxicol Pharmacol 2008; 52 (3): 264-289.

Dunovská L, Cajka T, Hajšlová J, Holadová K. Direct determination of acrylamide in food by gas chromatography-high-resolution time-of-flight mass spectrometry. Anal Chim Acta 2006; 578: 234–240.

Dunovska L. Hajslová J, Čajka T, Holadová K, Hájková K. Changes of Acrylamide Levels in Food Products during Technological Processing. Czech J. Food Sci 2004; 22: 283-286.

Dybing E, Farmer PB, Andersen M, Fennell TR, Lalljie SP, Muller DJ, et al. Human exposure and internal dose assessments of acrylamide in food. Food Chem Toxicol 2005; 43 (3): 365–410.

Dygert S, Li LH, Florida D, ThomaJA. Determination of reducing sugar with improved precision. Anal Biochem 1965; 13 (3): 367-374.

EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2013. Scientific Opinion on Acrylamide in Food. EFSA Journal, 303 pp. doi:10.2903/j.efsa.20YY.NNNN. (For public consultation).

EFSA. European Food Safety Authority. Update on acrylamide levels in food from monitoring years 2007 to 2010. EFSA Journal 2012; 10(10):2938-2976. Available online at: www.efsa.europa.eu/efsajournal.

Ehling S, Shibamoto T. Correlation of acrylamide generation in thermally processed model systems of asparagine and glucose with color formation, amounts of pyrazines formed, and antioxidative properties of extracts. J Agr Food Chem 2005; 53 (12): 4813-4819.

Ellenberg B. A General and Fast Methodology for the Extraction and GC-MS Analysis of Acrylamide from Carbohydrate-Rich Food Samples. 2004; Application Note 77. Germany: Varian.

EPA. National recommended water quality criteria. Washington, DC: Office of Water, Office of Science and Technology, U.S. Environmental Protection Agency. 2006. http://www.epa.gov/waterscience/criteria/wqcriteria.html.

European Commission. Special Eurobarometer nº246. Health and Food 2006. Available at: http://ec.europa.eu/health/eurobarometers/index_en.htm?Page=14

European Food Safety Authority, EFSA. (Accessed on 19-11-2013) http://www.efsa.europa.eu/en/topics/topic/acrylamide.htm

Fay LB, Brevard H. Contribution of mass spectrometry to the study of the Maillard reaction in food. Mass Spectrom Rev 2005; 24: 487– 507.

FDE. 2011. Food Drink Europe Acrylamide Toolbox. http://fooddrinkeurope.eu/uploads/publications_documents/Toolboxfinal260911.pdf

Fennell TR, Snyder RW, Sumner SC, Burgess J, Friedman MA. Kinetics of elimination of urinary metabolites of acrylamide in humans. Toxicol Sci 2006; 93 (2): 256-267.

Fennell TR, Sumner SC, Snyder RW, Burgess J, Spicer R, Bridson WE, et al. Metabolism and hemoglobin adduct formation of acrylamide in humans. Toxicol Sci 2005; 85 (1): 447-459.

Ferguson SA, Garey J, Smith ME, Twaddle NC, Doerge DR, Paule MG. Preweaning behaviors, developmental landmarks, and acrylamide and glycidamide levels after pre- and postnatal acrylamide treatment in rats. Neurotoxicol Teratol 2010; 32: 373-382.

Fernandes JO, Soares C. Application of matrix solid-phase dispersion in the determination of acrylamide in potato chips. J Chromatogr A 2007; 1175: 1-6.

Field EA, Price CJ, Sleet RB, Marr MC, Schwetz BA, Morrissey RE. Developmental toxicity evaluation of acrylamide in rats and mice. Fundam Appl Toxicol 1990; 14 (3): 502-512.

Fink M, Andersson R, Rosen J, Aman P. Effect of added asparagine and glycine on acrylamide content in yeast-leavened bread. CerealChem 2006; 83: 218–222.

Foot RJ, Haase NU, Grob K, Gondé P. Acrylamide in fried and roasted potato products: a review on progress in mitigation. Food Addit Contam 2006; 24 (1): 37-46.

Freisling H, Moskal A, Ferrari P, Nicolas G, Knaze V, Clavel-Chapelon F, et al. Dietary intake of adults in the European Prospective Investigationinto Cancer and Nutrition differs greatly according to geographical region. Eur J Nutr 2013; 52: 1369-1380.

Friedman M, Levin CE. Review of methods for the reduction of dietary content and toxicity of acrylamide. J Agr Food Chem 2008; 56 (15): 6113-6140.

Friedman M. Chemistry, biochemistry, and safety of acrylamide. A review. J Agr Food Chem 2003; 51 (16): 4504-4526.

Friedman MA, Tyl RW, Marr MC, Myers CB, Gerling FS, Ross WP. Effects of lactational administration of acrylamide on rat dams and offspring. Reprod Toxicol 1999; 13 (6): 511-520.

Fuhr U, Boettcher MI, Kinzig-Schippers M, Weyer A, Jetter A, Lazar A, et al. Toxicokinetics of acrylamide in humans after ingestion of a defined dose in a test meal to improve risk assessment for acrylamide carcinogenicity. Cancer Epidemiol Biomarkers Prev 2006; 15 (2): 266-271.

Gertz C, Klostermann S. Analysis of acrylamide and mechanisms of its formation in deep-fried products. Europ J Lipid Sci Technol 2002; 104: 762–771.

Ghanayem BJ, Witt KL, Kissling GE, Tice RR, Recio L. Absence of acrylamide-induced genotoxicity in CYP2E1-null mice: Evidence consistent with a glycidamide-mediated effect. Mutat Res 2005; 578: 284-297.

Girma KB, Lorenz V, Blaurock S, Edelmann FT. Coordination chemistry of acrylamide. Coordin Chem Rev 2005; 249 (11): 1283-1293.

Gokmen V, Kocadağlı T, Göncüoğlu N, Mogol BA. Model studies on the role of 5-hydroxymethyl-2-furfural in acrylamide formation from asparagine. Food Chem 2012; 132 (1): 168-174.

Gokmen V, Palazoglu TK. Measurement of evaporated acrylamide during frying of potatoes: Effect of frying conditions and surface area-to-volume ratio. J Food Eng 2009; 93 (2): 172-176.

Gokmen V, Senyuva H. Effects of some cations on the formation of acrylamide and furfurals in glucose–asparagine model system. Eur Food Res Technol 2007; 225: 815–820.

Gokmen V, Senyuva HZ, Acar J, Sarıoğlu K. Determination of acrylamide in potato chips and crisps by high-performance liquid chromatography. J Chromatogr A 2005; 1088: 193-199.

Gokmen V, Senyuva, H. Z. (2007). Acrylamide formation is prevented by divalent cations during the Maillard reaction. Food Chemistry, 103, 196–203.

Gómez-Alonso S, Hermosín-Gutiérrez I, García-Romero E. Simultaneous HPLC analysis of biogenic amines, amino acids, and ammonium ion as aminoenone derivatives in wine and beer samples. J Agr Food Chem 2007; 55 (3): 608-613.

Govaert Y, Arisseto A,Van Loco J, Scheers E, Fraselle S, Weverbergh E, et al. Optimization of a liquid chromatography-tandem mass spectrometry method for the determination of acrylamide in foods. Anal Chim Acta 2006; 556: 275-280.

Granby K, Fagt S. Analysis of acrylamide in coffee and dietary exposure to acrylamide from coffee. Anal Chim Acta 2004; 520: 177–182.

Granda C, Moreira R, Tichy S. Reduction of acrylamide formation in potato chips by low-temperature vacuum frying. J Food Sci 2004; 69:E405–E11.

Granda C, Moreira RG, Tichy SE. Reduction of Acrylamide Formation in Potato Chips by Low-temperature Vacuum Frying. J Food Sci 2004; 69: E405-E411.

Granvogl M, Jezussek M, Koehler P, Schieberle P. Quantitation of 3-aminopropionamide in potatoes - a minor but potent precursor in acrylamide formation. J Agr Food Chem 2004; 52: 4751-4757.

Guenther H, Anklam E, Wenzl T, Stadler RH. Acrylamide in coffee: review of progress in analysis, formation and level reduction. FoodAddit Contam 2007; 24 (1): 60–70.

Haase NU, Skog K, Alexander J. The formation of acrylamide in potato products. Acrylamide and other hazardous compounds in heat 2007; p. 41-59.

Haber LT, Maier A, Kroner OL, Kohrman RJ. Evaluation of human relevance and mode of action for tunica vaginalis mesotheliomas resulting from oral exposure to acrylamide. Regul Toxicol Pharmacol 2009; 53 (2): 134-149.

Hagmar L, Tornvist M, Nordander C, Rosén I, Bruze M, Kautiainen A, et al. Health effects of occupational exposure to acrylamide using hemoglobin adducts as biomarkers of internal dose. Scand J Work Environ Health 2001; 27 (4): 219-226.

Hagmar L, Wirfält E, Paulsson B, Törnqvist M. Differences in hemoglobin adduct levels of acrylamide in the general population with respect to dietary intake, smoking habits and gender. Mutat Res-Gen Tox En 2005; 580: 157–165.

Hamlet CG, Jayaratne SM, Sadd PA. Rapid,sensitive and selective analysis of acrylamide in cereal productsusing bromination and GC/MS/MS. Czech J Food Sci 2004; 22: 290–293.

Hamzahoglu A, Mogol BA, LumagaRB, Fogliano V, Gokmen V. Role of curcumin in the conversion of asparagine into acrylamide during heating. Amino acids 2013; 44 (6): 1419-1426.

Hargin KD. Using total diet studies to assess acrylamide exposure. In: Moy GG, Vannoort RW, editors. Total Diet Studies. London: Springer; 2013. p. 489-500.

He F, Zhang S, Wang H, et al. Neurological and electroneuro myographic assessment of the adverse effects of acrylamide on occupationally exposed workers. Scand J Work Environ Health 1989; 15 (2): 125-129.

HEATOX project, European Commission Research, http://ec.europa.eu/research/research-for-europe/agriculture-heatox_en.html

Hedegaard RV, Frandsen H, Granby K, Apostolopoulou A, Skibsted LH. Model studies on acrylamide generation from glucose/asparagine in aqueous glycerol. J Agr Food Chem 2007; 55: 486–492.

Hedegaard, R. V., Granby, K., Frandsen, H., Thygesen, J., ,Skibsted, L. H. (2008). Acrylamide in bread. Effect of prooxidants and antioxidants. European Food Research andTechnology, 227, 519–525.

Heudorf U, Hartmann E, Angerer J. Acrylamide in children-exposure assessment via urinary acrylamide metabolites as biomarkers. Int J Hyg Environ Health 2009; 212: 135-14.

Heuser F, Gerendas J, Sattelmacher B. Influence of N and K fertilization on contents of reducing sugars and free amino acids in potatoes. Significance for acrylamide contents in potato chips. Kartoffelbau, 2005; 56: 308-313.

Hirvonen T, Kontto J, Jestoi M, Valsta L, Peltonen K, Pietinen P, et al. Dietary acrylamide intake and the risk of cancer among Finnish male smokers. Cancer Cause Control 2010; 21 (12): 2223-2229.

Hoenicke K, Gatermann R, Harder W, Hartig L. Analysis of acrylamide in different foodstuffs using liquidchromatography–tandem mass spectrometry and gas chromatography–tandem mass spectrometry. Anal Chim Acta 2004; 520: 207–215.

Hoenicke K, Gatermann R. Studies on the stability of acrylamide in food during storage. J AOAC Int 2005; 8: 268–273.

Hogervorst JG, Schouten LJ, Konings EJ Goldbohm RA, van den Brandt PA. Dietary acrylamide intake and brain cancer risk. Cancer Epidemiol Biomarkers Prev 2009a; 18 (5): 1663-1666.

Hogervorst JG, Schouten LJ, Konings EJ, Goldbohm RA, van den Brandt PA. A prospective study of dietary acrylamide intake and the risk of endometrial, ovarian, and breast cancer. Cancer Epidemiol Biomarkers Prev 2007; 16 (11): 2304- 2313.

Hogervorst JG, Schouten LJ, Konings EJ, Goldbohm RA, van den Brandt PA. Dietary acrylamide intake and the risk of renal cell, bladder, and prostate cancer. Am J Clin Nutr 2008a; 87 (5): 1428-1438.

Hogervorst JG, Schouten LJ, Konings EJ, Goldbohm RA, van den Brandt PA. Dietary acrylamide intake is not associated with gastrointestinal cancer risk. J Nutr 2008b; 138 (11): 2229-2236.

Hogervorst JG, Schouten LJ, Konings EJ, Goldbohm RA, van den Brandt PA. Lung cancer risk in relation to dietary acrylamide intake. J Natl Cancer Inst 2009b; 101: 651-662

IARC. Acrylamide. In: IARC monographs of some industrial chemicals, summary of data reported and evaluation. Lyon, France: International Agency for Research on Cancer, 1994; 389-433.

Igisu H, Matsuoka M. Acrylamide encephalopathy. J Occup Health 2002; 44: 63-68.

JECFA. FAO/WHO Joint FAO/WHO Expert Committee on Food Additives, Seventy Second Meeting, Summary and Conclusions. 2010.

Jezussek M, Schieberle P. A New LC/MS-Method for the Quantification of Acrylamide Based on a Stable Isolute Dilution Assay and Derivatization with 2-Mercaptobenzoic Acid. Comparison with Two GC/MS Methods. J Agr Food Chem 2003; 51: 7866-7871.

Jiao J, Zhang Y, Ren Y, Wu X, Zhang Y. Development of a quantitative method for determination of acrylamide in infant powdered milk and baby foods in jars using isotope dilution liquid chromatography/electrospray ionization tandem mass spectrometry. J Chromatogr A 2006; 1099: 198-202.

Jin C, Wu X, Zhang Y. Relationship between antioxidants and acrylamide formation: A review. Food Res Int 2013; 51 (2): 611–620.

Jung MY, Choi DS, Ju JW. A novel technique for limitation of acrylamide formation in fried and baked corn chips and in French fries. J Food Sci 2003; 68: 1287–1290.

Kaplan O, Kaya G, Ozcan C, Ince M, Yaman M.Acrylamide concentrations in grilled foodstuffs of Turkish kitchen by high performance liquid chromatography-mass spectrometry, Microchem J 2009; 93: 173–179.

Kazantsev OA, Shirshin KV. Spontaneous polymerization of (meth) acrylamides in concentrated aqueous solutions. Polymer 2004; 45 (15): 5021-5029.

Keramat J, LeBail A, Prost C, Jafari M. Acrylamide in Baking Products: A Review Article. Food Bioprocess Technol 2011; 4: 530–543.

Kim CT, Hwang ES, Lee HJ. An improved LC-MS/MS method for the quantitation of acrylamide in processed foods. Food Chem 2007; 101: 401–409.

Kita A, Bråthen E, Knutsen S, Wicklund T. Effective ways of decreasing acrylamide content in potato crisps during processing. J Agr Food Chem 2005; 52: 7011–7016.

Klaunig JE. Acrylamide carcinogenicity. J Agric Food Chem 2008; 56 (15): 5984-5988.

Knol JJ, Van Loon WAM, Linssen JPH, Ruck AL, Van Boekel M, Voragen AGJ. Toward a kinetic model for acrylamide formation in a glucose–asparagine reaction system. J Agr Food Chem 2005; 53: 6133–6139.

Kolek E, Ŝimko P, Simon P. Inhibition of acrylamide formation in asparagine/D-glucose model system by NaCl addition. Eur FoodRes Technol 2006; 224: 283–284.

Konings EJM, Baars AJ, Van Klaveren JD, Spanjer MC, Rensen PM, Hiemstra M, et al. Acrylamide exposure from foods of the Dutch population and an assessment of the consequent risks. Food Chem Toxicol 2003; 41 (11): 1569-1579.

Kornbrust BA, Stringer MA, Lange NK, Hendriksen HV, Whitehurst RJ, van Oort M. Asparaginase-an enzyme for acrylamide reduction in food products. In: Whitehurst RJ, van Oort M. Enzymes in food technology 2nd Ed. Chichester: John Wiley and Sons; 2010. p. 59-87.

Kotsiou K, Tasioula-Margari M, Kukurová K, Ciesarová Z. Impact of oregano and virgin olive oil phenolic compounds on acrylamide content in a model system and fresh potatoes. Food Chem 2010; 123 (4): 1149-1155.

Koutsidis G, Elmore JS, Oruna-Concha MJ, Campo MM, Wood JD, Mottram DS. Water-soluble precursors of beef flavour: I. Effect of diet and breed. Meat Sci 2008; 79 (1): 124-130.

Kutlán D, Molnár-Perl I. New aspects of the simultaneous analysis of amino acids and amines as their o-phthaldialdehyde derivatives by high-performance liquid chromatography: Analysis of wine, beer and vinegar. J Chromat A 2003; 987 (1): 311-322.

Lagalante AF, Felter MA. Silylation of Acrylamide for Analysis by Solid-Phase Microextraction/Gas Chromatography/Ion-Trap Mass Spectrometry. J Agr Food Chem 2004; 54: 3744-3748.

Lantz I, Ternite R, Wilkens J, Hoenicke K, Guenther H, van der Stegen GHD. Studies on acrylamide levels in roasting, storage and brewing of coffee. Mol Nutr Food Res 2006; 50: 1039–1046.

Larsson SC, Akesson A, Wolk A. Dietary acrylamide intake and prostate cancer risk in a prospective cohort of Swedish men. Cancer Epidemiol Biomarkers Prev 2009e; 18 (6): 1939-1941.

Larsson SC, Akesson A, Wolk A. Long-term dietary acrylamide intake and risk of epithelial ovarian cancer in a prospective cohort of Swedish women. Cancer Epidemiol Biomarkers Prev 2009b; 18 (3): 994-997.

Larsson SC, Akesson A, Wolk A. Long-term dietary acrylamide intake and breast cancer risk in a prospective cohort of Swedish women. Am J Epidemiol 2009d; 169 (3): 376-381.

Larsson SC, Hakansson N, Akesson A, Wolk A. Long-term dietary acrylamide intake and risk of endometrial cancer in a prospective cohort of Swedish women. Int J Cancer 2009a; 124 (5): 1196-1199.

Larsson SC, Kesson A, Bergkvist L, Wolk A. Dietary acrylamide intake and risk of colorectal cancer in a prospective cohort of men. Eur J Cancer 2009c; 45: 513-516.

Lee S, Yoo M, Koo M, Kim HJ, Kim M, Park S-K, et al. In-house–validated liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for survey of acrylamide in various processed foods from Korean market. Food Sci Nut 2013; 1 (5): 402–407.

Leung KS, Lin A, Tsang CK, Yeung STK. Acrylamide in Asian foods in Hong Kong. Food Addit Contam 2003; 20 (12): 1105-1113.

Levine RA, Smith RE. Sources of variability of acrylamide levels in a cracker model. J Agr Food Chem 2005; 53 (11): 4410-4416.

Lichon MJ. Sample preparation. In Mollet, Leo, M.L., Ed. – Handbook of Food Analysis. Volume 1-Physical characterization and nutrient analysis. New York: Marcel-Dekker, 1996. ISBN: 0-8247-982-9. Chap 1.

Lim H-H, Shin H-S. Ultra trace level determinations of acrylamide in surface and drinking water by GC-MS after derivatization with xanthydrol. J Sep Sci 2013; 36: 3059–3066.

Lin Y, Lagergren J, Lu Yunxia. Dietary acrylamide intake and risk of exophageal cancer in a population-based case-control study in Sweden. Int J Cancer 2010; 128: 676-681.

Lindsay RC, Jang SJ. Chemical intervention strategies for substantial suppression of acrylamide formation in fried potato products. Adv Exp Med Biol 2005; 561:393–404.

Lineback DR, Coughlin JR, Stadler RH. Acrylamide in foods: a review of the science and future considerations. Annual Rev Food Sci Technol 2012; 3: 15-35.

LoPachin RM, Barber DS, Gavin T. Molecular mechanisms of the conjugated alpha, betaunsaturated carbonyl derivatives: Relevance to neurotoxicity and neurodegenerative diseases. Toxicol Sci 2008; 104 (2): 235-249.

LoPachin RM, Barber DS. Synapticcysteine sulfhydryl groups as targets of electrophilic neurotoxicants. Toxicol Sci 2006; 94: 240-255.

Lopachin RM, Decaprio AP. Protein adduct formation as a molecular mechanism in neurotoxicity. Toxicol Sci 2005; 86 (2): 214-225.

Lopes C, Oliveira A, Santos AC, Ramos E, Gaio AR, Severo M, Barros H.
Food Consumption in Porto. Faculdade de Medicina da Universidade do Porto 2006.Available at: www.consumoalimentarporto.med.up.pt. (In Portuguese).

Low MY, Koutsidis G, Parker JK, Elmore JS, Dodson AT, Mottram DS. Effect of citric acid and glycine addition on acrylamide and flavor in a potato model system. J Agr Food Chem 2006; 54: 5976–5983.

Low MY, Parker JK, Mottram DS. Mechanisms of alkylpyrazine formation in a potato model system containing added glycine. J Agr Food Chem 2007; 55: 4087–4094.

Mariotti S, Pedreschi F, Carrasco JA, Granby K. Patented techniques for acrylamide mitigation in high-temperature processed foods. Recent Pat Food Nutr Agric 2011; 3: 158–171.

Marková L, Ciesarová Z, Kukurová K, Zieliński H, Przygodzka M, Bednáriková A, et al. Influence of various spices on acrylamide content in buckwheat ginger cakes. Chem Pap 2012; 66 (10): 949-954.

Martin FL, Ames JM. Formation of Strecker aldehydes and pyrazines in a fried potato model system. J Agr Food Chem 2001; 49 (8): 3885-3892.

Matsuura-Endo C, Ohara-Takada A, Chuda Y, Ono H, Yada H, Yoshida M, e tal. Effects of storage temperature on the contents of sugars and free amino acids in tubers from different potato cultivars and acrylamide in chips. Biosci Biotechnol Biochemistry, 2006; 70 (5): 1173-1180.

Matthys C, Bilau M, Govaert Y, Moons E, De Henauw S, Willems JL. Risk assessment of dietary acrylamide intake in Flemish adolescents. Food Chem Toxicol 2005; 43 (2): 271–278.

Medeiros R, Mestdagh F, Van Poucke C, Kerkaert B, De Muer N, Denon Q. et al., Implementation of acrylamide mitigation strategies on industrial production of French fries: challenges and pitfalls. J Agr Food Chem 2011; 59: 898–906.

Medeiros Vinci R, Mestdagh F, De Meulenaer B. Acrylamide formation in fried potato products – present and future, a critical review on mitigation strategies. Food Chem 2012; 133: 1138–1154.

Mestdagh F, De Meulenaer B, Cucu T, Van Peteghem C. Role of water upon the formation of acrylamide in a potato model system. J Agr Food Chem 2006; 54: 9092–9098.

Mestdagh F, Maertens J, Cucu T, Delporte K, Van Peteghem C, De Meulenaer B. Impact of additives to lower the formation of acrylamide in a potato model system through pH reduction and other mechanisms. Food Chem 2008; 107: 26–31.

Michels KB, Rosner BA, Chumlea WC, Colditz GA, Willett WC. Preschool diet and adult risk of breast cancer. Int J Cancer 2006; 118 (3): 749-754.

Mizukami Y, Kohata K, Yamaguchi Y, Hayashi N, Sawai Y, Chuda Y. et al. Analysis of acrylamide in green tea by gas chromatography-mass spectrometry. J Agr Food Chem 2006; 54: 7370– 7377.

Mojska H, Gielecinska I, Szponar L, Oltarzewski M. Estimation of the dietary acrylamide exposure of the Polish population. Food Chem Toxicol 2010; 48: 2090-2096.

Molina-Garcia L, Santos CSP, Melo A, Fernandes JO, Cunha SC, Casal S. Acrylamide in Chips and French Fries: a Novel and Simple Method Using Xanthydrol for Its GC-MS Determination. Food Anal Met 2014; DOI 10.1007/s12161-014-0014-5.

Morales FJ, Mesias M. Analysis of Acrylamide in Coffee. In Preedy V. Coffee in healthy and disease prevention. New York: Elsevier Inc. Chap 111, 2015.

Mottram D, Wedzicha B, Dodson A. Food chemistry: Acrylamide is formed in the Maillard reaction. Nature 2002; 419: 448-449.

Mucci LA, Adami HO, Wolk A. Prospective study of dietary acrylamide and risk of colorectal cancer among women. Int J Cancer 2006: 118 (1): 169-173.

Mucci LA, Dickman PW, Steineck G, Adami H-O, Augustsson K. Dietary acrylamide and cancer of the large bowel, kidney, and bladder: Absence of an association in a population-based study in Sweden. Br J Cancer 2003; 88 (1): 84-89.

Mucci LA, Lindblad P, Steineck G, Adami H-O. Dietary acrylamide and risk of renal cell cancer. Int J Cancer 2004; 109 (5): 774-776.

Mucci LA, Sandin S, Balter K, Adami H-O, Magnusson C, Weiderpass E. Acrylamide intake and breast cancer risk in Swedish women. JAMA 2005; 293 (11): 1326-1327.

Murkovic M, Derler K. Analysis of amino acids and carbohydrates in green coffee. J Biochem Bioph Meth 2006; 69: 25–32.

Murkovic M. Acrylamide in Austrian foods. J Biochem Bioph Meth 2004; 61: 161-167.

Nielsen NJ, Granby K, Hedegaard RV, Skibsted LH. A liquid chromatography-tandem mass spectrometry method for simultaneous analysis of acrylamide and the precursors, asparagine and reducing sugars in bread. Anal Chim Acta 2006; 557: 211-220.

Normandin L, Bouchard M, Ayotte P, Blanchet C, Becalski A, Bonvalot Y, et al. Dietary exposure to acrylamide in adolescents from a Canadian urban center Food Chem Toxicol 2013; 57: 75-83.

Olesen PT, Olsen A, Frandsen H, Frederiksen K, Overvad K, Tjønneland A. Acrylamide exposure and incidence of breast cancer among postmenopausal women in the Danish Diet, Cancer and Health study. Int J Cancer 2008; 122 (9): 2094- 2100.

Olmez H, Tuncay F, Özcan N, Demirel S. A survey of acrylamide levels in foods from the Turkish Market. J Food Comp Anal 2008; 21: 564–568.

Ono H, Chuda Y, Ohnishi-Kameyama M, Yada H, Ishizaka M, Kobayashi H. Analysis of acrylamide by LC-MS/MS and GC-MS in processed Japanese foods. Food Addit Contam 2003; 20: 215–220.

Pabst K, Mathar W, Palavinskas R, Meisel H, Blüthgen A, Klaffke H. Acrylamide-occurrence in mixed concentrate feed for dairy cows and carry-over into milk. Food Addit Contam 2005; 22: 210-213.

Pastoriza S, Rufian-Henares JA, Morales FJ. Reactivity of acrylamide with coffee melanoidins inmodel systems. LWT–FoodSci Technol 2012; 45: 198–203.

Paulsson B, Grawe J, Tornqvist M. Hemoglobin adducts and micronucleus frequencies in mouse and rat after acrylamide or N-methylolacrylamide treatment. Mutat Res 2002; 516 (1-2): 101-111.

Pedreschi F, Granby K, Risum J. Acrylamide mitigation in potato chips by using NaCl. Food Bioprocess Technol 2009; 3: 917–921.

Pedreschi F, Kacrylamideck K, Granby K, Troncoso E. Acrylamide reduction under different pre-treatments in French fries. J Food Eng 2007; 79: 1287–1294.

Pedreschi F, Kacrylamideck K, Granby K. The effect of asparaginase on acrylamide formation in French fries. Food Chem 2008; 109: 386–392.

Pedreschi F, Mariotti S, Granby K, Risum J. Acrylamide reduction in potato chips by using commercial asparaginase in combination with conventional blanching. LWT–Food Sci Technol 2011; 44: 1473–1476.

Pelucchi C, Galeone C, Dal Maso L, Talamini R, Montella M, Ramazzotti V, et al. Dietary acrylamide and renal cell cancer. Int J Cancer 2007; 120 (6): 1376-1377.

Pelucchi C, Galeone C, Levi F, Negri E, Franceschi S, Talamini R, et al. Dietary acrylamide and human cancer. Int J Cancer 2006; 118 (2): 467-471.

Pelucchi C, Galeone C, Talamini R, Negri E, Polesel J, Serraino D, et al. Dietary acrylamide and pancreatic cancer risk in an Italian case-control study. Ann Oncol 2011; 22: 1910-1915.

Peng L. Rapid and Reproducible Extraction of Acrylamide in French Fries Using a Single Solid-Phase Sorbent. 2003; Application Note, American Laboratory, 10-14.

Perrault SD. Distinguishing between fertilization failure and early pregnancy loss when identifying male-mediated adverse pregnancy outcomes. Adv Exp Med Biol 2003; 518: 189-198.

Petersson EV, Rosén J, Turner C, Danielsson R, Hellenäs K-E. Critical factors and pitfalls affecting the extraction of acrylamide from foods: An optimization study. Anal Chim Acta 2006; 557: 287-295.

Pittet A, Périsset A, Oberson J-M. Trace level determination of acrylamide in cereal based foods by gas chromatography-mass spectrometry. J Chromatogr A 2004; 1035: 123-130.

Pollien P, Lindinger C, Yeretzian C, Blank I. Proton transfer reaction mass spectrometry, a tool for on-line monitoring of acrylamide formation in the headspace of Maillard reaction systems and processed food. Anal Chem 2003; 75 (20): 5488-5494.

Qiu YY, Qu XJ, Dong J, Ai SY, Han RX. Electrochemical detection of DNA damage induced by acrylamide and its metabolite at the graphene-ionic liquid-Nafion modified pyrolytic graphite electrode. J Hazard Mater 2011; 190: 480–485.

Quelhas I, Petisca C, Viegas O, Melo A, Pinho O, Ferreira IMPLVO. Effect of green tea marinades on the formation of heterocyclic aromatic amines and sensory quality of pan-fried beef. Food Chem 2010; 122: 98–104.

Reynolds T. Acrylamide and Cancer: Tunnel Leak in Sweden Prompted Studies. J Natl Cancer I 2002; 94 (12): 876-878.

Riboldi BP, Vinhas AM, Moreira JD. Risks of dietary acrylamide exposure: A systematic review. Food Chem 2014; 157: 310–322.

Riediker S, Stadler RH. Analysis of acrylamide by isotope-dilution liquid chromatography coupled with electrospray ionization tandem mass spectrometry. J Chromatogr A 2003; 1020: 121-130.

Robert F, Vuataz G, Pollien P, Saucy F, Alonso MI, Bauwens I. et al. Acrylamide formation from asparagine under low moisture Maillard reaction conditions. 2. Crystalline vs amorphous model systems. J Agr Food Chem 2005; 53: 4628–4632.

Rosén J, Hellenäs KE. Analysis of acrylamide in cooked foods by liquid chromatography tandem mass spectrometry. Analyst 2002; 127 (7): 880–882.

Rothweiller B, Kuhn E, Prest H. GC-MS Approaches to the Analysis of Acrylamide. PittCon 2003 Poster, Agilent Technologies.

Rufiàn-Henares J, Morales FJ. Determination of acrylamide in potato chips by a reversed phase LC-MS method based on a stable isotope dilution assay. Food Chem 2006; 97: 555-562.

Rydberg P, Eriksson S, Tareke E, Karlsson P, Ehrenberg L, Tornqvist M. Investigations of factors that influence the acrylamide content of heated foodstuffs. J Agr Food Chem 2003; 51: 7012–7018.

Rydberg P, Eriksson S, Tareke E, Karlsson P, Ehrenberg L, Törnqvist M. Factors that influence the acrylamide content of heated foods. In Chemistry and safety of acrylamide in food, pp. 317-328. Springer US, 2005.

Sadd PA, Hamlet CG, Liang L. Effectiveness of methods for reducing acrylamide in bakery products. J Agr Food Chem 2008; 56: 6154–6161

Schaller U. Experiences with acrylamide determination view from a retailer's laboratory, Presentation at the workshop Analytical methods for acrylamide determination in food, Oud-Turnhout, 2003, Belgium.

Schettgen T, Kutting B, Hornig M, Beckmann MW, Weiss T, Drexler H, et al. Trans-placental exposure of neonates to acrylamide-a pilot study. Int Arch Occup Environ Health 2004a; 77 (3): 213-216.

Schettgen T, Müller J, Fromme H, Angerer J. Simultaneous quantification of haemoglobin adducts of ethylene oxide, propylene oxide, acrylonitrile, acrylamide and glycidamide in human blood by

isotopedilution GC/NCI-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci 2010; 878 (27): 2467-2473.

Schettgen T, Rossbach B, Kutting B, Letzel S, Drexler H, Angerer J. Determination of haemoglobin adducts of acrylamide and glycidamide in smoking and non-smoking persons of the general population. Int J Hyg Environ Health 2004b; 207 (6): 531-539.

Schouten LJ, Hogervorst JG, Konings EJ, Goldbohm RA, van den Brandt PA. Dietary acrylamide intake and the risk of headneck and thyroid cancers: Results from the Netherlands Cohort Study. Am J Epidemiol 2009; 170: 873-884.

Schumacher JN, Green CR, Best FW, Newell MP. Smoke composition. An extensive investigation of the water-soluble portion of cigarette smoke. J Agric Food Chem 1977; 25 (2): 310-320.

Senyuva HZ, Gokmen V. Interference-free determination of acrylamide in potato and cereal-based foods by a laboratory validated liquid chromatography-mass spectrometry method. Food Chem 2006; 97: 539-545.

Senyuva HZ, Gokmen V. Survey of acrylamide in Turkish foods by an in-house validated LC-MS method, Food Addit Contam 2005; 22: 204-209.

Serpen A, Gokmen V. Evaluation of the Maillard reaction in potato crisps by acrylamide, antioxidant capacity and color. J Food Compos Anal 2009; 22 (6): 589-595.

Shipp A, Lawrence G, Gentry R, McDonald T, Bartow H, Bounds J, et al. Acrylamide: Review of toxicity data and dose-response analyses for cancer and noncancer effects. Crit Rev Toxicol 2006; 36 (6-7): 481-608.

Shojaee-Aliabadi S, Nikoopour H, Kobarfard F, Parsapour M, Moslehishad M, Hassanabadi H, et al. Acrylamide reduction in potato chips by selection of potato variety grown in Iran and processing conditions. J Sci Food Agr 2013; 93 (10): 2556-61.

Sickles DW, Stone JD, Friedman MA. Fast Axonal Transport: A Site of Acrylamide Neurotoxicity? NeuroToxicology 2002; 23: 223–251.

Smith EA, Oehme FW. Acrylamide and Polyacrylamide: A Review of Production, Use, Environmental Fate and Neurotoxicity. Reviews on Environmental Health 1991; 9 (4): 215-228.

Soares C, Fernandes J.O. MSPD Method to Determine Acrylamide in Food. Food Anal Method 2009; 2 (3): 197-203.

Soares C, Fernandes J.O. Screening of acrylamide in selected Portuguese foodstuffs and estimation of the dietary intake of the compound by the adult population of Porto, 2015

Soares C, Fernandes JO. MSPD Method to Determine Acrylamide in Food. Food Anal Method 2009; 2 (3): 197-203.

Soares CD, Cunha SC, Fernandes J.O. Determination of acrylamide in coffee and coffee products by GC-MS using an improved SPE clean-up. Food Add Cont 2006; 23: 1276-1282.

Soares CM, Alves RC, Casal S, Oliveira MB, Fernandes JO. Validation of a Matrix Solid-Phase Dispersion method to determine acrylamide in coffee and coffee surrogates. J Food Sci 2010 75 (3): T57-63.

Sohn M, Ho CT. Ammonia generation during thermal degradation of amino acids. J Agr Food Chem 1995; 43 (12): 3001-3003.

Sörgel F, Weissenbacher R, Kinzig-Schippers M, Hofmann A, Illauer M, Skotta A, et al. Acrylamide: Increased concentrations in home-made food and first evidence of its variable absorption from food, variable metabolism and placental and breast milk transfer in humans. Chemotherapy 2002; 48 (6): 267-274.

Stadler RH, Blank I, Varga N, Robert F, Hau J, Guy PA, et al. Acrylamide from Maillard reaction products. Nature 2002; 419: 449-450.

Stadler RH, Robert F, Riediker S, Varga N, Davidek T, Devaud S, et al. In-depth mechanistic study on the formation of acrylamide and other vinylogous compounds by the Maillard reaction. J Agr Food Chem 2004; 52 (17): 5550-5558.

Stadler RH, Scholz G. Acrylamide: an update on current knowledge in analysis, levels in food, mechanisms of formation, and potential strategies of control. Nut Rev 2004; 62 (12): 449-467.

Stadler RH, Verzegnassi L, Varga N, Grigorov M, Studer A, Riediker S, Schilter B. Formation of vinylogous compounds in model Maillard reaction systems. Chem Res Toxicol 2003; 16: 1242-1250.

Stockham K, Sheard A, Paimin R, Buddhadasa S, Duong S, Orbell JD, et al. Comparative studies on the antioxidant properties and polyphenolic content of wine from different growing regions and vintages, a pilot study to investigate chemical markers for climate change. Food Chem 2013; 140: 500-506.

Sumner SC, Fennell TR, Moore TA, Chanas B, Gonzalez F, Ghanayem BI. Role of cytochrome P450 2E1 in the metabolism of acrylamide and acrylonitrile in mice. Chem Res Toxicol 1999; 12 (11): 1110-1116.

Sumner SC, Macneela JP, Fennell TR. Characterization and quantitation of urinary metabolites of (1,2,3-13carbon)acrylamide in rats and mice using 13carbon nuclear magnetic resonance spectroscopy. Chem Res Toxicol 1992; 5 (1): 81-89.

Sumner SC, Williams CC, Snyder RW, Krol WL, Asgharian B, Fennell TR. Acrylamide: A comparison of metabolism and hemoglobin adducts in rodents following dermal, intraperitoneal, oral, or inhalation exposure. Toxicol Sci 2003; 75 (2): 260-270.

Taeymans D, Wood J, Ashby P, Blank I, Studer A, Stadler RH, et al. A review of acrylamide: an industry perspective on research, analysis, formation, and control. Crit Rev Food Sci 2004; 44 (5): 323-347.

Takahashi M, Shibutani M, Nakahigashi J, Sakaguchi N, Inoue K, Morikawa T, et al. Limited lactational transfer of acrylamide to rat offspring on maternal oral administration during the gestation and lactation periods. Arch Toxicol 2009; 83 (8): 785-793.

Tareke E, Heinze TM, Gamboa da Costa G, Ali S. Acrylamide formed at physiological temperature as a result of asparagine oxidation. J Agr Food Chem 2009; 57 (20): 9730-9733.

Tareke E, Rydberg P, Karlsson P, Eriksson S, Tornqvist M. Analysis of acrylamide, a carcinogen formed in heated foodstuffs. J Agric Food Chem 2002; 50 (17): 4998-5006.

Tareke, Eden, Beverly Lyn-Cook, Bonnie Robinson, and Syed F. Ali. "Acrylamide: a dietary carcinogen formed in vivo?." Journal of agricultural and food chemistry 56, no. 15 (2008): 6020-6023.

Taubert D, Harlfinger S, Henkes L, Berkels R, Schoming E. Influence of processing parameters on acrylamide formation during frying of potatoes. J Agric Food Chem 2004; 52: 2735–2739.

Tezcan F, Erim FB. On-line stacking techniques for the nonaqueous capillary electrophoretic determination of acrylamide in processed food. Anal Chim Acta 2008; 617: 196–199.

Tsukakoshi Y, Ono H, Kibune N, Isagawa S, Yamazaki K, Watai M, etal. Monitoring of acrylamide concentrations in potato chips in Japan between 2006 and 2010. Food Addit Contam 2012; 29: 1212–1218.

Twaddle NC, McDaniel LP, Gamboa da Costa G, Churchwell MI, Beland FA, Doerge DR. Determination of acrylamide and glycidamide serum toxicokinetics in B6C3F1 mice using LC-ES/MS/MS. Cancer Lett 2004; 207 (1): 9-17.

Tyl RW, Friedman MA, Losco PE,Fisher LC, Johnson KA, Strother DE, et al. Rat two-generation reproduction and dominant lethal study of acrylamide in drinking water. Reprod Toxicol 2000a; 14 (5): 385-401.

Tyl RW, Friedman MA. Effects of acrylamide on rodent reproductive performance. Reprod Toxicol 2003; 17 (1): 1-13.

Tyl RW, Marr MC, Myers CB, Ross WP, Friedman MA. Relationship between acrylamide reproductive and neurotoxicity in male rats. Reprod Toxicol 2000b; 14 (2): 147-157.

Urbancic A, olar MH, Dimitrijevic D, Demsar L, Vidrih R. Stabilisation of sunflower oil and reduction of acrylamide formation of potato with rosemary extract during deep-fat frying. LWT - Food Science and Technology 2014; 57: 671–678.

van Dijk-Looijaard AM, van Genderen J. Levels of exposure from drinking water. Food Chem Toxicol 2000; 38 (1): S37-S42.

Viegas O, Amaro LF, Ferreira IMPLVO, Pinho O. Inhibitory Effect of Antioxidant-Rich Marinades on the Formation of Heterocyclic Aromatic Amines in Pan-Fried Beef. J Agr Food Chem 2012; 60 (24): 6235-40.

Vinci RM, Mestdagh F, De Meulenaer B. Acrylamide formation in fried potato products–Present and future, a critical review on mitigation strategies. Food Chem 2012; 133 (4), 1138-1154.

Virk-Baker MK, Nagy TR, Barnes S, Groopman J. Dietary Acrylamide and Human Cancer: A Systematic Review of Literature, Nutr Cancer 2014; 66 (5): 774-790.

Vivanti V, Finotti E, Friedman M. Level of acrylamide precursors asparagine, fructose, glucose, and sucrose in potatoes sold at retail in Italy and the United States. Food Chem Toxicol 2006; 71: C81–C85.

Wenzl T, De La Calle B, Anklam E. Analytical methods for the determination of acrylamide in food products: a review. Food Addit Contam 2003; 20: 885-902.

Wenzl T, de la Calle B, Gatermann R, Hoenicke K, Ulberth F, Anklam E. Evaluation of the results from an inter-laboratory comparison study of the determination of acrylamide in crispbread and butter cookies. Anal Bioanal Chem 2004; 379: 449-457.

Wenzl T, Szilagyi S, Rosen J, Karasek L. Validation by collaborative trial of an isotope dilution liquid chromatographic tandem mass spectrometric method to determine the content of acrylamide in roasted coffee. Food Addit Contam 2009; A 26:1146–52.

Whittaker A, Marotti I, Dinelli G, Calamai L, Romagnoli S, Manzelli M, et al. The influence of tuber mineral element composition as a function of geographical location on acrylamide formation in different Italian potato genotypes. J Sci Food Agr 2010; 90 (12): 1968-1976.

WHO. Acrylamide in drinking-water. World Health Organization. 2003. WHO/SDE/WSH/03.04/71. http://www.who.int/water_sanitation_health/dwq/chemicals/acrylamide.pdf. August 3, 2011.

Williams JSE. Influence of variety and processing conditions on acrylamide levels in fried potato crisps. Food Chem 2005; 90 (4): 875-881.

Wilson KM, Balter K, Adami HO, Grönberg H, Vikström AC, Paulsson B, et al. Acrylamide exposure measured by food frequency questionnaire and hemoglobin adduct levels and prostate cancer risk in the cancer of the prostate in Sweden study. Int J Cancer 2009a; 124 (10): 2384-2390.

Wilson KM, Mucci LA, Cho E, Hunter DJ, Chen WY, Willet WC. Dietary acrylamide intake and risk of premenopausal breast cancer. Am J Epidemiol 2009b; 169 (8): 954-961.

Wilson KM, Mucci LA, Rosner BA, Willet WC. A prospective study on dietary acrylamide intake and the risk for breast, endometrial, and ovarian cancers. Cancer Epidemiol Biomarkers Prev 2010; 19 (10): 2503- 2515.

Wise LD, Gordon LR, Soper KA, Duchai DM, Morrissey RE. Developmental neurotoxicity evaluation of acrylamide in Sprague-Dawley rats. Neurotoxicol Teratol 1995; 17 (2): 189-198.

Yamazaki K, Isagawa S, Kibune N, Urushiyama T. A method for the determination of acrylamide in a broad variety of processed foods by GC-MS using xanthydrol derivatization. Food Addit Cont 2012; 29: 705–715.

Yanishlieva NV, Marinova E, Pokorný J. Natural antioxidants from herbs and spices. Eur J Lip Sci Technol 2006; 108 (9): 776-793.

Yasuhara A, Tanaka Y, Hengel M, Shibamoto T. Gas chromatographic investigation of acrylamide formation in browning model systems. J Agr Food Chem 2003; 51: 3999-4003.

Yaylayan V, Wnorowski A, Locas CP. Why Asparagine Needs Carbohydrates to Generate Acrylamide. J Agr Food Chem 2003; 51: 1753-1757.

Yaylayan VA, Perez Locas C, Wnorowski A, O'Brien J. Mechanistic pathways of formation of acrylamide from different amino acids. In: Friedman M, Mottram DS, editors. Chemistry and Safety of Acrylamide in Food. New York: Springer; 2005. p. 191-203.

Yaylayan VA, Perez Locas C, Wnorowski A, O'Brien J. The role of creatine in the generation of N-methylacrylamide: a new toxicant in cooked meat. J Agr Food Chem 2004; 52: 5559-5565.

Yaylayan VA, Stadler RH. Acrylamide formation in food: a mechanistic perspective. J AOAC Int 2005; 88: 262-267.

Yaylayan VA, Wnorowski A, Perez Locas C. Why asparagine needs carbohydrates to generate acrylamide. J Agr Food Chem 2003; 51 (6): 1753-1757.

Yusá V, Quintás G, Pardo O, Martí P, Pastor A. Determination of acrylamide in foods by pressurized fluid extraction and liquid chromatography-tandem mass spectrometry used for a survey of Spanish cereal-based foods. Food Addit Contam 2006; 23: 237-244.

Zamora R, Delgado RM, Hidalgo FJ. Conversion of 3-aminopropionamide and 3-alkylaminopropionamides into acrylamide in model systems. Mol Nut Food Res 2009; 53 (12): 1512-1520.

Zamora R, Delgado RM, Hidalgo FJ. Model reactions of acrylamide with selected amino compounds. J Agr Food Chem 2010; 58: 1708–1713.

Zamora R, Hidalgo FJ. Contribution of lipid oxidation products to acrylamide formation in model systems. J Agr Food Chem 2008; 56: 6075–6080.

Zeng X, Cheng KW, Jiang Y, Lin ZX, Shi JJ, Ou SY. Inhibition of acrylamide formation by vitamins in model reactions and fried potato strips. Food Chem 2009; 116: 34–39.

Zhang Gong, Huang G, Xiao L, Seiber J, Mitchell AE. Acrylamide formation in almonds (Prunus dulcis): influences of roasting time and temperature, precursors, varietal selection, and storage. J Agr Food Chem 2011; 59 (15): 8225-8232.

Zhang Y, Jiao J, Ren Y, Wu X, Zhang Y. Determination of acrylamide in infant cereal-based foods by isotope dilution liquid chromatography coupled with electrospray ionization tandem mass spectrometry. Anal Chim Acta 2005b; 55: 1150-158.

Zhang Y, Jiao JJ, Cai ZX, Zhang Y, Ren YP. An improved method validation for rapid determination of acrylamide in foods by ultra-performance liquid chromatography combined with tandem mass spectrometry. J Chromatogr A 2007; 1142: 194–198.

Zhang Y, Ren Y, Zhang Y. New research developments on acrylamide: analytical chemistry, formation mechanism, and mitigation recipes. Chem Rev 2009; 109(9): 4375-4397.

Zhang Y, Xu W, Wu X, Zhang X, Zhang Z. Addition of antioxidant from bamboo leaves as an effective way to reduce the formation of acrylamide in fried chicken wings. Food Addit Contam 2007; 24 (3): 242–251.

Zhang Y, Ying TJ, Zhang Y. Reduction of acrylamide and its kinetics by addition of antioxidant of bamboo leaves (AOB) and extract of green tea (EGT) in asparagine–glucose microwave heating system. J Food Sci 2008; 73 (2): 60–66.

Zhang Y, Zhang G, Zhang Y. Occurrence and analytical methods of acrylamide in heat-treated foods: Review and recent developments. J Chromatogr A 2005a; 1075: 1-21.

Zhang Y, Zhang Y. Effect of natural antioxidants on kinetic behavior of acrylamide formation and elimination in low-moisture asparagine–glucose model system. J Food Eng 2008; 85 (1): 105–115.

Zhang, Y., , Zhang, Y. (2007). Formation and reduction of acrylamide in Maillard reaction: A review based on the current state of knowledge. Critical Reviews in Food Scienceand Nutrition, 47, 521–542.

Zhou X, Fan L-Y, Zhang W, Cao C-X. Separation and determination of acrylamide in potato chips by micellar electrokinetic capillary chromatography. Talanta 2007; 71: 1541–1545.

Zhu F, Cai Y-Z, Ke J, Corke H. Compositions of phenolic compounds, amino acids and reducing sugars in commercial potato varieties and their effects on acrylamide formation. J Sci Food Agric 2010; 90: 2254–2262.

Zhu F, Cai Y-Z, Ke J, Corke H. Evaluation of the effect of plant extracts and phenolic compounds on reduction of acrylamide in an asparagine/glucose model system by RP-HPLC-DAD. J Sci Food Agric 2009; 89: 1674–1681.

Zhu YJ, Zeng T, Zhu YB, Yu S-F, Wang Q-S, Zhang L-P, et al. Effects of acrylamide on the nervous tissue antioxidant system and sciatic nerve electrophysiology in the rat. Neurochem Res 2008; 33 (11): 2310-2317.

Zyzak DV, Sanders RA, Stojanovic M, Tallmadge DH, Eberhart BL, Ewald DK, et al. Acrylamide formation mechanism in heated foods. J Agr Food Chem 2003, 51 (16): 4782-4787.

CHAPTER 2

Analytical Methodologies to Determine Acrylamide in Foodstuff

2.1. Introduction

In order to evaluate and identify the presence of acrylamide in the huge diversity of food matrices available to consumers, realiable analytical methods must be developed. An impressive number of papers dealing with methodologies to quantify acrylamide in foodstuff were presented since 2002. A not exhaustive compilation of the most notorious methods can be consulted in Chapter 1, sub-Chapter5- Analytical Methods for Acrylamide Determination in Foods.

The papers presented in this chapter represent our contribution to the volume of analytical methodologies discussed previously.

The paper "MSPD Method to Determine Acrylamide in Food" presents a methodology to determine acrylamide in carbohydrate-based foodstuffs using Matrix Solid phase Dispersion as extraction and simultaneous clean-up technique.

The second paper "Development and Validation of a Matrix Solid-Phase Dispersion Method to Determine Acrylamide in Coffee and Coffee Substitutes" presents the use of Matrix Solid Phase Dispersion and Solid Phase Extraction to extract acrylamide and eliminate interferences from coffee samples and coffee substitutes.

Chromatography and mass spectrometry (GC-MS) was used to separate and quantity acrylamide in food samples.

2.1. MSPD Method to Determine Acrylamide in Food

Abstract

The present work describes the development of an optimized MSPD procedure for the analysis of acrylamide in a variety of food matrices namely processed cereal products (bread, toasts, breakfast cereals, snacks, cookies and biscuits), chocolates and baby-foods. Briefly, 1 g of sample was dispersed with 4 g of C_{18} solid phase, the whole mixture was further packed in an empty SPE column and acrylamide was extracted with 6 + 6 ml of water with a soak step of 5 minutes each. The aqueous extract was then subject to a bromination procedure and acrylamide quantified by GC/MS in SIM mode.

Full validation of the method was conducted in samples representatives of each one of the food groups analysed including a comparison of the results obtained by using the reported method with those furnished by a previously developed analytical procedure based on a liquid extraction approach. The MSPD-GC/MS method presented a LOD of 5.2 μg/kg and a LOQ of 15.7 μg/kg and recoveries were in the range of 93-119% in all of the food matrices analysed.Furthermore, the bias of the method was tested with a certified toasted bread sample.

Keywords: Matrix Solid-Phase Dispersion (MSPD), acrylamide, food analysis, GC/MS

Food Anal. Methods (2009) 2:197–203
DOI 10.1007/s12161-008-9060-1

MSPD Method to Determine Acrylamide in Food

Cristina Maria Dias Soares · José Oliveira Fernandes

Received: 23 June 2008 / Accepted: 21 October 2008 / Published online: 8 November 2008

Abstract The present work describes the development of an optimized matrix solid-phase dispersion (MSPD) procedure for the analysis of acrylamide in a variety of food matrices, namely, processed cereal products (bread, toasts, breakfast cereals, snacks, cookies, and biscuits), chocolates, and baby foods. Briefly, 1 g of sample was dispersed with 4 g of C_{18} solid phase, the whole mixture was further packed in an empty SPE column, and acrylamide was extracted with 6+6 ml of water with a soak step of 5 min each. The aqueous extract was then subject to a bromination procedure and acrylamide quantified by gas chromatography–mass spectrometry (GC-MS) in SIM mode. Full validation of the method was conducted in samples representatives of each one of the food groups analyzed including a comparison of the results obtained by using the reported method with those furnished by a previously developed analytical procedure based on a liquid extraction approach. The MSPD-GC/MS method presented a limit of detection of 5.2 μg kg^{-1} and a limit of quantification of 15.7 μg kg^{-1}, and precisions were in the range of 1–7% in all of the food matrices analyzed. Furthermore, the bias of the method was tested with a certified toasted bread sample.

Keywords Matrix Solid-Phase Dispersion (MSPD) · Acrylamide · Food Analysis · GC-MS

C. M. D. Soares (✉) · J. O. Fernandes
REQUIMTE, Serviço de Bromatologia, Faculdade de Farmácia,
Universidade do Porto,
Rua Aníbal Cunha, 164,
4099-030 Porto, Portugal
e-mail: cristina.md.soares@gmail.com

Introduction

Acrylamide is a heat-generated food toxicant usually found in processed carbohydrate-rich foods that represent a potential health hazard for humans. The compound is a suspected human carcinogen, a severe neurotoxin, and causes irritation of the eyes, skin, and respiratory tract (Dybing and Sanner 2003). The dietary food groups where acrylamide was detected at higher levels were potato products (French fries, oven-baked chips, potato crisps, etc.) and the different cereal-based foods, e.g., breakfast cereals, cookies, biscuits, bread (especially toasted bread), pies, and cakes. Other foods of particular interest because they are highly consumed for some individuals include coffee, chicory, and other coffee substitutes, chocolates, teething biscuits, baby rusks, and other baby foods. The levels of acrylamide in the above-mentioned foodstuffs cover a wide range from more than 3,000 μg kg^{-1} in overcooked potato chips to 5 μg kg^{-1} or less in bread or baby foods (Castle 2006; Wenzl and Anklam 2007).

Gas chromatography–mass spectrometry (GC-MS; Soares et al. 2006; Zhang et al. 2005; Castle and Eriksson 2005; Robarge et al. 2003; Ono et al. 2003; Tareke et al. 2002) and liquid chromatography–mass spectrometry (LC-MS; Rosén and Hellenäs 2002; Riediker and Stadler 2003; Andrzejewski et al. 2004; Arisseto et al. 2008) are the first choice techniques for the determination of acrylamide in foodstuffs. Despite the requirement for a previous derivatization of the compound, not necessary when one uses LC-MS, the GC-MS has the advantages to use cheaper and more robust equipments and to be a sound established technique. In order to take full benefit of GC-MS technique, improved extraction procedures allowing both an efficient extraction of the analyte from the food matrix and the achievement of an extract clean enough to make it suitable

for gas chromatographic analysis are required. Recently, our group reported an analytical method based on matrix solid-phase dispersion (MSPD) to prepare potato chip samples for GC-MS acrylamide analysis (Fernandes and Soares 2007). MSPD technique greatly reduced sample manipulation, solvent usage and disposal, and working time. It also eliminated emulsification problems, and the elimination of fats before acrylamide extraction with water enhanced the extraction yield of the contaminant. The good results obtained and the simplicity of the method encouraged us the application to a wider range of food groups though the results were affected by the different composition of the matrices and the wide concentration range of acrylamide levels.

In this work, a slightly modified version of our previous method is presented for its application in the analysis of cereal products such as breakfast cereals, bread, toasted bread, salty snacks, biscuits and cookies, baby food, and chocolate bars, which generally contains low amounts (<100 μg/kg for most of the samples) of acrylamide. The method was validated in terms of limit of detection (LOD) and quantification (LOQ), precision and linearity for samples representatives of each of the above-named food groups. The performance of the method was assessed by simultaneously analyzing a total of 40 samples belonging to the mentioned food groups, randomly chosen, by using the developed methodology and a liquid-extraction method routinely used in our laboratory. A certified toasted bread sample was also analyzed by the two referred methods.

Experimental

Sampling

Thirty-nine samples were chosen to represent different food groups highly consumed such as breakfast cereals (eight samples), bread and toasted bread (five), biscuits and cookies (eight), baby food (five), salty snacks (five), and chocolates (eight). Samples were collected from several local supermarkets, grinded to a powder using a laboratory electric grinder (Moulinex, Ecully, France) and stored at 4 °C. The samples with a short lifetime, like bread, were frozen. The certified sample, toasted bread with 425±29 μg kg^{-1} of acrylamide with the reference ERM-BD 273, was acquired from the Institute of Reference Materials and Measurements (Geel, Belgium).

Chemicals and Standards

Acrylamide (AA) (purity, 99%) was from Aldrich (Steinheim, Germany). The internal standard acrylamide 1, 2, 3-C13 ($^{13}C_3$-AA) (purity, 99%) was acquired from Cambridge Isotope Laboratories (Andover, MA, USA) as a 1 mg ml^{-1} methanol solution. Working standard solutions of AA (at 2 and 4 mg l^{-1}) and of $^{13}C_3$-AA (at 20 mg l^{-1}) were prepared with acetonitrile and stored at 4 °C when not in use. MSPD experiments were carried out with a C_{18} sorbent 125 Å, 55–105 μm, from Waters (Milford, USA). All other chemicals used in this study were described in detail in a previous publication (Fernandes and Soares 2007).

Equipment and GC-MS Operating Conditions

All the MSPD extractions were made in a vacuum manifold model Visiprep Solid Phase Extraction Manifold with capacity for 12 columns from Supelco (Bellefont, PA, USA). GC-MS analyses were performed in a gas chromatograph (Agilent GC-6890N), equipped with a split–splitless injector and an automatic injector (Agilent 7683B), and coupled to a mass-selective detector (Agilent MSD-5975N; Agilent, Palo Alto, CA, USA). The analytical separation was performed in a capillary column MDN-12 (30 m×0.25 μm, 0.25 mm i.d.) from Supelco, and the MSD was used in the selected ion monitoring mode (SIM), using six selected fragments characteristics of derivatized AA (*m/z* 106, 150, and 152) and derivatized IS (*m/z* 110, 153, and 155). Other details of the GC-MS parameters can be found elsewhere (Fernandes and Soares 2007).

Sample Preparation

MSPD-GC/MS An aliquot of 1.0 g of sample (previously grinded), 4 g of C_{18} sorbent, and 0.50 μg of IS (25 μl of the 20 mg l^{-1} IS solution) were placed in a glass mortar and blended together using a glass pestle to obtain the complete disruption and dispersion of the sample on the solid support. When blending was completed, the sample was packed into an empty column containing a polyethylene frit at the bottom. A second frit was placed on the top of the sample before careful compression with a syringe plunger. The packed column was placed in a vacuum manifold, and acrylamide was extracted with 6 ml of water stopping the flow to permit a soaking step of 5 min. The water was eluted and completely collected into a vial. A second volume of 6 ml of water was added observing again a 5-min soaking step. The second elution volume was collected in the same vial, and the column was kept under vacuum aspiration during 5 min to collect all the water. The flow control was important to ensure reproducibility. All samples analyzed were prepared in duplicate.

LE-GC/MS To evaluate the performance of the MSPD method, all samples were also analyzed by applying our previously optimized liquid extraction procedure with hot

water. Briefly, acrylamide was extracted with water at 65 °C (2 g of sample+1 μg of IS to 20 ml of water) after a swelling time of 15 min. Protein precipitation was carried out by Carrez solutions followed by centrifugation. The supernatant was then collected, filtered under vacuum, and evaporated in a rotary evaporator to obtain 8 ml of the extract before derivatization. All samples were also prepared in duplicate.

Preparation of Standards for Calibration

MSPD-GC/MS Aliquots of the 2 mg l^{-1} working standard AA solution (equivalents to 0 to 1.5 μg of AA corresponding to 0 to 1,500 μg kg^{-1} in the samples) were placed in a glass mortar with 4 g of C_{18} and 0.50 μg of internal standard. After blending using a glass pestle, the mixtures were treated in parallel with the samples, according to the described procedure.

LE-GC/MS Aliquots of the 4 mg l^{-1} working standard AA solution (equivalent to 0 to 3 μg of AA corresponding to 0 to 1,500 μg kg^{-1} in the samples) were placed in 50-ml centrifugal tubes with 1 μg of IS, and the volume was made up to 20 ml with water. These solutions were treated in parallel with the samples, according to the described procedure.

Bromination of the Samples and Standards

To the aqueous extracts obtained in both methods 1 g of calcinated KBr was added. The solutions were then acidified with HBr until pH 1–3 (100–150 μl), and 2 ml of saturated bromine solution were added. The derivatization reaction took place in an ice bath, protected from light, for at least 1 h. The excess bromine was then decomposed by the addition of 1 mol l^{-1} $Na_2S_2O_3$ solution until the yellow color of the extracts disappeared (50–150 μl). The solutions obtained were saturated with 4 g of NaCl, and the acrylamide derivative was extracted twice with 10 and 5 ml portions of ethyl acetate/*n*-hexane 4:1 (*v*/*v*). The volume of the organic phase was reduced to 3 ml under a stream of nitrogen, and a small quantity of anhydrous Na_2SO_4 was added. Finally, it was centrifuged at 3,000 rpm during 3 min and the upper layer was transferred to another vial and evaporated to 0.5 ml under a gentle stream of nitrogen. The extracts were then injected twice in the gas chromatograph.

Results

In a recent work, we reported the development of a MSPD sample preparation method successfully applied to the determination of acrylamide in potato chips (Fernandes and Soares 2007). Briefly, 0.5 g of sample were dispersed with 2 g of the sorbent, the whole mixture was transferred for an empty cartridge fat was eliminated by elution with 20 ml of *n*-hexane, and finally, after drying the organic solvent, elution of acrylamide was carried out with water (two portions of 4 ml each, observing a 5-min soaking step for both additions).

When applied to other food matrices, such as bread, chocolate, baby foods, etc., the method showed a lack of sensitivity, making very difficult its application to samples with small amounts of the contaminant.

To improve the sensitivity of the method, some modifications were tried such as the use of different ratios sorbent/sample and the use of higher amounts of water in the elution step. Finally, we found that the best results were obtained when the amount of sample was doubled from 0.5 to 1.0 g, maintaining the 4:1 ratio of sorbent/sample and increasing the volume of water used to elute acrylamide from 4+4 ml to 6+6 ml. The deffating step has revealed to be unnecessary, which accounted for an important gain in terms of time and simplicity of execution and can be explained by the reduced amounts of fat presented in the studied samples when compared with potato chips. The chromatograms of a chocolate sample presented in Fig. 1 show some of the described modifications: Fig. 1a shows the improvement of the sensitivity when increasing both the quantity of sample and C18; Fig. 1b presents the effect of the deffating step, which, in this case, does not improve the results.

Finally, the method was applied to the samples. In Fig. 2, a chromatogram of a breakfast cereal with 134 μg l^{-1} of the compound is presented.

The method was validated in terms of linearity, precision, LOD, and LOQ for each one of the food groups studied in this work. The recent availability of a certified toasted bread sample furnished by the Institute of Reference Materials and Measurements also permitted to include the

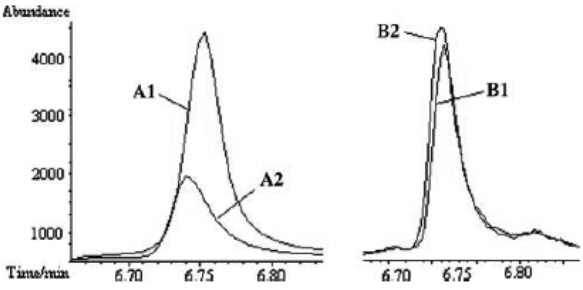

Fig. 1 Chocolate sample with 63 μg kg^{-1}: *A1* MSPD mixture with 1 g of sample and 4 g of C_{18} and *A2* MSPD mixture of 0.5 g of sample and 2 g of C_{18}; *B1* MSPD mixture with 1 g of sample and 4 g of C_{18} previously cleaned with *n*-hexane and *B2* MSPD mixture with 1 g of sample and 4 g of C_{18} without a previous clean up with *n*-hexane

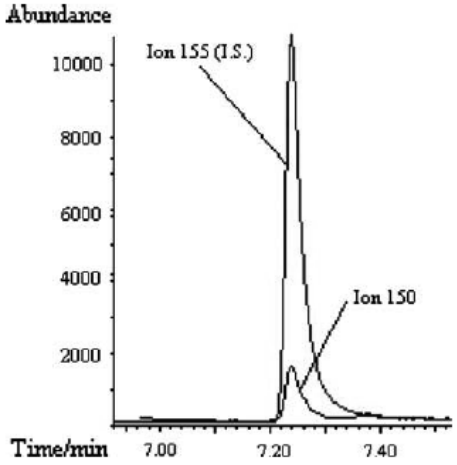

Fig. 2 Chromatogram of a cereal sample prepared with the reported MSPD-GC/MS method that presented 134 μg kg^{-1} of acrylamide

calculation of the bias of the method for this kind of samples.

The performance of the method was also evaluated by comparing the results obtained in the analysis of the different samples with the results obtained for the same samples analyzed by the liquid extraction method routinely used in our laboratory. The obtained results in the validation procedure are presented in the next sections and the results obtained from all the samples subject to analyses.

Linearity

Standards The linearity of the method was tested several times using standard (calibrating) solutions treated with the same method developed for the samples. Usually, seven standards were simultaneously prepared and treated in parallel with a set of samples. The range of concentrations corresponded to 0–1,500 μg l^{-1} AA in the sample. Calibration curves were constructed by plotting the AA/IS area ratio against the concentration of AA in the standard. The correlation coefficients were usually higher than 0.999.

Table 2 Intraday and interday precision for the different group of samples

Sample	Intraday precision		Interday precision	
	Average	RSD %	Average	RSD %
Breakfeast cereals	135	3	134	6
Bread	77	4	82	4
Biscuits and cookies	81	3	84	5
Baby food	139	2	136	4
Salty snacks	156	1	158	3
Chocolate bars	64	2	69	7

Real Samples To evaluate the linearity of the method in real samples, six aliquots (1 g) of a sample with a known amount of AA (one sample for each one of the six food groups considered) were prepared simultaneously. To the samples was added 0, 0.025, 0.050, 0.125, 0.250, and 0.500 μg of acrylamide (0, 25, 50, 125, 250, and 500 μl of the 2 mg l^{-1} AA solution) and 0.500 μg of IS. The samples were treated as described for the overall method and injected twice. The linearity of the method in real samples was evaluated by plotting the AA area/IS area ratio against the added amounts of AA. The correlation coefficients obtained were higher than 0.995. The values obtained are presented in Table 1.

Precision To study the intraday precision, three aliquots (1 g) of the same sample (one sample randomly chosen from each group of foodstuffs) were prepared simultaneously and submitted to the overall method and injected in triplicate in the same day. To study the interday precision of the method, three aliquots of the same sample were prepared and submitted to the overall developed method in three different weeks and injected in triplicate in different days. The intraday precision varied between 1% and 4% and the interday precision between 3% and 7%. The results obtained are presented in Table 2.

Bias The method bias was tested using a certified sample of toasted bread, which presents a certified value of 425 μg

Table 1 Linearity of the method in real samples—AA area/IS area vs added amount of AA

Sample	CAA initial (μg kg^{-1})	AA area/IS area vs added amount of AA		
		Equation	Correlation factor r	C_{AA} extrapolation (μg kg^{-1})
Breakfast cereals	134	$y=0.0022x+0.2872$	0.9997	131
Bread	78	$y=0.0024x+0.1620$	0.9993	68
Biscuits and cookies	82	$y=0.0025x+0.1815$	0.9982	73
Baby food	139	$y=0.0025x+0.2777$	0.9955	112
Salty snacks	157	$y=0.0023x+0.3710$	0.9998	161
Chocolate bars	63	$y=0.0025x+0.1713$	0.9997	69

kg^{-1} of acrylamide and an uncertainty of 29 μg kg^{-1}. The certified sample was prepared in triplicate using the MSPD method and injected in triplicate. The bias was calculated using the equation $\%Error = \frac{\text{obtained result} - \text{expected result}}{\text{expected result}} \times 100$. The obtained value was 440 μg kg^{-1} with a relative standard deviation of 1%. The percentage of error calculated using the given equation was 4%.

LOD and LOQ The LOD of the method was calculated using the calibration curve parameters (Ribani et al. 2004; Miller and Miller 1993). Therefore, the LOD=3.3×(*s*/*S*), where *s* is the standard deviation of the intercept and *S* is the slope of the curve. The LOQ was also calculated using this method applying the equation LOQ=10×(*s*/*S*). The LOD obtained for this method in the present conditions was 5.2 μg kg^{-1} and the LOQ was 15.7 μg kg^{-1}.

Comparison between MSPD-GC/MS and LE-GC/MS methods All the samples analyzed in this study were submitted to the described MSPD-GC/MS method and also to the LE-GC/MS method routinely used in our laboratory. The results obtained by both methods are presented in Table 3. The comparison of the two methods was made using a regression line (Fig. 3). By observing Table 3 and

Table 3 Results obtained for all the samples analysed using both methods described in this paper

Samples	Description	AA MSPD/GC-MS (μg kg^{-1})	AA LE/GC-MS (μg kg^{-1})
Breakfast cereals	Corn flakes	36	34
	Corn flakes	51	47
	Cereal mixture	134	129
	Cereals with nuts and honey	90	88
	Oats with honey	539	534
	Cereals with honey	63	56
	Whole cereals with honey	333	298
	Corn flakes	51	53
Bread	Broa de Avintes (maize and rye bread)	78	78
	Wholemeal bread	39	32
	Rye bread	43	40
	Wheat and rye bread	25	22
	White bread (wheat)	41	37
	Toasted bread (certified sample with 425±29 μg kg^{-1} of acrylamide)	440	427
Biscuits and cookies	Diet maize cookies	82	87
	Maize cookies	49	51
	Multicereals cookies	226	232
	Soy cookies	486	475
	Butter cookies	996	1,040
	Biscuits	495	490
	Toasted biscuits	363	365
	Butter biscuits	529	540
Baby food	Cereals with honey	139	112
	Cereals with 5 fruits	27	22
	Rice flakes	41	48
	Chocolate flakes	206	203
	Biscuits gruel	25	22
Salty snacks	Fried maize (SP)	64	56
	Fried maize	157	147
	Fried maize with cheese	28	23
	Fried maize with cheese	59	52
	Fried maize	174	167
Chocolate bars	Milk chocolate with fruits	63	78
	Milk chocolate with no sugar and jam	64	59
	Milk chocolate with no sugar	81	89
	Dark chocolate with no sugar	115	130
	Dark chocolate	134	126
	Milk chocolate with caramel	31	37
	White chocolate	15	12
	Milk chocolate with strawberry yoghurt	27	22

Fig. 3, a close similarity of the results obtained by both methods can be seen.

Discussion

The results reported in the method assessment procedure are a clear evidence that the developed method can be used with confidence in the determination of acrylamide in different foodstuffs. Not only the high recoveries and good precisions obtained for each sample group analyzed but also the small bias between the values obtained for the certified sample substantiate this conclusion. The LOD obtained for this method in the discussed terms was 5.2 μg kg^{-1}, and the LOQ was 15.7 μg kg^{-1}. These results are in the same range as other GC-MS methods reported in different studies (Robarge et al. 2003; Tareke et al. 2002; Wenzl et al. 2003; Claeys et al. 2005; Taeymans et al. 2004; Pittet et al. 2004; Nemoto et al. 2002).

Besides all the advantages described when using MSPD, such as the reduced sample manipulation, solvent usage and disposal, and good extraction efficiency, the proposed method also presents a reduced working time when compared to our previous LE-GC/MS method. The later one usually takes more than 1 h to prepare a sample until the derivatization step, while in the proposed MSPD-GC/MS method, less than 20 min was taken to reach the same point of analysis.

A brief analysis of the results obtained with the studied samples shows, for some of the food matrices, a large range of values among the various samples studied, which is in agreement with other published studies, difficulting the estimation of acrylamide daily ingestion based on food intake data. This is particularly evident for breakfast cereals, which presents acrylamide levels ranging from 36 to 539 μg kg^{-1} depending on the additives present in the samples. The highest values corresponded to cereals mixed with honey. For biscuit and cookies, the values ranged from 49 to 996 μg kg^{-1}, being the lowest results for biscuits prepared with maize flour and the highest for wheat products. The FDA (US Food and Drug Adminstration 2006) reported for breakfast cereals concentrations of acrylamide in the range of 25 to 534 μg kg^{-1} and for cookies 34 to 955 μg kg^{-1}. The European Union (EU/WHO

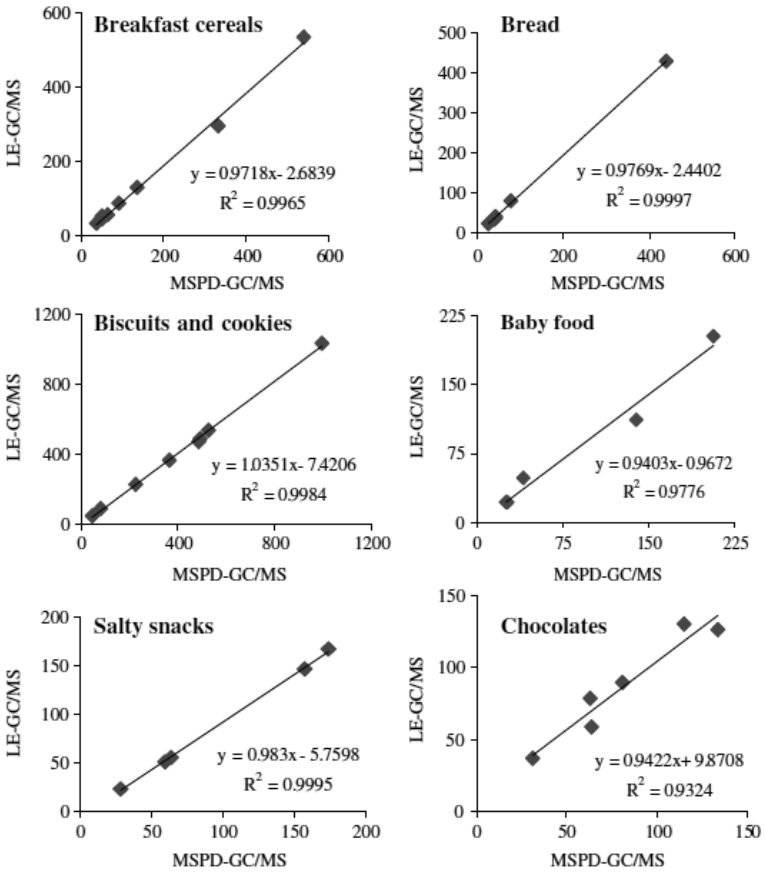

Fig. 3 Comparison of the results obtained with the two methods applied in this work for each one of the six food groups. The results are given in μg kg^{-1} of acrylamide

2006) published in an acrylamide monitoring database values of the compound in the range of 10 to 440 μg kg^{-1} for breakfast cereals and for cookies between 36 and 1,047 μg kg^{-1}. The other studied food matrices presents more homogeneous results, bread 39 to 78 $\mu g.kg^{-1}$, snacks 28 to 174 $\mu g.kg^{-1}$, baby food 25 to 206 $\mu g.kg^{-1}$, and chocolate 15 to 134 $\mu g.kg^{-1}$ of acrylamide. The results obtained are once more in line with the ones reported in the databases of FDA and EU.

Conclusions

MSPD extraction technique was applied to the extraction of acrylamide from foodstuff. The presented method used 1 g of sample disrupted in 4 g of C_{18}, and after blending, AA was extracted with 6+6 ml of water. After bromination of the extracts, the samples were analyzed by GC-MS. The practical limit of detection achieved was 5.2 μg kg^{-1}. The comparison of the results obtained for 40 samples of different foodstuffs using the MSPD method and a conventional liquid extraction method previously developed in our laboratory permits to conclude that they furnished similar results. However, the new method permits to carry out the analysis in an easier, simpler, and more expeditious manner, showing advantages over the generally employed liquid extraction methods.

Acknowledgments The authors are thankful to FCT for financial support in the framework of the project POCI/AGR/61543/2004 and for the PhD scholarship of Cristina Soares with the reference SFRH/BD/39360/2007.

References

Andrzejewski D, Roach JAG, Gay ML, Musser SM (2004) J Agric Food Chem 52:1996. doi:10.1021/jf0349634

Arisseto AP, Toledo MCF, Govaert Y, van Loco J, Fraselle S, Degroodt J-M (2008) Food Anal Methods 1:49

Castle L (2006) In: Acrylamide and other hazardous compounds in heat-treated foods, Ch. 6, 117. CRC, Boca Raton, ISBN: 978-0-8493-9096-8 (2006)

Castle L, Eriksson S (2005) J AOAC Int 88:274

Claeys WL, De Vleeschouwer K, Hendrickx ME (2005) Trends Food Sci Technol 16:181. doi:10.1016/j.tifs.2005.01.005

Dybing E, Sanner T (2003) Toxicol Sci 75:7. doi:10.1093/toxsci/kfg165

Fernandes J, Soares C (2007) J Chromatogr A 1175:1. doi:10.1016/j.chroma.2007.10.030

Miller JC, Miller JN (1993) Statistics for analytical chemistry, 3rd edn. Ellis Horwood Lda, Chichester, ISBN: 0-13-030990-7

Nemoto S, Takatsuki S, Sasaki K, Maitani T (2002) J Food Hyg Soc Jpn 43:371

Ono H, Chuda Y, Ohnishi-Kameyama M, Yada H, Ishizaka M, Kobayashi H, Yoshida M (2003) Food Addit Contam 20:215. doi:10.1080/0265203021000060887

Pittet A, Périsset A, Oberson J-M (2004) J Chromatogr A 1035:123. doi:10.1016/j.chroma.2004.02.037

Ribani M, Bottoli C, Jardim I, Melo L (2004) Quim Nova 27:771. doi:10.1590/S0100-40422004000500017

Riediker S, Stadler RH (2003) J Chromatogr A 1020:121. doi:10.1016/S0021-9673(03)00876-8

Robarge T, Phillips E, Conoley M (2003) LC-GC, Eur. The applications book. Thermo Electron Corp. Press, San Jose, CA, p. 2 (September)

Rosén J, Hellenäs K-E (2002) Analyst (Lond) 127:880. doi:10.1039/b204938d

Soares C, Cunha S, Fernandes J (2006) Food Addit Contam 23:1276. doi:10.1080/02652030600889608

Taeymans D, Wood J, Ashby P, Blank I, Studer A, Stadler RH, Gondé P, Van Eijck P, Lalljie S, Lingnert H, Lindblom M, Matissek R, Müller D, Tallmadge D, O'Brien J, Thompson S, Silvani D, Whitmore T (2004) Crit Rev Food Sci Nutr 44:323. doi:10.1080/10408690490478082

Tareke E, Rydberg P, Karlsson P, Eriksson S, Törnqvist M (2002) J Agric Food Chem 50:4998. doi:10.1021/jf020302f

US Food and Drug Adminstration (2006) Center for Food Safety and Applied Nutrition, Survey Data on Acrylamide in Food: Individual Food Products. http://www.cfsan.fda.gov/~dms/acrydata.html

European Union Joint Research Center/World Health Organization (EU/WHO) (2006) Monitoring database. http://irmm.jrc.ec.europa.eu/html/activities/acrylamide/EUacrylamidelevelmonitoringdatabase__statusJune2006.xls

Wenzl T, Anklam E (2007) Food Addit Contam 24:5. doi:10.1080/02652030701216479

Wenzl T, Beatriz de la Calle M, Anklam E (2003) Food Addit Contam 20:885. doi:10.1080/02652030310001605051

Zhang Y, Zhang G, Zhang Y (2005) J Chromatogr A 1075:1. doi:10.1016/j.chroma.2005.03.123

2.2- Development and Validation of a Matrix Solid-Phase Dispersion Method to Determine Acrylamide in Coffee and Coffee Substitutes

Abstract:

The present work describes the development and validation of a new method based on a Matrix Solid Phase Dispersion (MSPD) sample preparation procedure followed by GC/MS for determination of acrylamide levels in coffee (ground coffee and brewed coffee) and coffee substitute samples. Samples were dispersed in C_{18} sorbent and the mixture was further packed into a pre-conditioned custom-made ISOLUTE bi-layered SPE column (C_{18}/Multimode; 1 g+1 g). Acrylamide was subsequently eluted with water, and then derivatized with bromine and quantified by GC/MS in SIM mode. The MSPD-GC/MS method presented a LOD of 5 μg/kg and a LOQ of 10 μg/kg. Intra and interday precisions ranged from 2-4 % and 4-10%, respectively. To evaluate the performance of the method, 11 samples of ground and brewed coffee and coffee substitutes were simultaneously analysed by the developed method and also by a previously validated method based in a Liquid-Extraction (LE) procedure, and the results were compared showing a high correlation between them.

Keywords: Matrix Solid-Phase Dispersion (MSPD), acrylamide, coffee, coffee substitutes, GC/MS

JFS T: Toxicology and Chemical Food Safety

Journal of Food Science
THE Society for Food Science & Technology
A Publication of the Institute of Food Technologists

Development and Validation of a Matrix Solid-Phase Dispersion Method to Determine Acrylamide in Coffee and Coffee Substitutes

CRISTINA M. DIAS SOARES, RITA C. ALVES, SUSANA CASAL, M. BEATRIZ P.P. OLIVEIRA, AND JOSÉ OLIVEIRA FERNANDES

ABSTRACT: The present study describes the development and validation of a new method based on a matrix solid-phase dispersion (MSPD) sample preparation procedure followed by GC-MS for determination of acrylamide levels in coffee (ground coffee and brewed coffee) and coffee substitute samples. Samples were dispersed in C_{18} sorbent and the mixture was further packed into a preconditioned custom-made ISOLUTE bilayered SPE column (C_{18}/Multimode; 1 g + 1 g). Acrylamide was subsequently eluted with water, and then derivatized with bromine and quantified by GC-MS in SIM mode. The MSPD/GC-MS method presented a LOD of 5 μg/kg and a LOQ of 10 μg/kg. Intra and interday precisions ranged from 2% to 4% and 4% to 10%, respectively. To evaluate the performance of the method, 11 samples of ground and brewed coffee and coffee substitutes were simultaneously analyzed by the developed method and also by a previously validated method based in a liquid-extraction (LE) procedure, and the results were compared showing a high correlation between them.

Keywords: acrylamide, coffee, coffee substitutes, GC-MS, matrix solid-phase dispersion (MSPD)

Introduction

Coffee is one of the world's most popular beverages due to its attractive aroma, taste, and likely health benefits (Illy and Viani 2005). Accordingly, more than 7 million tons of coffee are produced and consumed per year around the world, making it one of the agricultural commodities of greater importance to the global scale (ICO 2009).

Coffee beans, which correspond to the seeds of crimson fruits from which the outer pericarp is completely removed, undergo several processes before they become the well-known roasted coffee. Once ripe, coffee berries are picked, processed, and dried. The beans are then roasted to varying degrees, undergoing several physical and chemical changes that influence the final flavor. They are then ground and brewed to create coffee drinks (Smith 1985; Clarke 1987). Coffee can be prepared and presented in a variety of ways depending on the regional and cultural preferences.

The most common ways to prepare coffee beverages correspond to the use of decoction methods (Turkish coffee, boiled coffee), steeping (French press), infusion (filter coffee), and Italian pressure methods (moka and espresso coffee) (Illy and Viani 2005). In Portugal, like other Mediterranean countries such as Spain and Italy, coffee is highly consumed as "espresso" which is prepared with a small amount of hot water (± 30 mL) percolated in a very short time at high pressure through a layer of ground roasted coffee (coffee cake: ±6 to 7 g) to produce efficiently a very concentrated brew (approximately 200 g of ground coffee per liter) (Nunes and others 1997).

It is now well established that coffee and other roasted materials used as coffee substitutes such as barley, chicory, figs, rye, corn, beetroot, and others are important sources of acrylamide, being among the highest contributors to the acrylamide intake in some developed countries (Dybing and others 2005; Seal and others 2008). Depending on the population's age, eating habits and processing preferences, the contribution of coffee to acrylamide exposure can reach close to 40% (Table 1).

To establish the exposure of consumers to acrylamide a special focus was put in the development of analytical techniques able to provide reliable data. The great majority of reported methods for acrylamide determination employed liquid chromatography tandem mass spectrometry (LC-MS/MS) or gas chromatography-mass spectrometry (GC-MS) for detection and quantification of the compound, presenting chiefly differences in the extraction and clean-up procedures (Zhang and others 2005). Although successfully applied to food matrices like potato and cereal products, many methods initially experienced major problems with the determination of acrylamide in more complex matrices, such as coffee (Riediker and Stadler 2003; Roach and others 2003). Nevertheless, over the past years adequate methods for acrylamide quantification in coffee brew and ground coffee were reported, most of all using LC-MS/MS and only a very few employing GC-MS (for references see Table 2). In general, authors are in agreement that additional clean-up steps are required to obtain an extract that provides final chromatograms almost free of interferences.

One of the 2 GC-MS methods for acrylamide analysis in coffee samples referred in Table 2 was previously reported by our research group (Soares and others 2006) (see Materials and Methods for a brief description). The method performed reasonably well in these kinds of matrices, presenting final chromatograms almost free of major interferences although complex and very time consuming. A solution to overcome a similar problem with a liquid extraction method applied to other food matrices but coffee was the use of matrix solid-phase dispersion (MSPD) to extract acrylamide from foodstuffs (Fernandes and Soares 2007; Soares and Fernandes 2009). The application of MSPD was, however, more challenging for coffee matrices due to the inevitability of extensive clean-up to eliminate important co-extractives.

MS 20090875 Submitted 9/7/2009, Accepted 1/7/2010. *Authors are with REQUIMTE, Serviço de Bromatologia, Faculdade de Farmácia, Univ. do Porto, Rua Antbal Cunha, 164, 4099-030 Porto, Portugal. Direct inquiries to author Dias Soares (E-mail: cristina.md.soares@gmail.com).*

Authors Dias Soares and Alves contributed equally to this study.

doi: 10.1111/j.1750-3841.2010.01545.x

Thus, the aim of the present study was to develop an MSPD procedure combined with a SPE clean-up in the determination of acrylamide in coffee products, avoiding the initial time-consuming liquid purification steps used in the liquid-extraction method previously published. The results obtained and the details concerning the development and validation of the method are discussed thoroughly in the next sections.

Materials and Methods

Sampling

Coffee samples were collected from several local supermarkets and stored at 4 °C. Samples included 1 roasted ground coffee, 1 decaffeinated coffee, 1 "torrefacto" coffee (coffee beans roasted with 15% of added sugar), 2 instant coffees, 1 coffee blend with cereals, and 2 roasted barley (soluble and insoluble).

Chemicals

Acrylamide (AA) with 99% purity grade was acquired from Aldrich (Steinheim, Germany). The internal standard acrylamide 1, 2, 3-C^{13} ($^{13}C_3$-AA) as a 1 mg/mL methanol solution with 99% purity grade was purchased from Cambridge Isotope Laboratories (Andover, Mass., U.S.A.). The solid-phase extraction (SPE) columns: ISOLUTE Multimode 1 g, ISOLUTE Multimode 3 g custom made, and the ISOLUTE C_{18}/Multimode (1 g + 1 g) bilayered SPE columns also custom made (see Figure 1A) were obtained from Biotage (Uppsala, Sweden). The bulk C_{18} sorbent 125 Å, 55 to 105 μm was obtained from Waters (Milford, Mass., U.S.A.). Before use, both bulk C_{18} (2 g per sample) and ISOLUTE columns were conditioned with 10 mL of methanol followed by 10 mL of water (holding the flow for a few minutes in each step). Care was taken to avoid drying the sorbents. All the other chemicals relevant for this study were described elsewhere (Soares and others 2006; Fernandes and Soares 2007).

Standards and reagents

A stock solution of AA (2 g/L) was prepared by dissolving the compound in acetonitrile, and appropriately diluted to prepare 2 working standard solutions at 1 and 4 mg/L that were used to prepare the calibrating solutions. Working standard solutions of internal standard (IS), $^{13}C_3$-AA, were prepared with acetonitrile at 10 and 40 mg/L. All solutions were stored at 4 °C. The saturated bromine–water solution and the Carrez reagents were prepared as previously described (Soares and others 2006).

Equipment and GC-MS operating conditions

GC-MS analysis were performed in a gas-chromatograph, model Agilent GC-6890N, equipped with a split-splitless injector and an automatic injector model 7683B Series for sample introduction, and coupled to a Mass Selective Detector model Agilent MSD-5975N (Agilent, Palo Alto, Calif., U.S.A.). The analytical separation was performed in a capillary column MDN-12 (30 m × 0.25 μm, 0.25 mm i.d.) from Supelco (Steinheim, Germany). MSD was used in the selected ion monitoring mode (SIM), using 6 selected fragments characteristics of derivatized AA (m/z 106, 150, and 152) and derivatized IS (m/z 110, 153, and 155). All the other equipment and further details of the operating conditions were described thoroughly elsewhere (Fernandes and Soares 2007).

Sample preparation

Matrix solid-phase dispersion (MSPD/SPE-GC/MS)

Ground coffee. A 0.5 g aliquot of ground coffee and 2 g of preconditioned C_{18} were placed in a glass mortar, added with 0.25 μg of IS (25 μL of the 10 mg/L IS solution) and thoroughly blended using a glass pestle during approximately 2 min. When blending was accomplished, the dispersed mixture was transferred to a preconditioned ISOLUTE C_{18}/Multimode (1 g + 1 g) bilayered SPE column. A frit was placed on the top of the sample mixture before careful compression with a syringe plunger. The packed column was placed in a vacuum manifold and acrylamide was eluted with 4 mL of water, holding the flow to guarantee a soaking step of 5 min. The water extract was collected on a 40-mL vial until reaching the top limit of the frit, to avoid drying the sorbents, and a 2nd portion of 4 mL of water was added, respecting again a 5-min soaking step. The 2nd elution volume was completely collected to the same vial keeping the column under vacuum during 5 min to collect all the water. All samples analyzed were prepared in duplicate.

Coffee brews (from ground coffees, instant coffees, and coffee substitutes). All coffee samples were brewed before analysis using 6 g of coffee product mixed with 30 mL of hot water (30 min, approximately 90 °C) in a GFL Shaking Water Bath 1083 (Millian Swiss Labware, Geneva, Switzerland). After cooling down to room temperature, coffee brews were centrifuged during 5 min at 5000 rpm and the supernatant transferred to another flask and kept in the refrigerator until acrylamide extraction. 2.5 mL of ground coffee brew or 1.25 mL of instant coffee or coffee substitute brew were treated as the overall MSPD method already described for ground coffee samples. The samples containing barley presented additional difficulties (see Discussion for details) during the MSPD extraction that demanded some changes in the procedure. No mortar and pestle were used and the dispersion was performed as follows: 1.25 mL of barley brew and 2 g of preconditioned C_{18} were put in a 15-mL corning centrifuge tubes and dispersed in a vortex during 2 min. The dispersed mixture was centrifuged at 5000 rpm (5 min) and only the supernatant transferred to the preconditioned ISOLUTE C_{18}/Multimode (1 g + 1 g) cartridges. The filtrate was further redispersed in the vortex with 1 mL of water which was also transferred to the SPE column. The elution was performed as described previously for the other samples.

Liquid extraction (LE/SPE-GC/MS). To evaluate the performance of the MSPD method, the samples were simultaneously analyzed by a previously optimized liquid extraction procedure (Soares and others 2006). Very briefly, coffee samples were extracted with hot water, followed by ethanol and Carrez solutions addition to promote the precipitation of polysaccharides and proteins,

Table 1 – Foodstuffs contribution to overall acrylamide exposure in different countries.

Country	Acrylamide exposure contribution			Population group	Reference
	Potato products	Bread	Coffee		
US	41%	9%	**7%**	>2 y	USFDA (2006)
The Netherlands	50%	10%	**13%**	Whole population	Konings and others (2003)
	59%	10%	0%	Children, adolescents (7 to 18 y)	
Norway	34%, 29%	21%, 24%	**27%, 29%**	Males, females (16 to 79 y)	Dybing and Sanner (2003)
Sweden	26%	17%	**39%**	Adults (18 to 74 y)	Svensson and others (2003)
Germany	10%, 13%	29%, 34%	**7%, 16%**	Boys, girls (15 to 19 y)	Hilbig and Kersting (2006)
Switzerland	—	—	**36%**	Males, females (16 to 57 y)	Brunner and others (2002)

Table 2—Chromatographic methods recently published to determine acrylamide in coffee products.

Sample preparation	Chromatogr. method LOD/LOQ (μg/kg)	Sample levels (μg/kg)	Reference
	Liquid chromatography		
Extraction: H_2O	LC-ESI-MS/MS	Ground coffee ($n = 47$): 37 to 374	Roach and others (2003)
SPE clean-up: Oasis HLB cartridge followed by a Bond Elut-Accucat cartridge	LOD: 4		
	LOQ: 10		
Extraction: H_2O	LC-MS/MS	Instant coffee ($n = 12$): 169 to 539	
SPE clean-up: Oasis HLB cartridge followed by a Bond Elut-Accucat cartridge	LOD: Ground/instant coffee: 10	Ground coffee ($n = 31$): 45 to 374	Andrzejewski and others (2004)
	Brewed coffee: 1[a]	Brewed coffee ($n = 40$): 6 to 11[a]	
Extraction: H_2O	LC-MS/MS	Ground coffee ($n = 2$): 203 to 312	
Clean-up: deproteination with Carrez I and II and DCM salting out with NaCl and extraction with EtAc	LOD: 9.2	Soluble coffee powder ($n = 1$): 771	Delatour and others (2004)
SPE clean-up: ISOLUTE Multimode cartridge	LOQ: 12.5	Coffee substitutes ($n = 3$): 214 to 4015	
Extraction: H_2O	LC-MS/MS	Brewed coffee ($n = 30$): 2 to 16[a]	Granby and Fagt (2004)
SPE clean-up: ISOLUTE Multimode cartridge	LOD: 2[a]		
Extraction: MeOH	LC-MS	Ground coffee ($n = 11$): 14 to 29	
Clean-up: deproteination with Carrez I and Carrez II	LOD: 2.0	Turkish coffee ($n = 5$): 29 to 75	Senyuva and Gökmen (2005)
SPE clean-up: Oasis HLB cartridge	LOQ: 10.0	Filter coffee ($n = 1$): 50 Instant coffee ($n = 3$): 42 to 338	
Extraction: H_2O	LC-MS/MS	Ground coffee ($n = 3$): 43.4 to 464.8	Águas and others (2006)
Clean-up: cleaning with DCM	LOQ: 12.3		
SPE clean-up: aminopropyl solid-phase extraction cartridge			
Extraction: H_2O	LC-MS/MS	Instant coffee ($n = 1$): 715	Arisseto and others (2008)
Clean-up: deproteination with Carrez I and Carrez II and cleaning with EtAc	LOD: 10	Ground coffee ($n = 1$): 206	
SPE clean-up: ISOLUTE Multimode cartridge	LOQ: 20		
Extraction: with H_2O after a defatting step with hx	LC-MS/MS	Ground coffee ($n = 12$): 299 to 762	Bagdonaite and others (2008)
SPE clean-up: Bond Elut-Accucat cartridge	LOD: 3.0		
Derivatization: 2-mercaptobenzoic acid at pH 8 during 3 h			
Extraction: H_2O	LC-MS/MS	Coffee surrogate ($n = 1$): 1492.4	Granvogl and Schieberle (2007)
Clean-up: hx and charcoal	LOD: 1.0 to 3.0	Coffee extract ($n = 1$): 806.9	
Derivatization: 2-mercaptobenzoic acid at pH 10 during 3 h		Ground coffee ($n = 1$): 267.7	
Extraction: H_2O	LC-MS/MS	Ground coffee ($n = 1$): 144.5	Mastovska and Lehotay (2006)
Clean-up: hx, MeCN, salting out with $MgSO_4$ and NaCl	LOQ: 10.0		
SPE clean-up: dispersive-SPE clean up with $MgSO_4$ and PSA			
Extraction: pressurized fluid extraction (PFE) with MeCN	LC-MS/MS	Ground coffee ($n = 20$): 62 to 287	Pardo and others (2007)
Clean-up: Florisil inside the PFE extraction cell	LOQ: 1.0		
	Gas chromatography		
Extraction: H_2O	GC-MS	Ground coffee ($n = 1$): 169	Nemoto and others (2002)
SPE clean-up: Florisil cartridge after derivatization	LOQ: 9.0	Coffee substitutes ($n = 2$): 195 to 520	
Derivatization: bromination during 90 min			
Extraction: H_2O	GC-MS	"Espresso" ($n = 18$): 11 to 36[a]	Soares and others (2006)
Clean-up: EtOH, followed by Carrez I and II	LOQ: 10.0[a]	Soluble coffee ($n = 5$): 47 to 95[a]	
SPE clean-up: ISOLUTE Multimode/C18 cartridges		Coffee substitutes ($n = 2$): 201 to 229[a]	
Derivatization: bromination during 1 h			

[a]LOD/LOQ and sample levels in μg/L.
DCM = dicholomethane; EtOH = ethanol; EtAc = ethyl acetate; hx = n-hexane; MeOH = methanol; MeCN = acetonitrile.

respectively. The resulting extract was centrifuged, filtrated and a partial evaporation of water under vacuum was performed until 5 mL of final solution was reached. The clean-up was performed using a solid-phase extraction (SPE) on ISOLUTE Multimode (3 g)/C_{18} (1 g) cartridges and the extracts brominated before GC-MS analysis in SIM mode.

Preparation of standards for calibration

MSPD/SPE-GC/MS. Aliquots of the 1 mg/L working standard AA solution (equivalents to 0 to 0.75 μg of AA) and 0.25 μg of internal standard (25 μL of the 10 mg/L solution) were used to prepare 7 standards with the concentrations of 0, 50, 100, 250, 500, 1000, and 1500 μg/kg according to the described procedure.

LE/SPE-GC/MS. Aliquots of the 4 mg/L working standard AA solution (equivalent to 0 to 3 μg of AA corresponding to 0 to 1500 μg/kg in the samples) and 1 μg of internal standard (25 μL of the 40 mg/L solution) were treated to prepare 7 standards as described previously (Soares and others 2006).

Bromination of the samples and standards

The aqueous extracts obtained in both methods were brominated and the derivatives extracted with hexane: ethyl acetate 1 : 4 (v/v) before GC-MS analysis as previously reported (Soares and others 2006).

Results and Discussion

Two MSPD procedures were previously optimized by our group directed to acrylamide quantification in potato chips (Fernandes and Soares 2007), and in other matrices such as cereal-based foods, baby foods, and chocolates (Soares and Fernandes 2009). The application of MSPD to more challenging matrices like coffee and coffee substitutes was developed to perform simultaneous extraction and purification of the analyte using the minimum sorbent and solvent consumption.

Ground coffee samples spiked with known amounts of AA and $^{13}C_3$-AA were used to perform the preliminary assays. Following the guidelines of our previous study, C_{18} (previously conditioned with 10 mL of methanol followed by 10 mL of water) was the sorbent chosen to perform the dispersion of the sample. Different sample/sorbent ratios were assayed: 1 : 6 (0.5 g of sample and 3 g of C_{18}); 1 : 4 (0.5 g of sample and 2 g of C_{18} and 1 g of sample and 4 g of C_{18}); 1 : 2 (1 g of sample and 2 g of C_{18}); and 1 : 1 (2 g of sample and 2 g of C_{18}). After transference for an empty SPE column, the dispersed sample was packed, fats were eliminated by elution with 20 mL of n-hexane, and acrylamide was extracted with different volumes of water, 6 + 6, 5 + 5, or 4 + 4 mL. Whatever the assayed combination, the resulting extracts were highly colored in all cases and the respective chromatograms presented too many interferences to allow quantification. Besides C_{18}, bulk ISOLUTE MultiMode and a 1 : 1 mixture of C_{18} and ISOLUTE were also assayed as sorbents to perform the MSPD extraction. Mixtures of 0.5 g of sample and 2 g of preconditioned sorbent were used and, after defatting with n-hexane, acrylamide was extracted with 4 + 4 mL of water. Again highly colored extracts were obtained and the chromatograms showed similar features like that obtained with C_{18}.

To overcome the problem an attempt was made employing methanol instead of water as acrylamide extraction solvent, taking into account the high solubility of the compound in this solvent (solubility 155 g/100 mL at 30 °C, Castle and Eriksson 2005). Mixtures of 0.5 g of sample and 2 g of preconditioned sorbent (C_{18} or ISOLUTE or 1 : 1 mixture of both sorbents) were prepared and extracted with 8 + 8 mL of methanol. The alcoholic extracts were evaporated in a rotary evaporator until 2 mL and then 5 mL of water were added before bromination. Although the final solutions were not so strongly colored as the water extracts previously referred and less interferences were present in the chromatogram, still an insufficient yield for acrylamide extraction was attained. A mixture of water/methanol (30/70) was also tried as the extraction solvent but no improvement was reached. It was concluded that further clean-up was mandatory to eliminate the major interferences that avoid acrylamide quantification.

To achieve the best additional SPE clean-up conditions, ISOLUTE multimode sorbent and C_{18} were tested alone or combined as a bilayered packing. Dispersed mixtures of 0.5 g of sample and 2 g of sorbent (preconditioned C_{18} or ISOLUTE) were packed in SPE columns containing 1, 2, and 3 g of sorbent or, alternatively, a bilayered sorbent composed by 1 g of each sorbent (A—1 g of C_{18} on top and 1 g of ISOLUTE at bottom; and B—1 g of ISOLUTE over 1 g of C_{18}) (Figure 1). Although lighter extracts were also obtained from the columns prepared with 2 and 3 g of C_{18} sorbent, the better results both at visual level or the chromatographic results were obtained from the bilayered columns, with advantage for the use

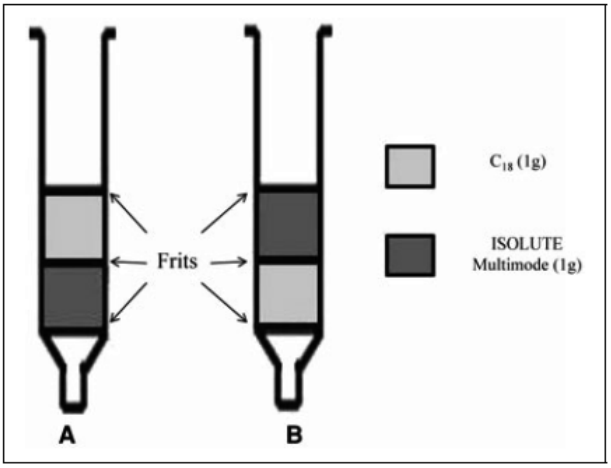

Figure 1 — SPE columns used for clean-up of coffee samples with 2 combinations of sorbents: (A) 1 g C_{18} over 1 g ISOLUTE and (B) 1 g ISOLUTE over 1 g C_{18}.

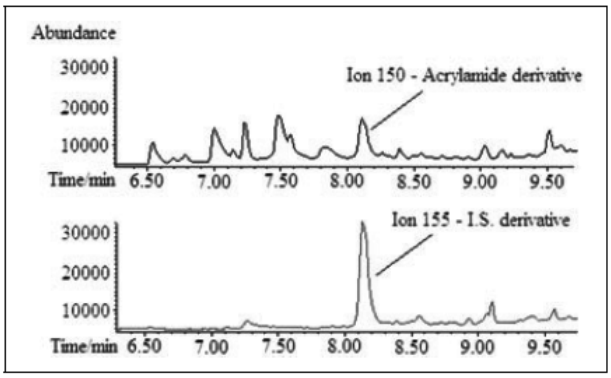

Figure 2 — Chromatogram of a ground coffee sample prepared with the final procedure: 0.5 g of sample and 2 g of preconditioned C_{18} packed over a 1 g C_{18} over 1 g ISOLUTE SPE column. The sample analyzed presented 210 μg/kg of acrylamide and 500 μg/kg of internal standard.

Table 3 – Final procedure used for MSPD/SPE-GC/MS extraction.

MSPD mixture	SPE column	C_{18} (MSPD) and SPE column conditioning	Acrylamide extraction	Results
0.5 g of ground coffee + 2 g C_{18}	1 g of C_{18} (top) + 1 g of ISOLUTE (bottom)	10 mL methanol + 10 mL water	4 + 4 mL water	Clean final extract (no color). Final chromatogram with no major interferences (Figure 2).

of the combination A, which provides uncolored extracts and chromatograms without major interferences (Figure 2).

Once established the better conditions for the extraction/clean-up of the dispersed mixtures, 2 parameters were again studied: the ratio sample/sorbent used in the initial MSPD process and the need to use n-hexane to eliminate fat. In what respect the first one, experiments were conducted at different spiking levels, with a ratio sample/sorbent 1 : 2 (1 g of sample and 2 g of C_{18}) to enhance the overall sensitivity of the method. No improvements were observed because the elution of all the AA present in the samples required more than 4 + 4 mL of water as elution solvent, resulting in final water extracts with roughly the same concentration of AA, and worst chromatographic performance. The influence of the defatting step with n-hexane was also tested, and results showed that no expressive differences were obtained when defatting is not performed. This can be explained by the low fat levels of this kind of sample. Therefore, to simplify the procedure, the defatting step was removed from the final analytical scheme.

As already noted, bulk C_{18} used for MSPD was tested after a previously conditioning procedure with 10 mL of methanol followed by 10 mL of water. The preconditioned bulk C_{18} exhibited improved cleaning properties in combination with SPE columns very likely because it also retains more effectively colored compounds that interfere with the analysis. Special care should be taken to avoid drying of the sorbent otherwise C_{18} would lose its potential to retain colored particles giving rise to highly contaminated chromatograms during GC-MS analysis, hindering an accurate separation/quantification of acrylamide. All SPE columns were also conditioned before use and care to avoid drying of the sorbents is also demanding. The volumes of solvents used to conditioning the sorbent and to perform the extractions were adapted to each quantity of bulk C_{18} used in the different experiments. In Table 3 is resumed the final procedure adopted to perform acrylamide extraction using MSPD from ground coffee.

Once established the better conditions to analyze ground coffee, the method was afterwards optimized for coffee brews prepared from ground coffee, instant coffee, and coffee surrogates. A small volume of the brews (2.5 mL) were subjected to the overall methodology described for ground coffee. Notwithstanding the small volume used, the high concentration of colored particles and polysaccharides in brews made from instant coffee and coffee surrogates make the MSPD extraction less efficient when compared with the ground coffee brews. To overcome this hindrance the solution successfully assayed was to reduce the volume of the instant and surrogates brewages from 2.5 to 1.25 mL. Besides this difficulty, a different problem was found with coffee surrogates containing barley. When performing the MSPD dispersion of a barley sample with C_{18} it was noticed that a gel-like mixture was produced having as unwanted consequence the inability to complete the elution procedure that follows, what is probably due to the obstruction of the SPE column frits. The solution adopted was to perform the dispersion of the barley-based matrices with C_{18} in a centrifugal tube using a vortex instead of using a mortar and pestle. After the vortex dispersion (approximately 1 min) only the supernatant was transferred to the SPE column surpassing the described problem. The troubles associated with the barley brews were probably due to the high viscosity of the aqueous extracts. The higher the content of roasted barley in the extracted mixture the higher the viscosity of the extract produced due to a high content of starch and dextrins. This can cause many difficulties in the extraction and filtration of the extracts (Pazola 1987).

The developed MSPD/SPE-GC/MS method was evaluated in terms of linearity, precision, recovery, and limits of detection and quantification. The linearity of the method was assessed using standard (calibrating) solutions treated with the same method developed for the samples. Seven standards were simultaneously prepared (range of concentrations corresponded to 0 to 1500 μg/kg AA in the sample) and calibration curves were constructed by plotting the AA/IS area ratio against the concentration of AA in the standard. The correlation coefficients were usually higher than 0.999.

One ground coffee, one coffee brew, and a surrogate brew were used to assess the linearity of the method in real samples, the intraday and the interday precisions. Linearity of the method in real samples was estimated by preparing 6 aliquots (0.5 g or 2.5 mL of brew) of a sample with a known amount of AA. To the samples were added 0, 0.025, 0.050, 0.125, 0.250, and 0.500 μg of acrylamide (0, 25, 50, 125, 250, and 500 μL of the 1 mg/L AA solution) and 0.25 μg of IS. The samples were treated as described for the overall method and injected twice. A plot of the AA area/IS area ratio against the added amounts of AA was constructed and the correlation coefficients calculated were higher than 0.990 (Figure 3).

The intraday precision was evaluated by triplicate analysis of the same sample, prepared simultaneously by the overall method, and injected in triplicate within the same day. To study the interday precision of the method, again 3 aliquots of the same sample were prepared and submitted to the overall developed method in 3 different weeks, and injected in triplicate in different days. The

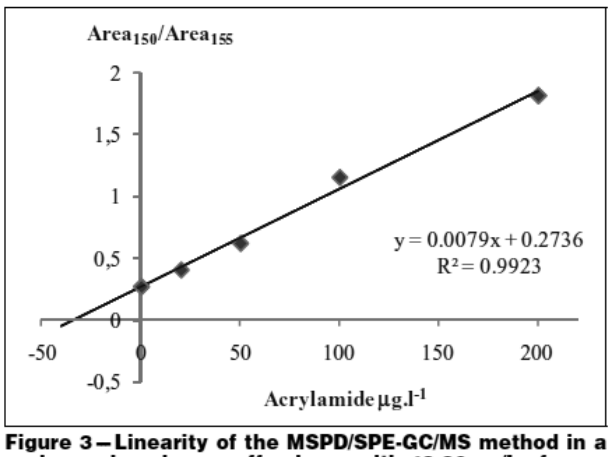

Figure 3 – Linearity of the MSPD/SPE-GC/MS method in a real sample using a coffee brew with 43.80 μg/L of acrylamide. The value calculated using the curve parameters was 42.75 μg/L.

intraday precisions were 2.5% for ground coffee, 3.6% for ground coffee brew, and 3.4% for soluble coffee brew and the interday precisions were 4.5%, 10.2%, and 10.4%, respectively.

The recovery of the extraction procedure was assessed by preparing 2 different sets of a coffee brew with 237 μg/kg (or 52.6 μg/L) of acrylamide. The 1st set was prepared by spiking the sample before extraction with 100, 200, and 500 μg/kg of acrylamide. The 2nd set was prepared by spiking the sample after the extraction procedure with the same levels of acrylamide. In both cases, internal standard was added after the extraction procedure. All samples were prepared simultaneously and the final extracts, obtained after derivatization, injected twice. Recoveries varied between 84% and 97%.

The limit of detection (LOD) of the method was calculated using the calibration curve parameters as in previous studies (Fernandes and Soares 2007; Soares and Fernandes 2009). The LOD obtained for this method was 5 μg/kg and the LOQ was 15 μg/kg.

All samples analyzed in this study were submitted to the described MSPD/SPE-GC/MS method and also to the more classical LE/SPE-GC/MS method routinely used in our laboratory. The validation parameters and the time necessary to prepare the samples until the derivatization step were compared for both methods (Table 4).

The acrylamide levels resulting from the application of both methods are presented in Table 5. For coffee drinks the results are presented in μg/L (assuming that 30 mL of water were used to prepare the brews, which also corresponds to the usual volume of an "espresso" cup). The comparison of the 2 methods was made using a regression line (Figure 4). In the *y*-axis are the results obtained with the conventional method and in the *x*-axis are the results for the MSPD method. Analyzing Table 5 and Figure 4 we can conclude that a close similarity of the results was obtained.

As mentioned previously (Table 2), several other groups had reported results about the presence of acrylamide in coffee and coffee substitutes. The amounts of acrylamide found varied widely in the range of 14 to 762 μg/kg for ground coffee and 6 to 229 μg/L for brewed coffee. The huge range of results is probably due to differences on the type of coffee samples analyzed, differences on the brewing techniques, and finally to the effect of the different methodologies employed to perform the determination. In this study, the values of acrylamide ranged from 210 to 330 μg/kg for ground coffee and from 23.6 to 66.7 μg/L for coffee brews. The coffee consumer's intake depends on the number of coffees that are drunk per day and on the type of coffee used to prepare the drink. Assuming a 30 mL drink as average, usually the volume of an "espresso" that is highly consumed in Portugal, the acrylamide ingestion will vary between 0.71 and 2.00 μg per cup, a value that could be worrying for the big consumers of coffee (Figure 5). As already observed in a previous study (Soares and others 2006), the presence of roasted cereals used as cheaper coffee substitutes increases acrylamide amounts. Accordingly, the highest values of acrylamide intake are therefore associated with the consumption of this kind of sample.

Table 4—Comparison of validation parameters for the developed MSPD/SPE-GC/MS method and the previous LE/SPE-GC/MS methodology (Soares and others 2006).

Method assessment	MSPD/SPE-GC/MS	LE/SPE-GC/MS
LOQ	15 μg/kg	10 μg/L
Linearity in standards (r^2)	0.999	0.997
Linearity in real samples (r^2)	0.990	0.980
Precision	2% to 10%	2% to 6%
Sample preparation time	30 min	2 h
Recovery (AA)	84% to 97%	95% to 108%

Table 5—Acrylamide levels in coffee and coffee substitute samples analyzed by MSPD/SPE-GC/MS and LE/SPE-GC/MS.

Samples	LE/SPE-GC/MS	MSPD/SPE-GC/MS
Ground coffee μg/kg		
Ground coffee	220	210
Decaffeinated coffee	235	237
"Torrefacto" coffee	305	330
Coffee brew μg/L		
Ground coffee	39.8	43.8
Decaffeinated coffee	47.0	52.6
"Torrefacto" coffee	52.8	58.4
Instant coffee	38.2	42.2
Instant coffee (100% arabica)	23.8	23.6
Coffee blend with cereals	66.2	65.4
100% instant barley	67.6	66.7
Roasted barley (insoluble)	47.4	47.4

Conclusions

The present study reports a new approach in sample preparation procedure to the GC/MS determination of acrylamide in

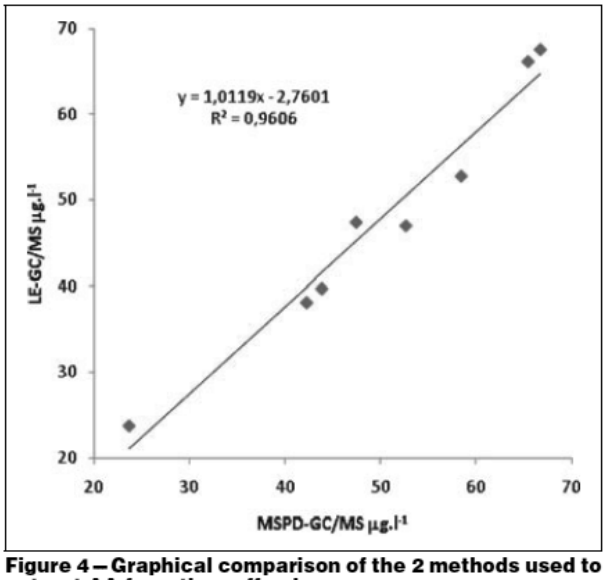

Figure 4—Graphical comparison of the 2 methods used to extract AA from the coffee brews.

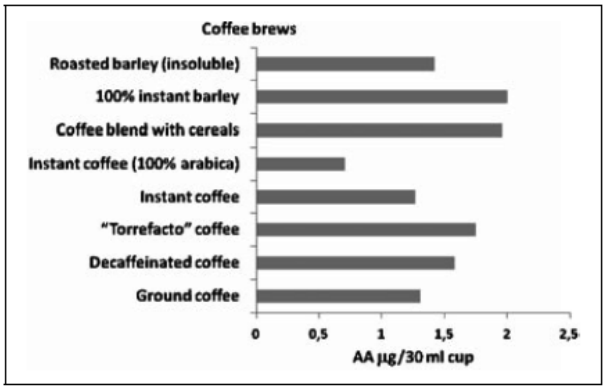

Figure 5—Acrylamide ingestion per 30 mL cup of coffee brew.

complex matrices such as coffee ground, coffee brews, and related beverages. An MSPD procedure combined with custom made SPE bilayered ISOLUTE Multimode/C_{18} cartridges was the technique employed, resulting in clean extracts that permitted efficient chromatographic analysis. The simplicity and ease of application of the reported method for the simultaneous extraction and purification of acrylamide in coffee samples has been demonstrated. The MSPD method compared favorably with more traditional multi-step procedures involving much manual handling, larger amounts of sample, sorbents, and organic solvents, and longer analytical times. The LOD of the method was 5 μg/kg, the LOQ was 15 μg/kg and precisions were in the range of 2% to 10%. All these results indicate that the new method should be considered as particularly suitable for routine analysis of acrylamide in complex foodstuffs such as coffee and similar beverages.

Acknowledgments

The authors are thankful to FCT for financial support in the framework of the project POCI/AGR/61543/2004 and for the PhD grant of Cristina Soares (SFRH/BD/39360/2007). Rita C. Alves is grateful to FCT for a PhD grant (SFRH/BD/22449/2005).

References

Aguas PC, Fitzhenry MF, Giannikopoulos G, Varelis P. 2006. Analysis of acrylamide in coffee and cocoa by isotope dilution liquid chromatography–tandem mass spectrometry. Anal Bioanal Chem 385:1526–31.

Andrzejewski D, Roach JAG, Gay ML, Musser SM. 2004. Analysis of coffee for the presence of acrylamide by LC-MS/MS. J Agric Food Chem 52:1996–2002.

Arisseto AP, Toledo MCF, Govaert Y, van Loco J, Fraselle S, Degroodt J-M. 2008. A modified sample preparation for acrylamide determination in cocoa and coffee products. Food Anal Methods 1:49–55.

Bagdonaite K, Derler K, Murkovic M. 2008. Determination of acrylamide during roasting of coffee. J Agric Food Chem 56:6081–6.

Brunner K, Dudler V, Reinhard H, Rhyn P, Rupp H, Sager F, Streule M, Zimmermann H, Zoller O. 2002. Preliminary communication: assessment of acrylamide intake by duplicate diet study. Bern, Switzerland: Swiss Federal Office of Public Health. Available from: http://www.bfr.bund.de/cm/208/assessment_of_acrylamide_intake_by_duplicate_diet_study.pdf. Accessed Jun 11, 2006.

Castle L, Eriksson S. 2005. Analytical methods used to measure acrylamide concentrations in foods. J AOAC Int 88:274–84.

Clarke RJ. 1987. Coffee: botany, biochemistry and production of beans and beverage. Chapter 10, Green coffee processing. London, U.K.: Croom Helm. p 230–50.

Delatour T, Périsset A, Goldmann T, Riediker S, Stadler RH. 2004. Improved sample preparation to determine acrylamide in difficult matrixes such as chocolate powder, cocoa, and coffee by liquid chromatography tandem mass spectroscopy. J Agric Food Chem 52:4625–31.

Dybing E, Sanner T. 2003. Risk assessment of acrylamide in foods. Toxicol Sci 75:7–15.

Dybing E, Farmer PB, Andersen M, Fennell TR, Lalljie SPD, Müller DJG, Olin S, Petersen BJ, Schlatter J, Scholz G, Scimeca JA, Slimani N, Törnqvist M, Tuijtelaars S, Verger P. 2005. Human exposure and internal dose assessments of acrylamide in food. Food Chem Toxicol 43:365–410.

Fernandes J, Soares C. 2007. Application of matrix solid-phase dispersion in the determination of acrylamide in potato chips. J Chromatogr A 1175:1–6.

Granby K, Fagt S. 2004. Analysis of acrylamide in coffee and dietary exposure to acrylamide from coffee. Anal Chim Acta 520:177–82.

Granvogl M, Schieberle P. 2007. Quantification of 3-aminopropionamide in cocoa, coffee and cereal products. Eur Food Res Technol 225:857–63.

Hilbig A, Kersting M. 2006. Dietary acrylamide exposure, time trends and the intake of relevant foods in children and adolescents between 1998 and 2004: results of the DONALD study. J Verbr Lebensm 1:10–8.

[ICO] Intl. Coffee Organization. Trade Statistics. 2009. London, U.K.: International Coffee Organization. Available from: http://www.ico.org/trade_statistics.asp. Accessed Jul 28, 2009.

Illy A, Vianni R, editors. 2005. Espresso coffee: the science of quality. 2nd ed. San Diego, Calif.: Elsevier.

Konings EJM, Baars AJ, van Klaveren JD, Spanjer MC, Rensen PM, Hiemstra M, van Kooij JA, Peters PWJ. 2003. Acrylamide exposure from foods of the Dutch population and an assessment of the consequent risks. Food Chem Toxicol 41:1569–79.

Mastovska K, Lehotay SJ. 2006. Rapid sample preparation method for LC-MS/MS or GC-MS analysis of acrylamide in various food matrices. J Agric Food Chem 54:7001–8.

Nemoto S, Takatsuki S, Sasaki K, Maitani T. 2002. Determination of acrylamide in foods by GC/MS using ^{13}C-labeled acrylamide as an internal standard. J Food Hyg Soc Jpn 43:371–6.

Nunes FM, Coimbra MA, Duarte AC, Delgadillo I. 1997. Foamability, foam stability, and chemical composition of espresso coffee as affected by the degree of roast. J Agric Food Chem 45:3238–43.

Pardo O, Yusà V, Coscollà C, León N, Pastor A. 2007. Determination of acrylamide in coffee and chocolate by pressurised fluid extraction and liquid chromatography–tandem mass spectrometry. Food Addit Contam 24:663–72.

Pazola Z. 1987. Coffee: related beverages (Volume 5). Chapter 3, The chemistry of cereal based beverages. New York: Elsevier Applied Science. p 59–104.

Riediker S, Stadler RH. 2003. Analysis of acrylamide in food by isotope-dilution liquid chromatography coupled with electrospray ionization tandem mass spectrometry. J Chromatogr A 1020:121–30.

Roach JAG, Andrzejewski D, Gay ML, Nortrup D, Musser SM. 2003. Rugged LC-MS/MS survey analysis for acrylamide in foods. J Agric Food Chem 51:7547–54.

Seal CJ, de Mul A, Eisenbrand G, Haverkort AJ, Franke K, Lalljie SPD, Mykkänen H, Reimerdes E, Scholz G, Somoza V, Tuijtelaars S, van Boekel M, van Klaveren J, Wilcockson SJ, Wilms L. 2008. Risk-benefit considerations of mitigation measures on acrylamide content of foods—a case study on potatoes, cereals and coffee. Br J Nutr 99:S1–46.

Şenyuva HZ, Gökmen V. 2005. Study of acrylamide in coffee using an improved liquid chromatography mass spectrometry method: investigation of colour changes and acrylamide formation in coffee during roasting. Food Addit Contam 22:214–20.

Smith AW. 1985. Coffee: chemistry (Volume 1). Chapter 1. Introduction. New York: Elsevier Applied Science. p 1–41.

Soares C, Fernandes JO. 2009. MSPD method to determine acrylamide in food. Food Anal Methods 2(3):197–203.

Soares C, Cunha S, Fernandes J. 2006. Determination of acrylamide in coffee and coffee products by GC-MS using an improved SPE clean-up. Food Addit Contam 23:1276–82.

Svensson K, Abramsson L, Becker W, Glynn A, Hellenäs K-E, Lind Y, Rosén J. 2003. Dietary intake of acrylamide in Sweden. Food Chem Toxicol 41:1581–6.

[USFDA/CFSAN] US Food and Drug Administration—Center for Food Safety and Applied Nutrition. Survey data on Acrylamide in food: Individual food products. 2006. New Hampshire, Md.: FDA. Available from: http://www.fda.gov/Food/FoodSafety/FoodContaminantsAdulteration/ChemicalContaminants/Acrylamide/ucm053549.htm. Accessed Jul 28, 2009.

Zhang Y, Zhang G, Zhang Y. 2005. Occurrence and analytical methods of acrylamide in heat-treated foods. Review and recent developments. J Chromatogr A 1075:1–21.

CHAPTER 3

Acrylamide in Coffee: Influence of Coffee Variety and Processing

3. Introduction

Coffee is highly consumed all over the world, and the risk of exposure to acrylamide through coffee consumption can be an issue for populations with a high comsumption of the drink.

In this Chapter, the influence of the variety chosen to prepare the drink, Arabica or Robusta, the coffee roast conditions and the volume of the brew ingested are going to be discussed in Paper 3. Acrylamide in Espresso Coffee: Influence of Species, Roast Degree and Brew Lenght.

The processing conditions of the coffee bean, also have an impact on the formation of acrylamide during roastin. Papers 4 and 5 deals with coffee bean processing after harvesting: Influence of unripe coffee fruit processing on acrylamide formation after roasting; and during roasting: Influence of the Roasting Conditions on the Formation of Acrylamide in Brazilian Coffee: Preliminary Results

3.1. Acrylamide in Espresso Coffee: Influence of Species, Roast Degree and Brew Lenght

Abstract

Espresso coffees were analysed for acrylamide contents by matrix solid-phase dispersion and GC/MS. The influence of coffee species, roast degree, and brew length were ascertained. Mean acrylamide contents of medium roasted espressos (30 mL) were 1.16 ± 0.25 and 2.31 ± 0.43 μg for pure arabica and robusta samples, respectively.

Espressos prepared from commercial blends contained an average acrylamide level of 1.26 ± 0.28 μg. A 25% decrease was observed when comparing espressos prepared with medium and dark roasted coffee. The extraction efficacy of acrylamide for standard espressos of 30 mL was near 80%, being only affected by brew volume, with long espressos (70 mL) containing practically all acrylamide of the coffee cake (99%), almost double that of short ones (20 mL). When compared with other common coffee beverages, espresso acrylamide concentration (μg/L) was higher. However, due to the small volume per cup, it may contribute less to acrylamide ingestion.

Keywords: acrylamide, espresso coffee, coffee brews, coffee species, roast, brew length

Food Chemistry 119 (2010) 929–934

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Acrylamide in espresso coffee: Influence of species, roast degree and brew length

Rita C. Alves *,[1], C. Soares [1], Susana Casal, J.O. Fernandes, M. Beatriz P.P. Oliveira

REQUIMTE/Serviço de Bromatologia, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha 164, 4099-030 Porto, Portugal

ARTICLE INFO

Article history:
Received 12 May 2009
Received in revised form 27 July 2009
Accepted 29 July 2009

Keywords:
Acrylamide
Espresso coffee
Coffee brews
Coffee species
Roast
Brew length

ABSTRACT

Espresso coffees were analysed for acrylamide contents by matrix solid-phase dispersion and GC–MS. The influence of coffee species, roast degree, and brew length were ascertained. Mean acrylamide contents of medium roasted espressos (30 mL) were 1.16 ± 0.25 and 2.31 ± 0.43 μg for pure arabica and robusta samples, respectively. Espressos prepared from commercial blends contained an average acrylamide level of 1.26 ± 0.28 μg. A 25% decrease was observed when comparing espressos prepared with medium and dark roasted coffee. The extraction efficacy of acrylamide for standard espressos of 30 mL was near 80%, being only affected by brew volume, with long espressos (70 mL) containing practically all acrylamide of the coffee cake (99%), almost double that of short ones (20 mL). When compared with other common coffee beverages, espresso acrylamide concentration (μg/L) was higher. However, due to the small volume per cup, it may contribute less to acrylamide ingestion.

1. Introduction

Acrylamide (2-propenamide), labelled by the International Agency for Research on Cancer (IARC, 1994) as probably carcinogenic to humans (Group 2A), is presently a focus of worldwide concern, especially since the announcement, in 2002, of its widespread occurrence in carbohydrate-rich cooked foods by the Swedish National Food Administration (SNFA, 2002). Several scientific initiatives have been launched in order to fully understand its chemistry and toxicology, focusing chiefly on its formation mechanism and possible human consequences.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2005) highlighted the importance of acrylamide occurrence data in foods consumed in developing countries, as a valuable tool in conducting intake assessments and mitigation approaches to reduce human exposure (a high priority for governments and industries) (Arisseto & Toledo, 2006).

A high relevance is being given to coffee, as an important dietary source of acrylamide, mainly in the Nordic European countries where it may contribute up to one third of total dietary intake (Dybing & Sanner, 2003; Guenther, Anklam, Wenzl, & Stadler, 2007; Svensson et al., 2003). Among other possible reaction pathways, the Maillard reaction represents the main route for acrylamide formation in coffee, being initiated by the condensation of asparagine and reducing carbohydrates or reactive carbonyls, when the beans are subjected to the high roasting temperature (Guenther et al., 2007). Acrylamide formation starts rapidly at the beginning of the roasting process and it decreases shortly after reaching a maximum level, probably due to physical and chemical losses (Bagdonaite, Derler, & Murkovic, 2008; Guenther et al., 2007; Lantz et al., 2006; Senyuva & Gökmen, 2005; Taeymans et al., 2004). Therefore, the degree of roasting will be a key factor in acrylamide content, with light roasted coffee attaining significantly higher amounts when compared with dark roasted counterparts (Bagdonaite et al., 2008; Guenther et al., 2007; Lantz et al., 2006; Senyuva & Gökmen, 2005; Taeymans et al., 2004). Moreover, when comparing the two coffee species of higher economical importance, namely *Coffea arabica* and *Coffea canephora* (also known as arabica and robusta coffees, respectively) increased levels of acrylamide are described for the latter (Bagdonaite et al., 2008; Guenther et al., 2007; Lantz et al., 2006; Summa, de la Calle, Brohee, Stadler, & Anklam, 2007). As a result, the reported levels for roasted coffee beans vary widely, usually within the range of 35–540 μg/kg of coffee (Aguas, Fitzhenry, Giannikopoulos, & Varelis, 2006; Andrzejewski, Roach, Gay, & Musser, 2004; Delatour, Périsset, Goldmann, Riediker, & Stadler, 2004; Friedman, 2003; Guenther et al., 2007; Hoenicke & Gatermann, 2005; Lantz et al., 2006; Murkovic, 2004; Roach, Andrzejewski, Gay, Nortrup, & Musser, 2003; Senyuva & Gökmen, 2005; Summa et al., 2007).

While the majority of published studies have focused on the assessment of acrylamide content in coffee beans, a research priority is to investigate the amount of it effectively ingested by consumers through coffee brews. Acrylamide is highly soluble in water and, thus, easily transferred from the coffee powder to the beverage (Andrzejewski et al., 2004). However, the chemical composition of coffee brew is highly dependent on several factors,

* Corresponding author. Fax: +351 222 003 977.
E-mail address: rita.c.alves@gmail.com (R.C. Alves).
[1] Both authors contributed equally to this project.

0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.07.051

including the amount of arabica and robusta used to prepare the blend, their degree of roasting, as well as the coffee/water ratio used, which depends on cultural and personal preferences (Alves, Casal, & Oliveira, 2007). Some studies have reported acrylamide levels in common coffee beverages (as plunger pot and filtered coffee) ranging between 2 and 25 μg/L (Andrzejewski et al., 2004; Dybing et al., 2005; Granby & Fagt, 2004; Pérez & Osterman-Golkar, 2003; Svensson et al., 2003).

Among all coffee brews, espresso is highly appreciated in Portugal, and its consumption is increasing worldwide. This brew is prepared by a special brewing technique in which a limited amount (20–50 mL) of hot water under high pressure (9 ± 2 atm, 90 ± 5 °C) is percolated in a very short time (30 ± 5 s) through a ground coffee cake (6.5 ± 1.5 g). The result is a concentrated and intensely flavoured brew covered by a dense foam layer, which should be tasted at the exact moment of extraction (Alves et al., 2007).

Lantz et al. (2006) reported that espresso coffee brewing incompletely extracts acrylamide from ground coffee, unlike other coffee brews (as plunger pot or filtered coffee) due to the short contact time with water. Therefore, it is of interest to study the technological parameters affecting acrylamide extraction into the espresso brew. Our group has already reported some preliminary data on acrylamide levels of standard espresso coffee (0.32–1.46 μg/30 mL or 10.7–48.7 μg/L) (Soares, Cunha, & Fernandes, 2006). The aim of the present work was to focus exclusively on this peculiar beverage, ascertaining the factors implicated in the acrylamide extraction, contributing to a better knowledge of the exposure levels of espresso consumers. The influence of coffee species and their degree of roasting, together with the extractability achieved by different percolation periods, are detailed. The acrylamide levels were also evaluated in several commercial samples, decaffeinated and servings included, and the values compared with those described for other common coffee beverages.

2. Materials and methods

2.1. Chemicals and reagents

Acrylamide was obtained from Aldrich (Steinheim, Germany). The internal standard $^{13}C_3$-acrylamide was purchased from Cambridge Isotope Laboratories (Andover, MA, USA) as a 1 mg/mL solution in methanol.

Preparative C_{18} sorbent (125 Å, 55–105 μm) was from Waters (Milford, MA, USA). The ISOLUTE C_{18}/Multimode layered solid-phase extraction (SPE) columns were from Biotage (2 g/15 mL, Uppsala, Sweden). The HPLC water was purified with a "Seral" system (SeralPur Pro 90 CN).

Potassium bromide (IR spectroscopy grade) and bromine (analytical grade) were from Merck (Darmstadt, Germany). Hydrobromic acid (48%) and sodium thiosulphate (1 mol/L) were from Riedel-de Häen (Seelze, Germany). A saturated bromine–water solution was prepared by adding bromine (~3 mL) to 200 mL of water until precipitation became visible (Fernandes & Soares, 2007).

Sodium chloride (analytical grade) was from J.T. Baker (Deventer, the Netherlands). *n*-Hexane, ethyl acetate (pesticide residue analysis grade), methanol and acetonitrile (ultrapure grade) were all from Fluka (Madrid, Spain). All other chemicals were of analytical grade.

2.2. Standards

A stock solution of acrylamide (2 g/L) was prepared by dissolving it in acetonitrile and then serially diluted to prepare working standard solutions. A working 10 mg/L solution of the internal standard was also prepared in acetonitrile. All stock and working solutions were stored at 4 °C.

2.3. Coffee samples

Commercial caffeinated ($n = 14$) and decaffeinated ($n = 6$) roasted beans, as well as servings ($n = 7$), were obtained in local supermarkets and cafeterias, and some supplied by a Portuguese industrial importer and roaster of coffee.

Green samples of *C. arabica* ($n = 8$) and *C. canephora* var. *robusta* ($n = 8$), from different geographical origins, were kindly supplied by the same industry. The arabica samples were from Hawaii, Costa Rica, Jamaica, Colombia, Ethiopia, Honduras ($n = 2$) and Brazil, and robusta samples were from India ($n = 2$), Uganda ($n = 2$), Cameroon ($n = 2$), Ivory Coast and Indonesia. All robusta samples were dry processed. Samples of arabica coffees were wet processed, except the one from Brazil, which was dry processed. Samples were individually roasted in a Probat L12 coffee roaster from Probat-Werke according to a standard method (210 °C, 10 min), usually practiced by the local industrial roaster. Additionally, four green coffee samples, two arabicas (Honduras and Brazil) and two robustas (Uganda and Ivory Coast), were also subjected to three different lengths of heat exposure (8–11 min, 210 °C, Probat Pré 1Z 2000, from Probat-Werke), in order to achieve three final roasting degrees (light, medium and dark), not exceeding the range of commercial espresso roasts usually practiced in Portugal. The roasting degree was determined by photometric analysis with infrared radiation Colorimeter Colorette 3 from Probat-Werke and also by the organic roast loss (ORL) evaluation in dry weight (% ORL dw) (Clarke, 1989; Illy & Viani, 2005). Sample moisture to calculate ORL was determined by drying at 103 ± 2 °C until a constant weight was reached. All samples were stored at 4 °C before analysis.

2.4. Brews preparation

All coffee beans were mechanically powdered to pass through a 0.75 mm sieve in the integrated grinder of a HL3854 *Espresso Professional* (Philips, The Netherlands). Espresso coffees (ECs) of 30 mL were prepared with deionized water (Amberlite MD 20) in the same espresso machine, using 6.5 g of ground coffee, exactly weighted. To evaluate extraction efficiency, an arabica (Honduras) and a robusta (Ivory Coast) sample were individually extracted (6.5 g) with different volumes of water ranging from 20 mL, a typical "ristretto" or "Italian", to 70 mL, the longest EC usually consumed in Portugal. ECs from paper coated servings were prepared in the same machine, by changing the filter chamber to one adapted to servings. ECs from aluminium coated servings were extracted in a Krups XN2105 (Germany).

2.5. Samples analysis

2.5.1. Matrix solid-phase dispersion

Acrylamide content and extractability were analysed using a previously optimised and validated methodology based on matrix solid-phase dispersion (Soares, Alves, Casal, Fernandes, & Oliveira, submitted for publication). Briefly, a 2.5 mL aliquot of beverage (or 0.5 g of ground coffee) was spiked with the internal standard (25 μL) and dispersed with C_{18} sorbent (previously conditioned with methanol and water). The mixture was then transferred to a preconditioned ISOLUTE C_{18}/Multimode SPE column and carefully compressed with a frit on the top. A Visiprep SPE Vacuum Manifold 57030-U (Supelco, Bellefonte, PA, USA) was used to manipulate the cartridges and the acrylamide elution was achieved with 4 + 4 mL of water, allowing soaking steps of 5 min. The sorbents dryness

was carefully avoided during the entire procedure. All samples were analysed in duplicate.

2.5.2. Bromination

The bromination step was performed as described elsewhere (Soares et al., 2006). Briefly, calcinated potassium bromide (1 g), hydrobromic acid (~150 μL) and saturated bromine solution (~2 mL) were added to the collected extract. The mixture was kept on ice, in the dark, at least for 1 h. Sodium thiosulphate (~150 μL) was used to neutralise the excess of bromine. After adding NaCl (~4 g), a double liquid–liquid extraction with ethyl acetate/*n*-hexane 4:1 (v/v) (10 + 5 mL) was performed. The collected organic extract was dried with anhydrous Na_2SO_4, centrifuged, concentrated (N_2, 60 °C) to about 0.5 mL and injected in the gas chromatograph.

2.5.3. Gas chromatographic–mass spectrometric analysis

The chromatographic analysis was carried out in a gas chromatograph (Agilent GC-6890N) equipped with a split–splitless injector and an automatic sampler (Agilent 7683B Series) coupled to a mass selective detector (Agilent MSD-5975N, Agilent, Palo Alto, CA, USA), according to Soares et al. (2006). The chromatographic separation was achieved on a capillary column MDN-12 (30 m × 0.25 μm, 0.25 mm i.d.) from Supelco (Bellefonte, PA, USA) and helium (1 mL/min, constant flow) was used as carrier gas. The sample injection volume was 1 μL (splitless, pulsed pressure 32 ψ, 60 s, 280 °C). The oven temperature was initially programmed at 85 °C for 1 min, increasing at 15 °C/min to 280 °C (10 min hold), and the transfer line set at 280 °C. The mass selective detector was set in the selected ion monitoring mode (SIM), selecting three characteristic fragments of each derivatized acrylamide (2,3-dibromopropionamide: *m/z* 106, 150, and 152) and derivatized internal standard (2,3-$^{13}C_3$-dibromopropionamide: *m/z* 110, 153, and 155). Peak identification was accomplished by retention time and comparison with standards. Quantification was performed on the basis of the internal standard using ions *m/z* 150 and 155, for 2,3-DBPA and 2,3-$^{13}C_3$-DMPA, respectively.

2.6. Statistical analysis

Data were recorded as mean ± standard deviation and analysed by the one-way ANOVA and Student's *t*-tests. All analyses were carried out with Microsoft Excel statistical software (Microsoft Office Excel 2003, Microsoft Corp., Redmond, WA).

3. Results and discussion

3.1. Variability within commercial samples

The acrylamide contents of caffeinated ECs (30 mL), prepared from commercial coffee blends, were highly variable, as can be observed in Table 1. The results are in accordance with those previously reported for standard espressos: 10.7–48.7 μg/L (Soares et al., 2006).

Table 1
Acrylamide contents of espresso coffees prepared from commercially available samples (caffeinated and decaffeinated).[a]

Commercial samples	*n*	μg/L			μg/EC (30 mL)		
		Mean	Min.	Max.	Mean	Min.	Max.
Caffeinated	14	41.9 a	27.4	58.5	1.26 a	0.82	1.76
Decaffeinated	6	33.2 a	24.8	49.5	1.00 a	0.74	1.49
Servings*	7	42.3 a	33.4	55.3	1.27 a	1.00	1.66

EC, espresso coffee; S.D., standard deviation; *, caffeinated samples.
[a] Data followed by the same letters within each column are not significantly different according to ANOVA ($p > 0.05$).

Although average levels obtained for decaffeinated ECs were lower, when compared with caffeinated samples (Table 1), the differences between both groups were not statistically significant ($p > 0.05$), suggesting that decaffeination process does not significantly affect acrylamide precursors in green coffee beans.

Servings are individual doses of ground coffee (about 6–7 g) coated with a paper layer, commercially available and produced to make an EC in a rapid, simple, and clean way, in adapted machines. Recently, new coffee servings have emerged which are coated with an aluminium layer instead of paper. Also, no significant differences ($p > 0.05$) were found when comparing the espressos prepared from servings with regular and decaffeinated ECs (Table 1). The great variability found in samples tested (Table 1), with some espressos containing twice the acrylamide amount than others, can be justified by consideration of several factors. According to some researchers, the acrylamide content of roasted coffee beans differs mainly with the coffee species (Bagdonaite et al., 2008; Lantz et al., 2006), degree of roasting (Bagdonaite et al., 2008; Lantz et al., 2006; Summa et al., 2007), and storage conditions (Andrzejewski et al., 2004; Delatour et al., 2004; Hoenicke & Gatermann, 2005; Lantz et al., 2006).

3.2. Influence of coffee species: arabica and robusta

Table 2 shows the influence of each coffee species (arabica and robusta) on the acrylamide content of EC. Although the samples were medium roasted by a standard procedure, the organic roast loss was different within each species, as a consequence of the beans intrinsic characteristics.

Significantly higher amounts ($p < 0.001$) of acrylamide were found in robusta samples, with levels per cup (30 mL) ranging between 1.71 and 2.92 μg. For arabica coffee, the levels were half lower, varying from 0.87 and 1.52 μg/EC.

These results are in agreement with those described for coffee beans (Bagdonaite et al., 2008; Lantz et al., 2006). Lantz et al. (2006) reported average levels of 378 and 251 μg/kg, for robusta and arabica medium roasted coffees, respectively. This difference seems to be associated with an increased content of asparagine amount in robusta raw beans (Bagdonaite et al., 2008; Lantz et al., 2006), in comparison with arabica beans.

Arabica and robusta coffees, the two main species used to prepare brews, have different chemical and sensory properties. The quality of the beverage is usually dependent on the proportion of both in the blend, arabica being considered a higher value product. In Portugal, the majority of the commercially available coffee brands are mixtures of both arabica and robusta, with the latter usually at no more than 30% of the blend. However, some blends containing higher amounts of robusta can also be found in the market. The addition of this coffee species to the blend aims to increase the body and improve the espresso foam, together with some economical saving, since robusta price is lower.

Results shown in Table 2 are, therefore, indicative of the minimum and maximum levels of acrylamide that can be found in EC prepared with medium roasted coffee beans.

Although subjected to a different postharvest treatment, no significant ($p > 0.05$) differences were found between the dry-processed sample from Brazil and other wet-processed arabicas.

3.3. Influence of degree of roasting

When coffee beans are subjected to the high temperatures of roasting, innumerous chemical reactions occur as well as physical modifications that might influence the extraction of some compounds to the brew (Illy & Viani, 2005). In order to observe the

Table 2
Acrylamide contents in espresso coffees prepared with arabica or robusta medium roasted beans.[a]

Coffee species and geographical origin	% ORL	Color	μg/L	μg/EC(30 mL)
			Mean ± S.D.	Mean ± S.D.
Arabica				
Hawaii	5	132	29.1 ± 0.8	0.87 ± 0.02
Costa Rica	11	127	38.0 ± 0.3	1.14 ± 0.01
Jamaica	11	126	33.3 ± 0.2	1.00 ± 0.01
Colombia	7	122	32.7 ± 0.2	0.98 ± 0.01
Ethiopia	12	128	44.6 ± 1.5	1.34 ± 0.04
Honduras (1)	9	123	49.4 ± 0.5	1.48 ± 0.01
Honduras (2)	9	130	50.8 ± 1.2	1.52 ± 0.04
Brazil	10	138	32.8 ± 0.3	0.99 ± 0.01
Total mean (n = 8)	9	128	38.8 ± 8.4 a	1.16 ± 0.25 a
Robusta				
India (1)	10	118	56.9 ± 1.4	1.71 ± 0.04
India (2)	11	133	84.1 ± 3.4	2.52 ± 0.10
Uganda (1)	9	122	75.5 ± 0.8	2.27 ± 0.02
Uganda (2)	11	122	73.5 ± 1.4	2.20 ± 0.04
Cameroon (1)	16	124	80.5 ± 1.5	2.41 ± 0.05
Cameroon (2)	12	132	89.4 ± 3.7	2.68 ± 0.11
Ivory Coast	10	131	97.3 ± 3.9	2.92 ± 0.12
Indonesia	9	125	58.0 ± 0.2	1.74 ± 0.01
Total mean (n = 8)	11	126	76.9 ± 14.2 b	2.31 ± 0.43 b

ORL, organic roast loss; EC, espresso coffee; S.D., standard deviation.
[a] Data followed by different letters within each column are significantly different according to ANOVA at $p < 0.001$.

influence of the degree of roasting on the acrylamide content and extractability, four green coffee samples (two arabicas, Brazil and Honduras, and two robustas, Uganda and Ivory Coast) were roasted at three different roasting degrees as described in the experimental section. Organic roast loss and colours achieved are summarised in Table 3, together with the results for acrylamide. The total acrylamide content was calculated for 6.5 g of ground coffee (cake weight) and the acrylamide extractability obtained by the following formula: espresso content/cake content × 100.

In a general way, acrylamide levels in ground coffee (both arabica and robusta) significantly decreased ($p < 0.05$) with increased roasting period, for each individual sample. In fact, very high levels of acrylamide were detected in the lightest roasted coffee samples, with maximum of 1240 and 2190 μg/kg, for arabica and robusta, respectively (Table 3). Also Taeymans et al. (2004) reported that levels of about 2000 μg/kg were observed at the early stages in the process of coffee beans roasting. Moreover, the amount present in dark roasts (Table 3) corresponds only to 15% and 23% of that present in light roasts, for arabica and robusta, respectively. Therefore, considering all the samples together, mean loss of about 80% occurred from the light roast to the dark one. Taeymans et al. (2004), based on experiments with isotope-labelled acrylamide, reported that more than 95% of the total acrylamide generated by roasting is further degraded during the process and is no longer found in the final product. Comparing the two coffee species analysed (Table 3), the average acrylamide contents of the compound were always significantly higher ($p < 0.05$) for robusta ground coffee, in all roasting stages.

The results obtained for espresso coffees follow a similar profile (Table 3): significant decreases ($p < 0.05$) of acrylamide were observed during roasting; pure robusta espressos contained approximately double amounts of acrylamide of arabica, for all degrees of roasting; and mean decreases of 30% and 20%, for arabica and robusta, respectively, were found when comparing medium roasted ECs with dark roasted counterparts. Thus, ECs prepared from dark roasted commercial blends might have about 25% less acrylamide than medium roasted brews.

Concerning the extraction efficacy of acrylamide, no significant differences ($p > 0.05$) were found between different degrees of roasting in each sample analysed. Also, no differences ($p > 0.05$) existed when arabica and robusta groups were compared. Therefore, although coffee species and degree of roasting influence the acrylamide content of the brew they do not affect acrylamide extractability (mean extraction of 80%, in a standard Portuguese espresso of 30 mL).

3.4. Influence of EC volume

The mean EC volume, usually consumed in Portugal, is around 30–40 mL. However, it may vary from a "ristretto" (20 mL or less) to a "lungo" (50 mL or more), according to the consumer's preference. Espresso percolation was described by Lantz et al. (2006) as the only brewing procedure that incompletely extracted acrylamide from ground coffee when compared with other coffee brews, due to the short contact time between coffee and water. In order to study the influence of the water volume on the amount of acrylamide of espresso, two coffee samples (one arabica and one robusta) were used to prepare ECs of different lengths (20, 30, 50 and 70 mL). The results are shown in Fig. 1.

The arabica cake under test (6.5 g) contained 1.82 ± 0.03 μg of acrylamide, while the robusta contained 3.67 ± 0.03 μg. The behaviour of acrylamide extraction was very similar in both coffee species, showing that an increase in the water volume that percolates through the coffee cake is responsible for a higher extraction of the compound. The extraction percentage variation according to the brew volume was also very similar: from 59% to

Table 3
Acrylamide contents and extractability in espresso coffees at different roast degrees.[a]

Sample	Roast degree	ORL (%)	Color	Ground coffee (μg/kg) Mean ± S.D.	μg/L Mean ± S.D.	μg/EC (30 mL) Mean ± S.D.	Extraction (%) Mean ± S.D.
Arabica							
Brazil	Light	7	200	782.78 ± 33.71 a	135.6 ± 5.7 a	4.07 ± 0.17 a	79.9 ± 0.1 a
	Medium	10	138	183.37 ± 8.35 b	32.8 ± 0.3 b	0.99 ± 0.01 b	82.7 ± 2.9 a
	Dark	13	119	131.79 ± 5.75 c	22.8 ± 1.3 c	0.68 ± 0.04 c	79.7 ± 1.1 a
Honduras	Light	6	200	1243.14 ± 67.34 a	216.0 ± 11.0 a	6.48 ± 0.33 a	80.2 ± 0.3 a
	Medium	9	130	279.26 ± 4.09 b	50.8 ± 1.2 b	1.52 ± 0.04 b	84.0 ± 3.3 a
	Dark	12	112	185.54 ± 1.24 c	33.8 ± 0.5 c	1.01 ± 0.01 c	84.1 ± 1.7 a
Robusta							
Uganda	Light	7	200	1384.50 ± 69.02 a	236.6 ± 0.5 a	7.10 ± 0.01 a	79.0 ± 3.8 a
	Medium	11	122	454.85 ± 2.67 b	73.5 ± 1.4 b	2.20 ± 0.04 b	74.6 ± 1.0 a
	Dark	13	97	383.46 ± 18.93 c	68.5 ± 2.1 b	2.05 ± 0.06 b	82.6 ± 6.5 a
Ivory Coast	Light	6	200	2191.18 ± 20.37 a	390.0 ± 16.6 a	11.70 ± 0.50 a	82.1 ± 4.3 a
	Medium	10	131	564.06 ± 25.05 b	97.3 ± 3.9 b	2.92 ± 0.12 b	79.6 ± 0.3 a
	Dark	13	110	441.91 ± 6.44 c	72.8 ± 1.7 c	2.18 ± 0.05 c	76.0 ± 0.6 a

ORL, organic roast loss; EC, espresso coffee; S.D., standard deviation.
[a] Data followed by different letters within each column, for each geographical origin, are significantly different according to Student's t-tests at $p < 0.05$.

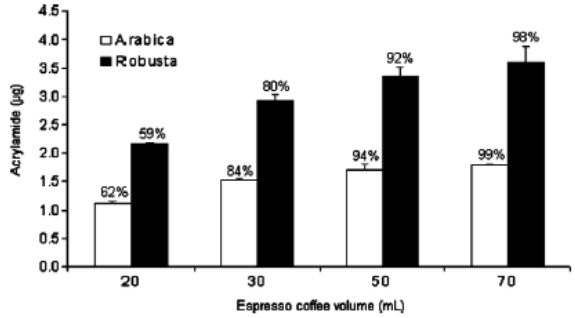

Fig. 1. Influence of volume on espresso coffee acrylamide content. Percentages on each bar represent acrylamide extractability.

98%, for robusta, and from 62% to 99%, for arabica. Therefore, a "lungo" EC practically contains all the acrylamide initially present in coffee cake (almost the double than a "ristretto"). Although the final content of acrylamide increases with volume (Fig. 1), the brew concentration (in ng/mL) simultaneously decreases, as expected, due to a reduction in the coffee/water ratio: from 108.2 ± 1.5 to 50.1 ± 3.9 µg/L, for robusta ECs, and from 56.3 ± 1.3 to 24.2 ± 0.3 µg/L, for arabica.

3.5. Comparison of EC with other coffee brews

The highly water-soluble acrylamide is easily extracted from the ground coffee to the liquid phase of the beverage (Andrzejewski et al., 2004). Some studies reporting acrylamide levels in common coffee beverages (as plunger pot and filtered coffee) have already been published, reporting values between 2 and 25 µg/L. The acrylamide concentrations reported in this study, for standard espressos (30 mL), are higher than those reported by other authors for other coffee brews. Indeed, considering all caffeinated samples presented in Table 1 ($n = 21$) the mean acrylamide concentration was 40 ± 9 µg/L. Moreover, acrylamide levels of medium roasted ECs could vary between 38 ± 8 and 77 ± 14 µg/L, when robusta percentage in blend ranges from 0% to 100%, respectively. These high concentrations (compared with other beverages) depend essentially on the coffee/water ratio used to prepare the brew, factor that varies with consumers' preferences and geographical habits: 20 g/L in USA (Andrzejewski et al., 2004) and 40 g/L in Northern Europe (Granby & Fagt, 2004), while in Portuguese espressos may range from 325 ("ristretto") to 93 g/L ("lungo") (Alves et al., 2007).

Considering the final acrylamide content per cup, it will obviously depend on the ingested amount of beverage. While EC is, generally, a very short beverage, higher volumes per cup of other coffee beverages are usually consumed. For example, a cup of filter coffee may achieve 200 mL, because it is considered a light brew (Alves et al., 2007). Therefore, according to the concentrations reported by other authors, one can estimate that a cup of 200 mL may contain up to 5 µg of acrylamide. This value was never achieved in our study, not even in the longest pure robusta ECs, because although very concentrated, EC is a very small beverage. Thus, the acrylamide intake through espresso brews will mainly depend on the consumption habits, considering type, strength and volume of beverage, and intake frequency, factors that are influenced by cultural and personal preferences of consumers.

4. Conclusions

Acrylamide intake through espresso coffee brew is mainly dependent on the type of coffee used to prepare the blend (arabica or robusta) and their degree of roasting, with the lowest amounts found in dark arabica roasted samples. The acrylamide extraction efficiency for standard espressos approached 80% and this value was only affected by brew volume increment: "lungo" ECs (70 mL) practically contained all the acrylamide initially present in coffee cake (almost the double than a "ristretto" of 20 mL). When compared with other common coffee beverages, EC is a very concentrated brew. However, its acrylamide content per cup may be lower, due to its small volume.

With the results obtained from commercial ECs (30 mL) it is possible to estimate that a moderate espresso consumer (3 to 5 doses per day) will ingest about 4–6 µg of acrylamide per day via this beverage. There are very limited processes available to reduce acrylamide level without affecting the quality of the brew, especially in relation to its sensory properties. A complementary option to reduce the amount of acrylamide ingested trough EC is to select commercial blends with higher arabica percentages and darker degrees of roasting and, simultaneously, prefer shorter brews instead of long ones, but this will obviously depend on the consumers' preferences.

Acknowledgments

The authors are thankful to Fundação para a Ciência e a Tecnologia for financial support in the framework of the project POCI/AGR/61543/2004 and for the Ph.D. grants of Rita Alves (SFRH/BD/22449/2005) and Cristina Soares (SFRH/BD/39360/2007). We also thank BICAFÉ for providing coffee samples.

References

Aguas, P. C., Fitzhenry, M. J., Giannikopoulos, G., & Varelis, P. (2006). Analysis of acrylamide in coffee and cocoa by isotope dilution liquid chromatography–tandem mass spectrometry. *Analytical and Bioanalytical Chemistry, 385*, 1526–1531.

Alves, R. C., Casal, S., & Oliveira, B. P. P. (2007). Factors influencing the norharman and harman contents in espresso coffee. *Journal of Agricultural and Food Chemistry, 55*, 1832–1838.

Andrzejewski, D., Roach, J. A., Gay, M. L., & Musser, S. M. (2004). Analysis of coffee for the presence of acrylamide by LC–MS/MS. *Journal of Agricultural and Food Chemistry, 52*, 1996–2002.

Arisseto, A. P., & Toledo, M. C. F. (2006). Acrylamide in foods: A review. *Brazilian Journal of Food Technology, 9*, 123–134.

Bagdonaite, K., Derler, K., & Murkovic, M. (2008). Determination of acrylamide during roasting of coffee. *Journal of Agricultural and Food Chemistry, 56*, 6081–6086.

Clarke, R. J. (1989). Roasting and grinding. In R. J. Clarke & R. Macrae (Eds.). *Coffee: Technology* (Vol. 2, pp. 73–107). Great Yarmouth, UK: Elsevier Applied Science.

Delatour, A., Périsset, T., Goldmann, T., Riediker, S., & Stadler, R. H. (2004). Improved sample preparation to determine acrylamide in difficult matrixes such as chocolate powder, cocoa, and coffee by liquid chromatography tandem mass spectroscopy. *Journal of Agricultural and Food Chemistry, 52*, 4625–4631.

Dybing, E., Farmer, P. B., Andersen, M., Fennell, T. R., Lalljie, S. P., Müller, D. J., et al. (2005). Human exposure and internal dose assessments of acrylamide in food. *Food and Chemical Toxicology, 43*, 365–410.

Dybing, E., & Sanner, T. (2003). Risk assessment of acrylamide in foods. *Toxicological Sciences, 75*, 7–15.

Fernandes, J., & Soares, C. (2007). Application of matrix solid-phase dispersion in the determination of acrylamide in potato chips. *Journal of Chromatography A, 1175*, 1–6.

Friedman, M. (2003). Chemistry, biochemistry, and safety of acrylamide. *A review. Journal of Agricultural and Food Chemistry, 51*, 4504–4526.

Granby, K., & Fagt, S. (2004). Analysis of acrylamide in coffee and dietary exposure to acrylamide from coffee. *Analytica Chimica Acta, 520*, 177–182.

Guenther, H., Anklam, E., Wenzl, T., & Stadler, R. H. (2007). Acrylamide in coffee: review of progress in analysis, formation and level reduction. *Food Additives and Contaminants, 24*, 60–70.

Hoenicke, K., & Gatermann, R. (2005). Studies on the stability of acrylamide in food during storage. *Journal of AOAC International, 88*, 268–273.

International Agency for Research on Cancer (IARC) (1994). *Some industrial chemicals* (Vol. 60, pp. 389–441). Lyon: IARC monographs on the evaluation of carcinogenic risk to humans.

Illy, A., & Viani, R. (2005). *Espresso coffee: The science of quality*. London, UK: Academic Press.

Joint FAO/WHO Experts Committee on Food Additives (JECFA) (2005). Summary and conclusions of the sixty-fourth meeting of the Joint FAO/WHO Experts Committee on Food Additives (JECFA), Rome, 8–17 February; JECFA/64/SC.

Lantz, I., Ternité, R., Wilkens, J., Hoenicke, K., Guenther, H., & van der Stegen, G. H. (2006). Studies on acrylamide levels in roasting, storage and brewing of coffee. *Molecular Nutrition and Food Research, 50*, 1039–1046.

Murkovic, M. (2004). Acrylamide in Austrian foods. *Journal of Biochemical and Biophysical Methods, 61*, 161–167.

Pérez, H. L., & Osterman-Golkar, S. (2003). A sensitive gas chromatographic–tandem mass spectrometric method for detection of alkylating agents in water: Application to acrylamide in drinking water, coffee and snuff. *Analyst, 128*, 1033–1036.

Roach, J. A., Andrzejewski, D., Gay, M. L., Nortrup, D., & Musser, S. M. (2003). Rugged LC–MS/MS survey analysis for acrylamide in foods. *Journal of Agricultural and Food Chemistry, 51*, 7547–7554.

Senyuva, H. Z., & Gökmen, V. (2005). Study of acrylamide in coffee using an improved liquid chromatography mass spectrometry method: Investigation of colour changes and acrylamide formation in coffee during roasting. *Food Additives and Contaminants, 22*, 214–220.

Swedish National Food Administration (2002). Information about acrylamide in food, 24 April 2002. <http://www.slv.se>.

Soares, C., Cunha, S., & Fernandes, J. (2006). Determination of acrylamide in coffee and coffee products by GC–MS using an improved SPE clean-up. *Food Additives and Contaminants, 23*, 1276–1282.

Soares, C., Alves, R. C., Casal, S., Fernandes, J. O., & Oliveira, B. P. P. (submitted for publication). Validation of a matrix solid-phase dispersion method to determine acrylamide in coffee and coffee surrogates. *Food Additives and Contaminants.*

Summa, C. A., de la Calle, B., Brohee, M., Stadler, R. H., & Anklam, E. (2007). Impact of the roasting degree of coffee on the in vitro radical scavenging capacity and content of acrylamide. *LWT – Food Science and Technology, 40*, 1849–1854.

Svensson, K., Abramsson, L., Becker, W., Glynn, A., Hellenäs, K.-E., Lind, Y., et al. (2003). Dietary intake of acrylamide in Sweden. *Food and Chemical Toxicology, 41*, 1581–1586.

Taeymans, D., Wood, J., Ashby, P., Blank, I., Studer, A., Stadler, R. H., et al. (2004). A review of acrylamide: An industry perspective on research, analysis, formation, and control. *Critical Reviews in Food Science and Nutrition, 44*, 323–347.

3.2. Influence of unripe coffee fruit processing on acrylamide formation after roasting

Abstract

Natural coffee produced in Brazil shows a pattern of highly variable quality, which affects the marketability of the grain. The presence of the green defect is associated with factors arising from the harvesting, processing and drying techniques. Green coffee resulting from this process has a lower commercial value because it contains defective grains. Peeling immature fruit is viewed as a promising method of minimizing the negative impact of these defective grains in the quality and commercial value of coffee. During post-harvest coffee processing, techniques such as piling and immersion in water may be used to store the coffee prior to pulping. These processes result in coffee with a different physical-chemical composition and higher sensory quality than the green grains that result from natural processing. The amino acid asparagine is the main precursor of acrylamide, a potentially harmful substance to human health. Asparagine levels become relevant in coffee composition because asparagine concentration is higher in immature fruit. Peeling of unripe coffee enables a reduction in asparagine levels. Different post-harvest processing and degrees of roasting were compared with the aim of minimizing acrylamide formation in roasted unripe beans. It was concluded that pulped unripe fruits present lower levels of acrylamide. Levels are even smaller for dark-roasted beans, showing promising results in the control and formation of this substance.

Key-words: unripe coffee, pulped and natural processing, acrylamide, roasting

Influence of unripe coffee fruit processing on acrylamide formation after roasting

Eduardo C. Dias [a], Flávio M. Borém [a], Rosemary G. F. A. Pereira [a], Cristina Soares [b], José O. Fernandes [b].

Universidade Federal de Lavras, Departamento de Ciência dos Alimentos, Lavras, Minas Gerais, CEP 37200-000 Tel: 00 55 35 38291392.

[a] Doutor em Ciência dos Alimentos, Departamento de Ciência dos Alimentos, Universidade Federal de Lavras, MG - ecdias5@gmail.com.

[a] Professora, Dra., Departamento de Ciência dos Alimentos, Universidade Federal de Lavras, MG - rosegfap@hotmail.com

[a] Professor, Dr., Departamento de Engenharia - Universidade Federal de Lavras. MG - flavioborem@deg.ufla.br

[b] Doutoranda, Requimte, Departamento de Ciências Químicas, Laboratório de Bromatologia e de Hidrologia, Faculdade de Farmácia, Universidade do Porto, Portugal - cristina.md.soares@gmail.com

[b] Professor, Dr., Requimte, Departamento de Ciências Químicas, Laboratório de Bromatologia e de Hidrologia,, Faculdade de Farmácia, Universidade do Porto, Portugal - josefer@ff.up.pt

Abstract

Natural coffee produced in Brazil shows a pattern of highly variable quality, which affects the marketability of the grain. The presence of the green defect is associated with factors arising from the harvesting, processing and drying techniques. Green coffee resulting from this process has a lower commercial value because it contains defective grains. Peeling immature fruit is viewed as a promising method of minimizing the negative impact of these defective grains in the quality and commercial value of coffee. During post-harvest coffee processing, techniques such as piling and immersion in water may be used to store the coffee prior to pulping. These processes result in coffee with a different physical-chemical composition and higher sensory quality than the green grains that result from natural processing. The amino acid asparagine is the main precursor of acrylamide, a potentially harmful substance to human health. Asparagine levels become relevant in coffee composition because asparagine concentration is higher in immature fruit. Peeling of unripe coffee enables a reduction in asparagine levels. Different post-harvest processing and degrees of roasting were compared with the aim of minimizing acrylamide formation in roasted unripe beans. It was concluded that pulped unripe fruits present lower levels of acrylamide. Levels are even smaller for dark-roasted beans, showing promising results in the control and formation of this substance.

Key-words: unripe coffee, pulped and natural processing, acrylamide, roasting

Introduction

The presence of a high amount of immature fruit in Brazilian coffee is one of the reasons for the low quality of a final product. There is no doubt that factors such as grade and homogeneity of the material affect the attributes of this commodity. Although the majority of fruit are ripe at harvest, there will always be a significant proportion of immature and overripe fruit because most of the coffee in Brazil is harvested by strip-picking, in which all kinds of fruits are processed together. There are three methods of processing coffee: the wet, dry and pulped natural methods. The so-called washed or wet process requires a raw material composed of only ripe cherries. After passing through the washer-separators and before pulp removal, the separation of the green immature fruit from the ripe fruit can be performed using differences in pressure in the separator. In this method, the cherries are

pulped and the mucilage is removed from the parchment mechanically or by the use of fermentation. In the dry or natural process, whole ripe and unripe cherries (bean, mucilage and pulp) are dried on patios after harvest. A third process called “pulped natural” is an intermediate between natural and wet processing. The coffee cherries are pulped, and the bean surrounded by the mucilage is dried. Fermentation for the removal of the mucilage is not used in this process (Illy & Viani, 2005).

The pulpers used in the preparation of pulped coffees are equipped with pressure separators to divide immature grains from mature ones. The immature fruit must be dried separately; coffee in parchment with unremoved mucilage must be immediately dried (Illy & Viani, 2005). However, after this operation, the batch consisting predominantly of green fruit has a low potential of producing high-quality coffee (Borém, 2008). Moreover, due totheoperational capabilityof the equipmentforpulpingimmaturecoffee inthe same day,the temporary storage of the coffee fruitinwatercanassist in post-harvest processingwithout necessarily compromising the final quality of thecoffee (Machado, 2005).

Applying correct post-harvest processing techniques can improve immature fruit quality (Illy & Viani, 2005). Pulpingofthe green fruithas emerged asaway toimprove coffee qualityand addvalue toimmature coffee beans. Grainsresulting from thisprocesshave a higher quality than those obtained by drying the entire green fruit. In addition to shorteningthefermentation process and encouraging amoreuniform and faster drying, the immature peeled coffeehasfewerdefects, reducing the percentageofPVA[black (P), green (V)andsour (A)] (Borém, 2008).

It is well known that the mode of coffee processing (i.e., the wet or dry method) determines the quality of the corresponding green coffees and establishes characteristic flavor differences. The biggest difference between the chemical composition of washed and natural coffee is in the soluble solids content, which is higher in the case of natural coffee. However, differencesinthe constitutionofcoffee beansmay berelated tothe presence or absenceofskin tissues,as reportedbyBytof et al. (2005). Pulped natural coffee offers an intermediate position (Illy & Viani, 2005). Furthermore, specific and low molecular flavor precursors, i.e., carbohydrates (Knopp et al., 2006) and free amino acids (Bytof et al., 2005), are different in differently processed green coffees.

Among the amino acids present in raw coffee grain, asparagine is the major precursor of acrylamide, a potentially carcinogenic substance. During coffee roasting, amino acids and reducing sugars react to form pirazines due to the Maillard reaction. Pirazines are important for aroma development, but other compounds are also formed, including substances such as acrylamide. The presence of acrylamide in foods, including coffee, may present risks to human health. The toxicological potential of acrylamide in foods

is related to the concentrations of its precursors (reducing sugars and asparagine) in the raw material, which may vary significantly between species, cultivation practices and processing techniques. Qualitative analysis has shown that asparagine is the main amino acid present in immature coffee grains (Mazzafera, 1998). More recent work has shown that the pulping of immature fruit allows a reduction in asparagine levels (Dias et al, 2011). Both the low yield of the asparagine/acrylamide reaction and the significant reduction (loss) of acrylamide during roasting may be the reason that the correlation between asparagine and acrylamide in coffee is less prominent than in other foods. Although coffee beans are roasted at high temperatures, the amount of acrylamide found in roasted beans and ground coffee is reported to be low (Friedman, 2003). Reports on commercial market samples of roasted coffee confirm lower levels of acrylamide for dark-roasted in comparison to lighter-roasted coffees (Granby & Fagt, 2004; Senyuva & Gokmen, 2005). As aroma is a result of the roasting process and is related to the chemical composition of coffee beans, each change in raw material or in the roasting process can result in different characteristics of the final product (Bagdonaite, 2008). The color of the beans after roasting is often considered a quality criterion. Therefore, optimizing roasting conditions to reduce acrylamide formation while maintaining product quality needs to be investigated (Guenther et al., 2007).

Interventions in coffee roasting have been largely unsuccessful to date due to important changes in organoleptic properties that occur when roasting conditions are changed. Dark roasts contain less acrylamide than lighter-roasted coffee because acrylamide is destroyed during the roasting process. This market range, however, cannot be used as a mitigation scenario unless consumers are educated to drink darker-roast coffee. Furthermore, coffee is a single-ingredient product; thus, additives cannot be used. Coffee varieties from different origins are usually blended to obtain a constant product quality; therefore, any measures taken on one part of the blend will be diluted in the final blend. Processes based on reaction mechanisms causing a loss of acrylamide during storage are currently the only imaginable option for lowering acrylamide, but they present quality issues and have, therefore, not been used as a method of lowering acrylamide to date (Seal et al., 2008). The range of best practice recommendations focusing on the reduction of acrylamide is still only possible for *agronomical*practices. The first harvests of young coffee plants are usually possible in the plants' 3rd and 5th years of age. In addition, cultivation conditions are very specific and restricted to certain climatic conditions, making the implementation of interventions into agricultural practice a difficult and lengthy process. However, some post-harvest practices were reported to interfere with the free amino acid content of green coffee beans, which might offer the opportunity for further investigations (CIAA, 2006).

The aim of this study is to assess the effect of post-harvest coffee processing on acrylamide formation in immature coffee, relating it to asparagine concentrations in the raw material. The post-harvest processing conditions of immature coffee beans are also related to the degree of roasting and acrylamide formation in order to improve the quality and value of the final product. Other quality parameters will be discussed in other studies.

Materials and methods

Coffee samples. Coffee fruits (*Coffea arabica L.)* of Topázio cultivar (2006/2007 crop) were grown in the UFLA site. After cleaning and hydraulic separation, the proportion formed by cherry and green fruits was pulped without a counter-weight. This method regulates the pressure of the drum pulper, allowing no more than 10% of cherry fruits to exit with the green fruits. The pulped cherry beans were dried and the rejected mixture with 10% cherries was used in this study. A part of this portion was naturally processed and used as control (A). Another part of the mixture was pulped using a counter-weight to regulate the pressure, resulting in a pulped parcel (B) and a natural parcel (C) that were also dried. A third part of the mixture with 10% cherry fruits was put in two boxes and allowed to rest for 12 hours. One of the boxes was filled with water. After the resting period, both portions were pulped with a counter-weight, giving rise to pulped and natural immature coffee samples after resting in water (D and E) and pulped and natural immature coffee samples after resting in the open air (F and G) (Borém, 2008). A schematic representation of this procedure is shown in Figure 1. The control and other experimental parcels were dried on patios until the water content of the coffee beans was about 10-12% (wb).

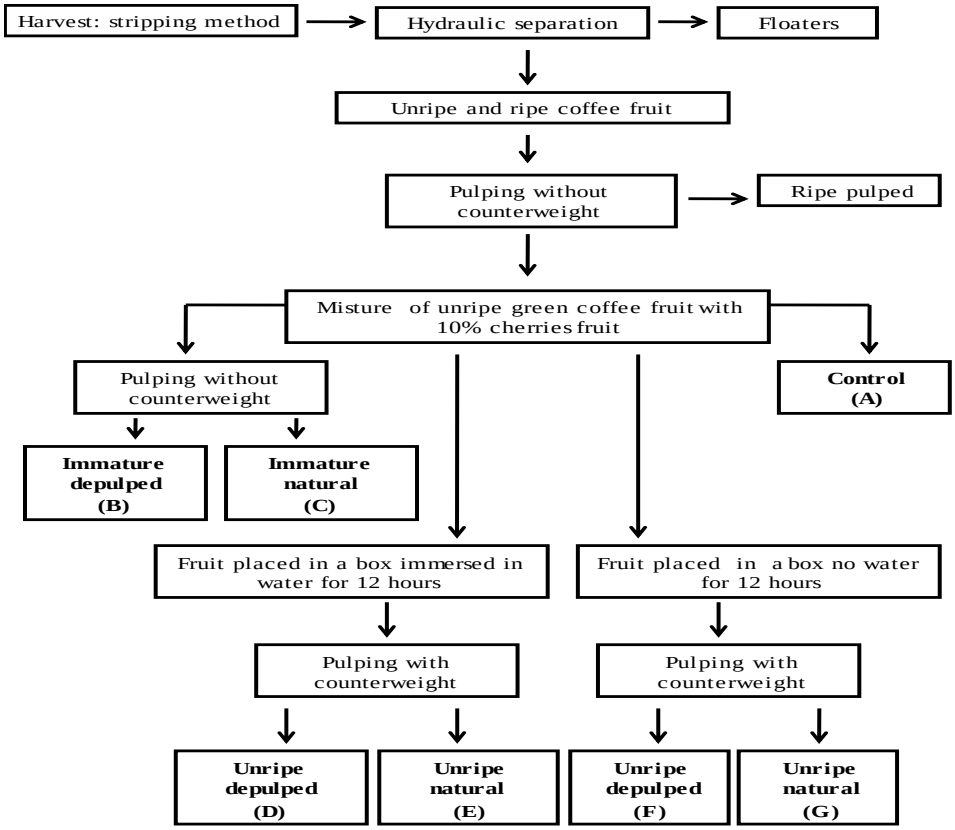

Figure 1- Schematic representation of coffee bean processing

A – Control; B – Unripe pulped; C – Unripe natural; D – Unripe pulped 12 hours in water; E – Unripe natural 12 hours in water; F – Unripe pulped 12 hours piled ; G – Unripe natural 12 hours piled

Chemicals. Acrylamide (AA) of 99% purity grade was acquired from Aldrich (Steinheim, Germany). Acrylamide 1,2,3-C13 ($^{13}C_3$-AA, IS) 99% in methanol was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The ISOLUTE C_{18}/Multimode (1g/1g) layered SPE columns were from Biotage (Uppsala, Sweden). The bulk C_{18} sorbent 125 Å, 55-105 μm was from Waters (Milford, USA). Both SPE columns and bulk C_{18} were conditioned with methanol and water and used without drying. The n-hexane and ethyl-acetate were of pesticide residue analysis grade and acetonitrile was of ultrapure grade, all from Fluka. The potassium bromide for IR spectroscopy grade and the bromine analytical grade were from Merck (Darmstad, Germany). Analytical grade sodium chloride was from J.T. Baker (Deventer, Holland). Hydrobromic acid 48% and sodium tiosulphate volumetric solution 1 mol L^{-1} were from Riedel-de Häen (Seelze, Germany).

Standards and reagents. A stock solution of AA (2 g L^{-1}) was prepared by dissolving the compound in acetonitrile and appropriately diluted to prepare a working standard solution at 1 mg L^{-1}. A stock solution of internal standard (IS) $^{13}C_3$-AA was prepared in the same way, with the difference that the working standard solution was made at 10 mg L^{-1}. All stock and working solutions were stored at 4°C. The saturated bromine–water solution was prepared by adding bromine (~3 mL) to 200 ml of water until precipitation became visible.

Asparagine analysis. The amino acid present in unroasted coffee was analyzed by reversed phase chromatography after derivatization with phenylisothiocyanate and ultraviolet detection. A full description of the methodology and discussion of the results can be seen in Dias (2011).

Roasting process. Coffee samples were roasted in a batch roaster (Probat model BRZ 6 - Brazil) using medium and dark roast (Pittia et al., 1996; Pizzirani et al., 1996). The roasting process started at 150°C, and the final point was determined by visual and instrumental examination with a colorimeter (Chromameter-2 Reflectance, Minolta, Osaka, Japão). The average temperature reached was 220°C after 9 minutes. The roasted beans were vacuum packed in aluminum packages, sealed and stored until analysis. The roasted beans were ground in an electric grinder at 20 mesh to perform the analysis (Moulinex, Model A843, Ecully, France).

Equipment. GC/MS analyses were performed in a gas chromatograph, model HP GC-6890, split-splitless injector, coupled to a Mass Selective Detector model Agilent MSD-5973N (Agilent, Palo Alto, CA, USA). The analytical separation was performed in a capillary column MDN-12 (30 mm x 0.25 mm x 0.25 μm DI) from Supelco (Steinheim, Alemanha). The centrifugations were made in a Heraeus Sepatek, model Labofuge Ae (Osterode, Germany) at 3000 g. The MSPD extractions were made in a vacuum manifold model Visiprep Solid Phase Extraction Manifold from Supelco (Taufkirchen, Germany) with capacity for 12 columns. Evaporation under a stream of nitrogen was carried out on a Pierce, model Reacti-therm 18790 (Rockford, IL, USA) with capacity for 9 vials.

GC/MS operating conditions. Gas chromatography. Carrier gas: helium (constant flow at 1 mL $minute^{-1}$). Sample injection volume: 1 μL (splitless, pulsed pressure 32 psi, 60 sec). Injector temperature: 280°C. Oven temperature: 85°C (1 minute), 15°C $minute^{-1}$ to

280ºC, hold 10 minutes (24 minutes), transfer line, 280 ºC. Mass-spectrometry. Electron energy, 70 eV (EI mode). Mode of acquisition: selected ion monitoring (SIM), ions m/z 106, 150 and 152 for 2,3-dibromopropionamide (2,3-DBPA) and ions m/z 110, 153, 155 for 2,3-$^{13}C_3$- dibromopropionamide (2,3-$^{13}C_3$-DBPA). The ions m/z 150 for 2,3-DBPA, m/z 155 for 2,3-DBPA($^{13}C_3$) were used for quantification and the others for confirmation. AA was determined with the internal standard, using the ratio of peak area of 2,3-DBPA to 2,3-$^{13}C_3$-DBPA. The identity of the peak was confirmed by retention time and by comparing the relative abundance ratios of the confirmatory ions to those of the standard solution.

Sample preparation using MSPD. A 0.5 g aliquot of ground coffee and 2 g of preconditioned C_{18} were placed in a glass mortar, spiked with 0.25 μg of IS (25 μl of the 10 mg/L IS solution) and blended together using a glass pestle for ≈ 2 minutes. When the blending was complete, the dispersed mixture was transferred to a pre-conditioned ISOLUTE C_{18}/Multimode (1g/1g) layered SPE column (Soares et al., 2010). A frit was put on top of the sample mixture before careful compression with a syringe plunger. The packed column was placed in a vacuum manifold and acrylamide was extracted with 6 mL of water, holding the flow to guarantee a soaking step of 5 minutes. The water solution was collected until it reached the top limit of the frit to avoid drying the sorbents, and a second aliquot of 6 mL of water was added, requiring another 5-minute soaking step. The second elution volume was collected in a single vial and the column was kept under vacuum for 5 minutes to collect all the water. All samples analyzed were prepared in duplicate.

Calibration standard preparation using MSPD. Aliquots of the 1 mg L^{-1} working standard AA solution (equivalent to 0 to 0.75 μg of AA corresponding to 0 to 1500 μg kg^{-1} in the samples) were placed in a glass mortar with 2 g of C_{18} and 1 μg of internal standard. These mixtures were blended together and treated according to the overall procedure described for the samples.

Bromination of the samples and standards. One g of calcinated KBr was added to each of the aqueous extracts. The solutions were then acidified with HBr until a pH of 1-3 (100-150 μL) was reached, when 2 mL of saturated bromine solution were added. The solutions were allowed to stand in an ice bath and kept from the light for at least 1 hour. The excess bromine was decomposed by the addition of 1 mol L^{-1} sodium tiossulphate solution until the yellow color of the extracts disappeared (50-150 μL). The solutions obtained were saturated with 2 g of NaCl and the acrylamide derivative extracted twice with 10 mL and 5

mL portions of ethyl acetate/n-hexane 4:1 (v/v). The volume of the organic phase was reduced to 3 mL under a stream of nitrogen, and a small quantity of anhydrous Na_2SO_4 was added. Finally, it was centrifuged at 3,000 rpm for 3 minutes. The upper layer was transferred to another vial and evaporated to 0.5 mL under a gentle stream of nitrogen. The acrylamide derivative extracts were then injected into the gas chromatograph.

Statistical analysis. All data were subject to analysis of variance (ANOVA). The GLM (General Linear Models) procedure of the SAS (Statistical Analysis System – SAS Institute Inc., North Carolina, USA - 2001) was used to evaluate the statistical significance of the differences among mean values. The F-test was also applied. The Tukey test was used to compare means, with 5% considered the level of significance.

Results and discussion

One factor that could contribute to relatively higher acrylamide levels is the number of defective beans used in coffee production. Defective coffee beans, and immature beans in particular, are characterized by significantly higher amounts of free asparagine than mature beans (Mazzafera, 1998). However, this parameter is a central component of quality control and in most cases the green fruits are classified accordingly. In coffee, acrylamide formation does not appear to be affected by a reduction in sugars content and only correlates with asparagine concentration in the green beans (Lantz et al., 2006).

Arnold and Ludwig (1996) identified considerable differences in free amino acid composition during post-harvest treatment and after model drying, fermentation and storage of green arabica beans. Acrylamide does not accumulate during coffee roasting. Rather, its formation and degradation occur simultaneously, and only 20% of the initially formed acrylamide survives the roasting process. Post-harvest changes in precursor concentrations will affect the potential for acrylamide formation as the relative concentrations change.

The results obtained from this work are shown in Table 1.

Table 1- Asparagine and acrylamide concentrations for unripe coffee beans submitted to different post-harvest procedures. The data are mean values of 3 analyses.

	Procedures	Asparagine (g/100g green coffee)[a]	Acrylamide (µg/kg)	
			Medium roast	Dark roast
A	Control	1.06 ± 0.35	435.7 ± 73.5	371.2 ± 40.1
B	0 h pulped	0.84 ± 0.22	346.5 ± 24.2	259.6 ± 60.2
C	0 h natural	1.18 ± 0.41	558.6 ± 177.9	336.2 ± 100.3
D	12 h pulped/water	1.34 ± 0.39	352.7 ± 50.6	401.5 ± 37.2
E	12 h natural/water	1.04 ± 0.28	610.1 ± 26.3	355.0 ± 37.6
F	12 h pulped/rest	0.97 ± 0.15	421.2 ± 32.2	217.0 ± 76.8
G	12 h natural/rest	1.29 ± 0.13	482.5 ± 39.6	347.6 ± 26.7

[a]Asparagine results obtained from Dias 2008.

Storage procedures: A – Control; B – Unripe pulped; C – Unripe natural; D – Unripe pulped 12 hours in water; E – Unripe natural 12 hours in water; F – Unripe pulped 12 hours piled; G – Unripe natural 12 hours piled.

The acrylamide concentrations are presented for the two roasting levels tested, as well as the asparagine levels obtained from each procedure applied to the unripe coffee. Higher amounts of asparagine correspond to increased levels of acrylamide in roasted coffee, as shown in Figure 2. In general, the coffee beans presenting higher levels of acrylamide are those submitted to medium roast and obtained from natural or dry process for all procedures (Figure 3): control (A), natural 0 hours (C), 12 hours immersed in water (E) and 12 hours without water (G). This fact may be related to the higher amounts of asparagine present in natural coffee compared with pulped coffee, as demonstrated by Dias et al. (2011).

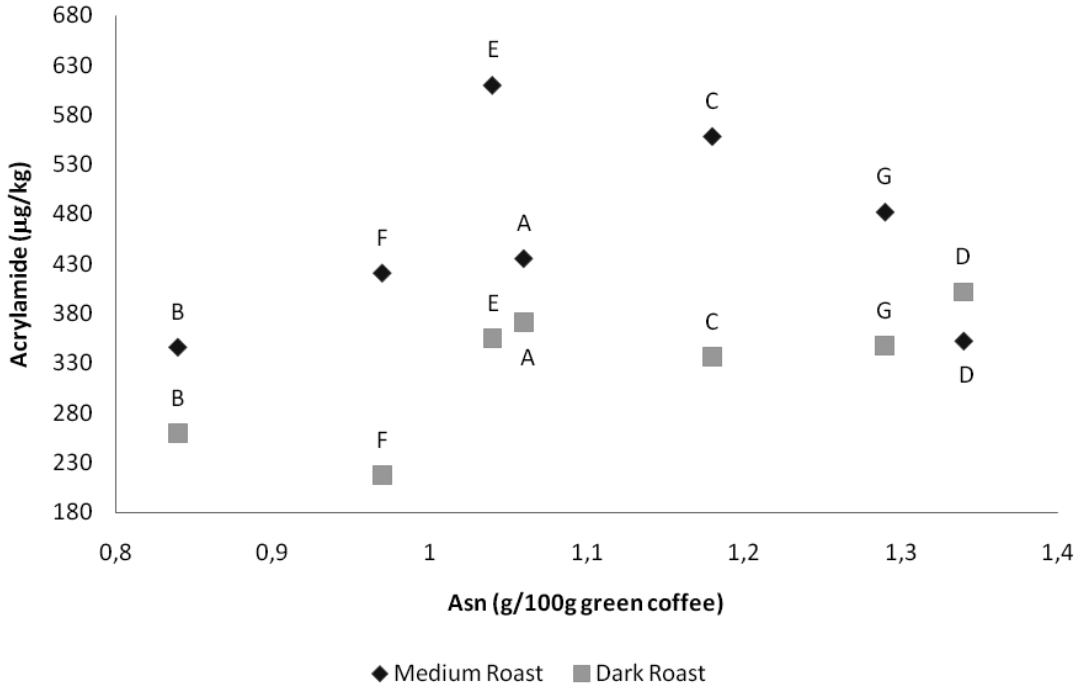

Figure 2- Correlation between acrylamide levels in dark- and medium-roasted coffee and asparagine (g/100g of green coffee) obtained with processed green beans. Storage procedures: A – Control; B – Unripe pulped; C – Unripe natural; D – Unripe pulped 12 hours in water; E – Unripe natural 12 hours in water; F – Unripe pulped 12 hours piled; G – Unripe natural 12 hours piled.

The lowest levels of asparagine were present in immature grains resulting from pulped coffee processing, corresponding to the presence of minimal amounts of acrylamide after roasting. It was observed that the procedures pulped 0 hours (B) and 12 hours (F) resulted in lower levels of acrylamide during dark roasting of the grains compared to the same procedures submitted to medium roasting (Figure 3). An exception was observed for procedure D (pulped for 12 hours and immersed in water), which showed a higher amount of acrylamide in the dark-roasted grains. In immature coffee fruits, higher levels of asparagine and acrylamide were found when the storage procedure included water for 12 hours. This effect is probably due to physiological changes caused by the water absorption by seeds by altering the concentration of the amino acid precursor (Bytof et al., 2005). However, an increase in the content of this amino acid may also be caused by induced stress, such as drying (Lea et al., 2006).

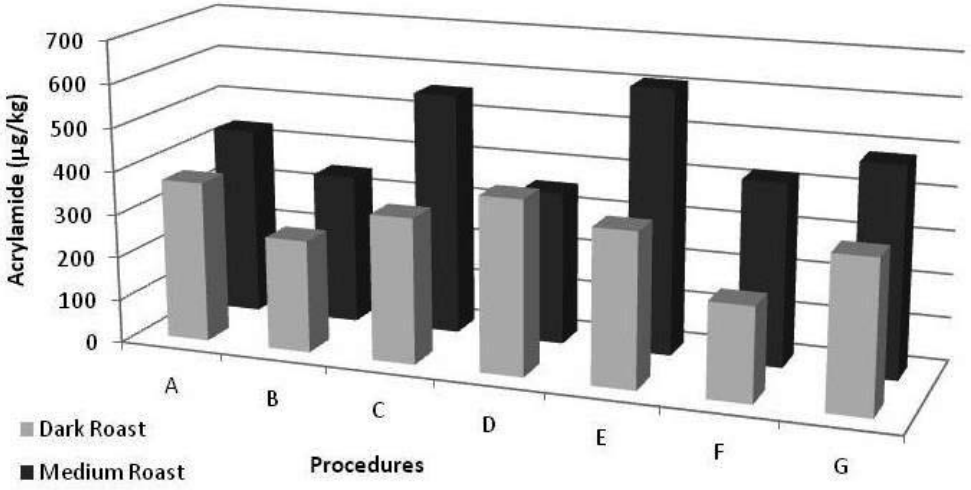

Figure 3- Formation of acrylamide in unripe coffee differently processed and roasted at medium and dark degrees. Storage procedures: A – Control; B – Unripe pulped; C – Unripe natural; D – Unripe pulped 12 hours in water; E – Unripe natural 12 hours in water; F – Unripe pulped 12 hours piled; G – Unripe natural 12 hours piled.

Unripe natural coffee grains submitted to medium roasting showed higher levels of acrylamide comparedwithunripe, pulped beans. These levels are directly related to asparagine quantities. The results are in accordance with other studies (Lantz et al., 2006), in which asparagine was reported as a limiting factor in acrylamide formation during the roasting process. However, no significant differences in acrylamide levels were detected in the dark roasts of natural and pulped immature coffee (Table 2).

Table 2- Acrylamide levels (µg/kg) in immature coffee beans submitted to different postharvest procedures (natural and pulped natural) after medium and dark roasting. The data are mean values of 9 analyses.

Post-harvest processing	**Acrylamide levels (µg kg $^{-1}$)**	
	Medium roast	**Dark roast**
Unripe natural	550.41 a	346.26 b
Unripe pulped	373.45 b	292.71 b

Values followed by the same letter are not significantly different, according to the F-test (p>0.05).

A comparison of storage conditions for both pulped and natural unripe coffee (Table 3) concludes that storage conditions have no influence on the acrylamide formation of medium-roasted coffees. For darker roasts, however, the storage of unripe fruits in water for 12 hours shows an increase in acrylamide levels in comparison with other storage

conditions. A decrease in acrylamide levels was observed among all of the procedures when submitted to dark roasting processes.

Table 3- Acrylamide levels (µg/kg) in unripe natural and pulped fruit subjectedtodifferent storage procedures aftermedium anddarkroasting. The data are mean values of 3 analyses.

Unripe coffee storage procedures	**Acrylamide levels (µg kg $^{-1}$)**	
	Medium roast	Dark roast
Fruit without rest	452.53 a	297.92 bc
Fruit stored in a box during 12 hours	451.85 a	282.28 b
Fruit stored in water during 12 hours	481.42 a	378.25 c

Values followed by the same letter are not significantly different, according to the Tukey test (p>0.05).

In this experiment, the highest concentrations of acrylamide were obtained at low temperatures (220°C) and a short roasting time (9 minutes). During more intense roasting, the acrylamide degrades until it can no longer be detected. Coffee is typically roasted at temperatures in the range of 200–230°C. Roasting time and speed impact the coffee's sensorial properties (aroma/taste). These are carefully fine-tuned to provide the characteristic profile and clear identity of the coffee product. Experiments have shown that acrylamide is degraded or eliminated during roasting, and the profile of acrylamide formation during the roasting of coffee reflects this effect very clearly (Taeymans et al., 2004). In coffee, acrylamide is formed at the beginning of the roasting step. Toward the end of the roasting cycle, acrylamide loss seems to dominate. Therefore, light-roasted coffees may contain relatively higher amounts of acrylamide than dark-roasted beans. However, higher roasting as a potential method of reducing acrylamide could generate undesirable compounds and negatively impact the taste and aroma of the product. Consequently, no practical solutions are available today to both reduce acrylamide levels and concomitantly retain the coffee's quality characteristics because the roasting step cannot be fundamentally changed (Taeymans et al., 2004).

However, the roasting time necessary to obtain an optimal product depends mainly on the type of bean (composition, humidity and size) and has to be optimized for each green coffee. Roasting is an important point to be considered because it facilitates the formation of substances important to the quality attributes of coffee. Through the Maillard and Strecker reactions, the effects of various biochemical reactions contribute to the formation of substances such as acrylamide during coffee roasting (Granby & Fagt, 2004; Senyuva & Gokmen, 2005). Therefore, most intense roasting process or dark grains help decrease the content of acrylamide but have negative effects on the sensory attributes of the product, decreasing acidity, reducing sweetness and intensifying bitterness due to some components' carbonization (Theurillat et al., 2006; Pereira, 2003).

The average amount of acrylamide found in immature coffee beans was 458.2 μg kg^{-1} for medium roasting and 326.8 μg kg^{-1} for dark roasting. Roasted coffee beans can produce levels of acrylamide between 40 and 400 μg kg^{-1} with a mean value of 200 μg kg^{-1} (Hoenicke & Gatermann, 2005; Murkovic, 2004). At the end of the roasting process, according to data from the European Commission, the beans present an average level of acrylamide between 265 and 290 μg/kg (Guenther et al., 2007). In dark-roasted immature coffee beans, the mean levels found are close to those made public by the European Commission.

It can conclusively be said that the food industry, in a joint collaborative effort, could show moderate success in the relative reduction of acrylamide by establishing several collective measures for the treatment of certain foods, e.g., raw material selection, adapting processing parameters and guidance on final food preparation. Alterations in post-harvest coffee will define the potential formation of acrylamide from changes in concentration of its precursor. Acrylamide content is altered by the levels of asparagine and the type of processing, showing a significant difference after coffee roasting. Nobre (2009) showed that pulped immature coffee presents a closer chemical composition to ripe coffee in comparison with natural, unripe coffee. The author found that the pulped unripe coffee presented fewer defects, with only 2.8% PVA (black, green and sour grains).

Conclusion

Currently, there are no options available for acrylamide reduction in the process of roasting coffee beans. Controlling the levels of acrylamide's precursors in the raw material seems to be the most effective way to reduce acrylamide during roasting. The pulping of immature coffee contributes to decreased asparagine levels, and consequently acrylamide levels, for both levels of roasting. Whatever the processing type, acrylamide contentislowerafter dark roastingwhenimmaturecoffee beansare processed inthe same dayor when the fruits are stored piled in a box for 12 hours. The procedures discussed in this work can form the scientific basis for the control and generation of new technologies in post-harvest coffee treatments, minimizing the potential formation of harmful chemicals such as acrylamide and improving the quality, safety and economic viability of immature coffee.

References

Arnold U, Ludwig E. Analysis of free amino acids in green coffee beans. II. Changes of the amino acid content in arabica coffees in connection with post-harvest model treatment. Z Lebensm Unters For 1996; 203:379–384.

Bagdonaite K. Determination of acrylamide during roasting of coffee. J Agr Food Chem 2008; 56(15):6081-6086.

Bytof G. Einfluss der nacherntebehandlung auf die qualit_tsauspr_gung bei arabica-kaffee (*Coffea arabica L.*). Thesis (Ph.D.) - TU Brau, 2003.

Bytof G, Knopp SE, Schieberle P, Teutsch I, Selmar D. Influence of processing on the generation of g-aminobutyric acid in green coffee beans. Eur Food Res Technol 2005; 220:245-250.

Borém FM. Pós-colheita do café. Lavras: UFLA, v.1. 2008 631 (In Portuguese).

Confederation of the Food and Drink Industries of the EU (CIAA) The CIAA Acrylamide "Toolbox", pp. 1–35. Brussels: CIAA AISBL, 2006.

Dias EC, Borém FM, Pereira RGFA, Guerreiro MC.Amino acid profiles in unripe Arabica coffee fruits processed using wet and dry methods, Eur Food Res Technol 2012; 234:25-32.

Friedman M. Chemistry, biochemistry and safety of acrylamide. A review. J Agr Food Chem 2003; 51:4504-4526.

Guenther H, Anklam E, Wenzl T, Stadler RH. Acrylamide in coffee: Review of progress in analysis, formation and level reduction. Food Addit Contam 2003; 24(1):60-70.

Granby K, Fagt S. Analysis of acrylamide in coffee and dietary exposure to acrylamide from coffee. Anal Chim Acta 2004; 520:177–182.

Hoenicke K, Gatermann R. Studies on the stability of acrylamide in food during storage. J. AOAC Int 2005; 88:268-273.

Illy A, Viani R. Espresso Coffee: the Science of Quality. 2nd ed. Amsterdam, London, New York: Elsevier Academic Press, 2005.

Lantz I, Ternite R, Wilkens J, Hoenicke K, Guenther H, van der Stegen G. Studies on acrylamide levels in roastings, storage and brewing of coffee. Mol Nut Food Res 2006; 50:1039–1046.

Lea PJ, Sodek L, Parry MAJ, Shewry PR, Halford NG. Asparagine in plants. Ann Appl Biol 2007; 150:1-26.

Knopp SE, Bytof G, Selmar D. Influence of processing on the content of sugars in green Arabica coffee beans. Eur Food Res Technol 2006; 223:195–201.

Machado MC. Viabilidade da técnica de imersão para armazenagem temporária dos frutos de café. Tese (doutorado) – Universidade Federal de Viçosa, MG Viçosa: UFV. 90 p, 2005. (In portuguese)

Mazzafera P. Chemical composition of defective coffee beans. J Food Chem 1998; 64:547-554.

Murkovic M. Acrylamide in Austrian foods. J Biochem Biophys 2004; 61:161-167.

Nobre GW. Processamento e qualidade dos frutos verdes de café arábica. Tese (Doutorado em Fitotecnia) - Universidade Federal deLavras, Lavras, 85 p., 2009. (In portuguese).

Pereira RGFA. Tecnologia e qualidade de café, raízes e tubérculos. Lavras: UFLA/FAEPE 54 p. 2003 (In portuguese).

Pittia P, Dalla Rosa M, Pinnavaia M, Massini R. Evoluzione di alcune caratteristiche fisiche del caffè durante la torrefazione. Industrie Alimentari 1996; 35(351):945-950. (In itallian)

Pizzirani S, Romani S, Anese M, Barbanti D. Studio sulle caratteristiche chimiche e chimico-fisiche del caffè torrefatto e della bevanda di estrazione. Industrie Alimentari 1996; 34(1):658-663. (In itallian)

Seal CJ, De Mul A, Eisenbrand G, Haverkort AJ, Franke K, Lalljie SPD, et al. Risk-benefit considerations of mitigation measures on acrylamide content of foods – A case study on potatoes, cereals and coffee. Brit J Nut 2008; 99:S1–S47.

Senyuva HZ, Gokmen V. Study of acrylamide in coffee using an improved liquid chromatography mass spectrometry method: investigation of colour changes and acrylamide formation in coffee during roasting. Food Addit Contam 2005; 22(3):214-220.

Soares C, Alves RC, Casal S, Fernandes JO, Oliveira BPP. Validation of a Matrix Solid-Phase Dispersion method to determine acrylamide in coffee and coffee surrogates. J Food Sci 2010; 75:T57-T63.

Statistical Analysis SystemInstitute. *The SAS system for windows: release 8.02*. Cary, 842 p 2001.

Taeymans D, Wood J, Ashby P, Blank I, Studer A, Stadler RH, et al. A review of acrylamide: an industry perspective on research, analysis, formations, and control. Crit Rev Food Sci Nutr 2004; 44:323–347.

Theurillat V, Leloup V, Liardon R, Heijmans R, Bussmann P. Impact of roasting conditions on acrylamide formation in coffee. In: Proceedings of the 21st Association for Science and Information on Coffee International Conference, 2006, Montpellier (CD-ROM).

3.3. Influence of the Roasting Conditions on the Formation of Acrylamide in Brazilian Coffee: Preliminary Results

Summary

The present work reports a study concerning the formation of acrylamide in Brazilian coffee, using different roasting conditions. Temperature and roasting time were kept constant and the flow of hot air in a fluidized bed roaster was set to three different velocities.

Acrylamide contents were determined using a MSPD-GC/MS method recently developed and applied for coffee sample. The results obtained so far, clearly show that the content of acrylamide increases as the velocity of hot air also increases in all samples analysed.

More studies are being performed in order to determine the best conditions to roast coffee with the least possible formation of acrilamydes .

Influence of the Roasting Conditions on the Formation of Acrylamide in Brazilian Coffee: Preliminary Results

In: 22nd International Conference on Coffee Science, September 14th - 19th, 2008, Campinas (Brasil).

C. SOARES*, A. FARAH**, A. TOCI**, F. FERNANDES***, J. O. FERNANDES*

*REQUI*M*TE/ Serviço de Bromatologia, FFUP, Porto, Portugal

** Laboratório de Bioquímica Nutricional e de Alimentos, Instituto de Química, UFRJ, Rio de Janeiro, Brazil.

*** Cia Cia. Lilla de Máquinas Ind. e Com., São Paulo, Brazil

Summary

The present work reports a study concerning the formation of acrylamide in Brazilian coffee, using different roasting conditions. Temperature and roasting time were kept constant and the flow of hot air in a fluidized bed roaster was set to three different velocities.

Acrylamide contents were determined using a MSPD-GC/MS method recently developed and applied for coffee sample. The results obtained so far, clearly show that the content of acrylamide increases as the velocity of hot air also increases in all samples analysed.

More studies are being performed in order to determine the best conditions to roast coffee with the least possible formation of acrilamydes .

Introduction

In 2002, Swedish scientists reported the discovery of large amounts of acrylamide in starch-rich foods that had been cooked at high temperatures. Although the mechanism of acrylamide formation remains unclear, it is believed that this compound is a by-product of the Maillard reaction through a mechanism involving asparagine and glucose. Acrylamide has been demonstrated to cause cancer in animals and it is thought that its presence in foods may be potentially harmful to people's health (Tareke, 2002).

Since the Swedish discovery, a global effort has been undertaken to gather data involving this compound. According to several studies on the contribution of the nutritional habits to acrylamide intake, coffee contributes to about 40 % of the total acrylamide exposure in Sweden and about 33 % in Switzerland, making this beverage a significant contributor to acrylamide intake in dietary sources. The level of acrylamide in a cup of coffee depends on how concentrated it is and how the beans are roasted. Concentrations of acrylamide in instant and ground coffee are reported to be very similar — both about 290 μg/kg — because this compound is a product of the roasting of the beans rather than of any subsequent processing (Soares et al., 2006; Dybing et al., 2005; Granby et al., 2004).

Acrylamide levels in roasted coffee are dependent on the formation and elimination reactions that take place during the roasting process; the profile of acrylamide formation reflects this effect very clearly. Acrylamide formation reactions are dominant at the beginning of the roasting cycle, leading to increased levels at this stage, and then steeply decline toward the end of the roasting cycle. Therefore, light roasted coffees may contain relatively higher amounts of acrylamide than very dark roasted beans. Reports on commercial market samples of roasted coffee showed lower levels of acrylamide for darker coffee roasts in comparison to lighter roasts, confirming these predictions. However, darker roasting as a potential option to reduce acrylamide could potentially generate other undesirable compounds like furan and polycyclic aromatic hydrocarbons (PAH´s) and will definitively have an impact in the taste/aroma of the product. Consequently, no practical solutions are available that would reduce acrylamide levels and concomitantly retain the quality characteristics of coffee, since the roasting step cannot be fundamentally changed (Guenther et al., 2007).

The objective of this work was to study the roasting conditions that would lead to a decrease in the acrylamide formation. Different roasting conditions were set and some preliminary results of this study are presented, particularly the effect of the velocity of the hot air flow used in a fluidized bed roaster.

Experimental

Sampling: A total of 4 roasted coffee samples of different cup qualities were tested: Espresso (good quality) and 3 blends of *C. arabica* and C. *canephora*.

Acrylamide extraction: A 0.5 g aliquot of ground coffee and 2 g of C_{18} sorbent were placed in a glass mortar, spiked with 0.50 μg of IS (25 μl of the 10 mg l^{-1} IS solution) and blended together using a glass pestle to obtain the complete disruption and dispersion of the sample on the solid support. When blending was complete, the sample was transferred to an Isolute C18/Multimode 2 g/15 mL layered SPE column. A frit was placed on the top of the sample mixture before careful compression with a syringe plunger. The packed column was placed in a vacuum manifold and acrylamide was extracted with 4 ml of water holding the flow to permit a soaking step of 5 min. This water was collected until the limit of the frit to avoid drying of sorbents and a second aliquot of 4 ml of water was added observing again a 5 min soaking step. The second elution volume was completely collected to the same vial and the column was kept under vacuum aspiration during 5 minutes to collect all the water. The aqueous extracts obtained were derivatized with bromine and after the derivative's extraction with an organic solvent the total volume was reduced to 0.5 ml under a gentle stream of nitrogen. The extracts were then injected in the gas-chromatograph.

Results and discussion

The results obtained for the acrylamide analysis are presented in figure 1. The analysis of the data permits to conclude that the content of the compound increases as the velocity of hot air also increases in all samples analysed, independently of the quality of the coffee beans. Probably this result is a consequence of differences between heat transfer among the coffee beans and the hot air velocity.

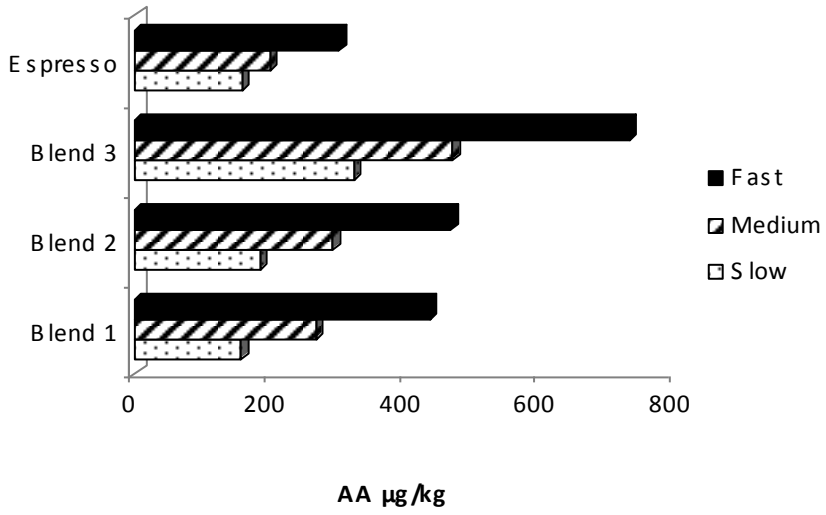

Figure 1- Concentration of acrylamide in the different coffee samples related to the hot air velocity inside the roaster

To support these findings, more studies are being performed using different roasting conditions. The final objective is to determine and propose a new approach of coffee roasted in fluidized bed and similar type roasters to obtain coffee with appropriated organoleptic properties and small amounts of acrylamide.

Conclusion

The change in roasting conditions, namely the hot air flow velocity, is a potential contributor to the preparation of coffee with less acrylamide. But in order to maintain the desired qualities of the coffee drink, more studies are needed . Other roasting parameters and samples are being tested and the conclusions will be reported shortly.

Acknowledgements: Cristina Soares and José O. Fernandes are thankful to FCT for financial support in the framework of the project POCI/AGR/61543/2004 and for the PhD grant of Cristina Soares with the reference SFRH/BD/39360/2007.

References

Dybing E, Farmer PB, Andersen M, Fennell TR, Lalljie SP, Müller DJ, et al. Human exposure and internal dose assessments of acrylamide in food. Food Chem Toxicol 2005; 43:365-410.

Granby K, Fagt S. Analysis of acrylamide in coffee and dietary exposure to acrylamide from coffee. Anal Chim Acta 2004; 520:177-182.

Guenther H, Anklam E, Wenzl T, Stadler RH. Acrylamide in coffee: review of progress in analysis, formation and level reduction. Food Addit Contam 2007; 24:60-70.

Soares C, Cunha S, Fernandes J. Determination of acrylamide in coffee and coffee products by GC/MS using an improved SPE clean-up. Food Addit Contam 2006; 23:1276 -1282.

Tareke E, Rydberg P, Karlsson P, Eriksson S, Tornqvist M. Analysis of acrylamide, a carcinogen formed in heated foodstuffs. J AgrFood Chem 2002; 50:4998–5006.

CHAPTER 4

Mitigation Strategies to Reduce the Formation of Acrylamide in Home- Made Food

4.1. Introduction

The efforts of the scientific community regarding the mitigation of acrylamide in foods has been oriented to industrial processing. Most of the databases presenting levels of the compound in foods are about commercial foods.

The home preparation of meals has been neglected and the intention of this chapter is to present possible mitigation strategies to reduce the intake of acrylamide through home made food.

4.1.1. Strategies to mitigate the formation of acrylamide in home prepared food

Abstract

Mitigation of acrylamide in home-cooked foods without affecting final product specifications and quality, has been a challenge for food scientists. Two different approaches to reduce acrylamide formation during home-cooking of meat and fried potatoes are presented. For meat, the effect of marinades with white wine, beer, or green tea, alone or added with herbs and spices, were studied in pan-fried beef, using non-marinated beef as control. Cooking experiments were performed under controlled time and temperature, and samples were analyzed for acrylamide by GC-MS. Best results were obtained with beer or beer with herbs marinade during 4 hours, with a reduction of 72 % and 59 %, respectively. For potatoes, the importance of potato cultivar for frying was evaluated. Samples of 5 different cultivars available in the Portuguese market were fried under controlled time and temperature conditions. Lowest levels of acrylamide were obtained with Agria and Marabel varieties, which could be justified by its lower amounts of reducing sugars.Taking these highly consumed food as example, the combined effect of selecting adequate potato varieties for frying with the effect of marinades before meat cooking can contribute significantly to reduced acrylamide ingestion from home-made food cooking.

Strategies to mitigate the formation of acrylamide in home prepared food

Soares C.*, Fernandes, J.O.

REQUIMTE - Laboratory of Bromatology and Hydrology, Faculty of Pharmacy, University of Porto, Rua Jorge de Viterbo Ferreira 228, 4050-313, Porto, Portugal

*Corresponding author e-mail: cristina.md.soares@gmail.com

Abstract

Mitigation of acrylamide in home-cooked foods without affecting final product specifications and quality, has been a challenge for food scientists. Two different approaches to reduce acrylamide formation during home-cooking of meat and fried potatoes are presented. For meat, the effect of marinades with white wine, beer, or green tea, alone or added with herbs and spices, were studied in pan-fried beef, using non-marinated beef as control. Cooking experiments were performed under controlled time and temperature, and samples were analyzed for acrylamide by GC-MS. Best results were obtained with beer or beer with herbs marinade during 4 hours, with a reduction of 72 % and 59 %, respectively. For potatoes, the importance of potato cultivar for frying was evaluated. Samples of 5 different cultivars available in the Portuguese market were fried under controlled time and temperature conditions. Lowest levels of acrylamide were obtained with Agria and Marabel varieties, which could be justified by its lower amounts of reducing sugars.Taking these highly consumed food as example, the combined effect of selecting adequate potato varieties for frying with the effect of marinades before meat cooking can contribute significantly to reduced acrylamide ingestion from home-made food cooking.

Keywords: acrylamide, reducing sugars, asparagine, marinades, beer, wine, green tea, potato varieties, mitigation,

Introduction

Since the finding of acrylamide in foods back in 2002 there has been an extensive effort to understand its formation and possible ways to reduce it. Early research focused mainly in: a) the development of adequate analytical methods to assess acrylamide content in a wide range of food matrices (Rosén and Hellenäs, 2002; Ahn et al., 2002; Fernandes and Soares, 2007; Soares and Fernandes, 2009), b) determination of formation routes of acrylamide (Mottram et al., 2002; Stadler et al., 2002, 2004; Zyzak et al., 2003) and c) find possible mitigation strategies to reduce consumers exposure. The more successful mitigation options that have been proposed and tested so far have been collected by the Food and Drink Europe (FDE, formed termed Confederation of the Food and Drink Industries of European Union (CIAA)) in a guidance document, the so-called "toolbox" for acrylamide which latest edition was updated at 2011 (FDE, 2011).

For ready to eat foods, strategies reported in the literature to reduce acrylamide content focus in three main aspects: decrease precursor'samounts by careful choice or modification of raw materials, optimization of processing conditions (pH, temperature and heating time), and addition of additives, including acidulants, salts, antioxidants and enzymes.

For raw materials, the objective is to control the content of the carbonyl source and/or asparagine responsible for acrylamide formation (Jin et al., 2013). Contrary to what happens with most of cereals, asparagine levels in potatoes are in excess compared with reducing sugars contents, so is the sugar level that constitute the limiting factor in acrylamide formation as firstly reported by Amrein et al. (2003), and further confirmed by several other authors (Rydberg et al., 2003; Williams, 2005). Selecting cultivars that contain naturally lower levels of reducing sugarsis an obviously option,although asparagine levels can also be important for acrylamide levels in finished fried products (Friedman, 2003).

The extension of the classical Maillard reaction between reducing sugars and free amino acids is the main responsible for potato coloration and flavor. However, during the same reaction, sugars interact with asparagine to produce acrylamide (Amrein et al., 2003). Therefore, during processing is advisable to set appropriate heating temperature and avoid long-time processing of the foodstuff (Mottram et al., 2002).

Regarding exogenous additives, some substances reported to be effective for reducing acrylamide formation, include some organic acids (Cook and Taylor, 2005), amino acids (Claeys et al., 2005; Zamora et al., 2010), vitamins (Zeng et al., 2009) and some mono-

and divalent cations (Levine and Smith, 2005; Gokmen and Senyuva, 2007). The use of antioxidants from natural sources was also described as a potential alternative to mitigate the formation of acrylamide levels in foods. Some examples include the use of bamboo leaves and green tea extracts by Zhang et al. (2008)with a significant reduction in acrylamide formation in asparagine–glucose model systems.Rosemary, a medicinal and aromatic plant with known antioxidant properties, has also been proposed as an effective inhibitor of acrylamide formation in potato slices fried in corn or olive oil (Becalski et al., 2003). Similar results were obtained by Urbancic et al. (2014) that added rosemary extracts to sunflower oil and reported a stabilization of the oil and a lower acrylamide formation in fried potato slices. Hedegaard et al. (2008) confirmed the reducing effects of rosemary while adding aqueous rosemary extract, rosemary oil and dried rosemary leaves to a bread model, reducing the content of acrylamide by 62, 67 and 57%, respectively, compared to bread without rosemary. However, Zhu et al. (2009) tested several natural antioxidants and some phenolic compounds and reported that not all antioxidants had a mitigation effect. Instead, some compoundsenhanced acrylamide formation while others had no effect. Similar conclusions were obtained by several other authors (Bassama et al., 2010, Becalski et al., 2010, Ehling and Shibamoto, 2005, Serpen and Gokmen, 2009). Kotsiou et al. (2010) concluded that phenolic compounds without aldehydic groups in their structures are more effective in acrylamide reduction. Supplementary factors associated to the addition of antioxidants, such as pH decrease, or the presence of amino acids present in the extracts, could influence acrylamide contents and therefore hinder the comparison of results between different studies (Mestdagh et al., 2008).

It has been found that in lipid-rich systems, lipid oxidation products can convert asparagine into acrylamide in the absence of reducing sugars. Capuano et al. (2010) reported that lipid oxidation had a positive influence in the formation of acrylamide, especially in sugar-free systems where lipids became the main sources of carbonyl groups. Under this frame, antioxidants can inhibit acrylamide formation by preventing lipid oxidation, limiting the accumulation of carbonyls, for example in meat samples (Zamora and Hidalgo, 2008).

The capacity of marinades containing alcoholic beverages and mixtures of aromatic herbs to minimize the formation of heterocyclic aromatic amines (HAAs) was already established by our research group (Quelhas et al., 2010; Viegas et al., 2012). Therefore, taking into account these results, this study aimed to realize if identical mitigating effect is observed for acrylamide when using antioxidant rich marinades containing beer and white wine and a mixture of herbs commonly used as meat flavoring (garlic, ginger, thyme, rosemary, red-pepper) under household cooking conditions. Simultaneously, frying tests

were performed with potatoes from diverse cultivarsin order to evaluate its influence in the final acrylamide content, testing different frying times and temperatures.

Experimental methodology

Meat samples. Meat samples, from *Longissimus dorsi* muscle of middle-aged bovine carcasses, were obtained from a butchery in Porto, Portugal. Samples were chilled overnight in a cold storagechamber (4 ± 1˚C) before processing. Meat wassliced into steaks weighing about 100 g each.

Preparation of beer and wine marinades.Five marinades were tested: beer, beer withherbs, wine, wine with herbs and water. Plain steaks, unmarinated, were used as control. Pilsner beer (5.2% alcohol) and white wine (13.5% alcohol content from Douro valley region) were purchased at localsupermarkets. Herb marinades were prepared immediately before useby dispersing 2.8 g of ginger (Zingiber officinale), 2.9 g of garlic (Allium sativum), 0.4 g of rosemary (Rosmarinus officinallis), 0.25 g of thyme (Thymus vulgaris), and 0.1 g of red chili pepper (Capsicum annumm) in 100 mL of beer or wine. The ratio between the amount of meat and the volume of marinade was 1:1, i.e., 100 mL of marinade to 100 g of steak. One group of three meat samples remained unmarinated (control meat samples).Meat samples were marinated during 4 hunder refrigeration (4ºC) before being drained and pan-fried in a Teflon-coated pan during 3 min on each side, without adding oil. The temperature on the surface of the meat was monitored continuously during cooking with a digital thermometer, rounding 180 °C. Meat samples were weighed before and after cooking to calculate weight losses, usually around 40%. Each sample was grounded in a kitchen blender (Moulinex, France) to produce a uniform sample and frozen at -20 °C until analysis. Full details of the experimental conditions can be found at Viegas et al. (2012).

Preparation of green tea marinades. Green tea (Gorreana, Azores) infusions were prepared with 2 g of green tea per 250 ml (a cup), using boiling tap water for 10 min. Samples (n=2) were marinated during 1, 2, 4, and 6 h under refrigeration and finally grilled after being drained, as described above. Control beef samples were treated identically to the test samples, except that they were not marinated. Meat was weight before and after cooking to calculate the percent loss of weight with cooking. Average cooking losses of around 39-40% and 45-49% were observed for unmarinated and marinated samples, respectively. After

grilling beef steaks were grinded and stored at -20ºC until analysis. Full details of these conditions can be found at Quelhas et al. (2010).

Potato samples. Five varieties of potato commonly used in Portugal were acquired in local markets and prepared as soon as possible in order to avoid changes in the composition due to storage conditions. Selected varieties were Agria (AG), Monalisa (MO), Asterix (AS), Marabel (MA) and Rodeo (RO). Besides raw potatoes, 2 brands of pre-fried potatoes were also acquired and kept in the freezer (-20 °C) until analysis. Agria is a semi-late variety with yellow skin and flesh suitable for frying because of the low concentration of reducing sugars. Monalisa has good storage characteristics being usually sold for baking. Asterix presents excellent boiling qualities and industrialization characteristics. Marabel is a variety intended for fresh consumption, "*in natura*", with a very good flavor and pulp texture suitable for boiling, salad, mashed or other similar forms of preparation. Rodeo is characterized by small tubers, and isindicated for roasting (Agroportal, 2015). No indication of the potato variety was found on the pre-fried potatoes.

Preparation of potato samples. Fresh potatoes were peeled, cut into regular sticks (1 x 1 x 4 cm), rinsed in running water and the excess water removed with paper towels. Then, 200 g potato sticks were fried in anelectric fryer (Kenwood, Spain)containing 1 liter of peanut oil. Oil temperature was set to 180 °C (178-183 °C). Potatoes were fried during the following time periods: 3, 5, 7 and 9 min. The same procedure was followed for pre-fried potatoes despite the package instructionsto fryonly for 3-4 min at a temperature of 180 °C.After frying, potatoes were cooled, crushed and frozen until analysis.

Acrylamide analysis.

Acrylamide analysis in potato samples was carried out by a previous MSPD (Matrix Solid-Phase Dispersion) extraction followed by separation and quantitation by GC-MS according to previous reports (Fernandes and Soares, 2007). Briefly, 0.5 g of potato sample and 2 g ofC_{18} and internal standard (I.S.), were dispersed together in a container. Fats were removed with n-hexane and acrylamide extracted with water. All aqueous extracts were derivatized with bromine and acrylamide derivative was extracted with ethyl acetate/n-hexane (4:1). The extract was evaporatedunder a gentle nitrogen stream (40ºC) and the resulting sample extracts were reconstituted in 0,5 ml of the same solvent mixture and analyzed by GC-MS in SIM Mode. For meat and fish samples 2 g of beef sample was placed in a centrifuge tube with 20 mL water and homogenized by vortexing. I.S. was added and placed in a water bath

at 65 ° C for 20 minutes. Upon cooling,0.15 mL of CH_3COOH were added, followed sequentially by 1 mL of each Carrez I and Carrez II, used to eliminate most proteins from the extracts. Samples were placed at -20 ° C for 30 min and centrifuged at 9000 rpm for 15 min at 4 ° C. After filtration of the supernatant, the residue was extracted a second time with an additional 10 mL water amount. After homogenization, samples were centrifuged again (conditions above) the extracts combined, filtered and reduced to about 5 mL under vacuum and the resulting solutions derivatized with bromine. After extracting the derivative with ethyl acetate/n-hexane and evaporation under a nitrogen stream, samples were analyzed by GC-MS in SIM mode.

Asparagine analysis

To extract asparagine from potatoes, 1 g of sample was suspended in 5 mL of 0.2 M perchloric acid. The mixture was homogenized in a vortex for 1 min, kept in an ultrasonic bath for 30 min, and then centrifuged at 9000 g for 20 min. The upperlayer was transferred to another flask and a second volume of 5 mL of 0.2 M perchloric acid was added to the residue. The mixture was homogenized in a vortex and then mixed in a orbital mixer for 5 min. The solution was centrifuged again at 9000 g during 20 min and the supernatant added to the same flask. The final extract was filtered through a 0.45 µm membrane before dilution (when needed) and derivatization. For derivatization, 50 µL of the extract were added to 200 µL borate buffer (0.2 M boric acid solution adjusted to pH 8.5 with 30% potassium hydroxide solution). After addition of 200 µL (fluorenylmethyloxycarbonyl chloride) FMOC-Cl reagent (3 mM in acetone), the solution was mixed during 5 min at room temperature in a orbital mixer. Then,100 µL of heptylamine reagent (1 mL heptylamine and 5 mL acetonitrile, adjusted to pH 7-8with 60 mL 0.1 M HCl) were added to remove the excess of FMOC-Cl reagent. After 5 min of mixing, 80 µL of the reaction solution were diluted in 320 µL of eluent A, detailed below. Then 20 µL were injected for HPLC analysis. Asparagine was analyzed by HPLC together with other extracted amino acids, separated by gradient elution using a Jasco HPLC system equipped with an intelligent HPLC pump PU-1580, a 4-line degasser DG-1580-54, a quaternary gradient unit LG-1580-04, an intelligent sampler AS-950 and an intelligent fluorescence detector FP-920 (Jasco Analytical Instruments, Easton, Maryland, USA). The HPLC column used was a Waters Spherisorb ODS2 (4.6 mm× 150 mm 3µm) (Waters, Milford, Massachussetts, USA). The mobile phase was a mixture of (A) citrate buffer pH 6 (4.8 g citric acid + 200 µL triethylamine + water to 1000 mL) and (B) methanol (HPLC grade). The gradient elution started with 60% A for 2 min, changing to 50% A in 3 min , to 40% A at 7 min, 30% A at 20 min, 0% A at 23 min,

10% A at 25 min and finally returning to 60% A at 28 min and maintained until the end of the run. The injected volume was 20 μL, the flow rate 1.0 mL/min and the total running time 31 min. Asparagine was detected by fluorescemce, at λ_{ex}=260 nm/λ_{em}=310 nm. Asparagine concentration was calculated by reference to calibration curves with a pure standard (Sigma) and expressed as g/kg fresh weight (fw).

Reducing sugars determination through the neocuproine method

As described by Dygert et al. (1965),1 g of food was placed in a Falcon tube and fats are extracted with 20 mL of n-hexane. The sample was homogenized with the Ultra-Turrax, centrifuged at 9000 rpm for 10 min and n-hexane was decanted, 20 mL of 80% ethanol solution was added to the defatted sample and after homogenization; the tubes were placed in a water bath at 80 ° C with constant agitation for 15 min. The tubes were centrifuged at 9000 rpm for 20 min and the supernatant was filtered (and interferences eliminated) through an activated carbon column (20 g of prewashed granulated charcoal) in order to keep the entire sample into contact with the charcoal for 5 min. The filtration flow rate was about 1 drop/sec. After the passage of the entire sample, coal was washed with 2 x 50 mL of hot water (~ 60 °C) followed by 20 mL ethanol. 5 mL of the supernatant was placed in a Falcon tube with 1 mL of 25% HCl and heated in a water bath (100ºC) for 30 min to invert sucrose. The solution was neutralized with a few drops of 25% NaOH and 1 mL of the samples and standard solutions were placed in tubes and 3 ml of copper solution (0.5 L water: 20 g of anhydrous Na_2CO_3; 8 g of glycine; 0.225 g $CuSO_4.5H_2O$) was added, followed by 3 mL of neocuproine solution (0.5 L: 0.60 g of neocuproine dissolved in 100 mL ethanol + 400 mL water; stored in dark glass). After stirring the resulting solutions were placed in a boiling water bath for 15 min and then cooled in running water. Finally, 1 mL of the resulting solution was diluted 10 times and analyzed by UV-Vis spectroscopy at 455 nm. The calibration curve was constructed with a glucose solution prepared in 80% ethanol.

Results and discussion

Meat mitigation results

The ability of green tea marinades and marinades containing alcoholic beverages and aromatic herbs to minimize the formation of HAAs in meat samples was recently reported by Quelhas et al. (2010) and Viegas et al. (2012), respectively. Under the present work, the same grilled samples were also tested for acrylamide in order to evaluate the potential

mitigation effects. The results obtained are presented in Table 3 and Table 4. The herbs chosen are commonly used as meat flavoring (garlic, ginger, thyme, rosemary, red-pepper) in home cooking preparations.

Table 1- Mitigation effects of alcoholic beverages marinades and herbs in acrylamide formation in grilled beef. All samples were prepared in duplicate and analyzed also in duplicate.

Marinade(4h)	AA* levels µg/kg	Effect on AA %
Control	150 ±	
Water	73 ±	- 51
Whitewine	105 ±	- 30
Whitewine and herbs	161 ±	+ 7
Beer	42 ±	- 72
Beer and herbs	62 ±	- 59

*AA: acrylamide

The analysis of the results presented in Table 3 permits to conclude that beer with and without herbs presents the highest mitigation potential for acrylamide in meat samples. A possible explanation on the mitigation effects of beer in acrylamide formation could be the presence of several antioxidants like sulfite, catechin, phenolic acids and ascorbic acid in the drink (Andersen et al., 2000). As previously mentioned, Zhu et al. (2009) tested several extracts of natural antioxidants and 11 phenolic compounds in Asparagine-Glucose model systems. They reported a reduction range from 16 to 60% of acrylamide levels in the presence of the phenolic compounds with catechin alone presenting a reducing effect of 23%. White wine did not show such a large ability to lower acrylamide formation despite of the presence of important levels of quercetin and resveratrol (Stockham et al., 2013). These phenolic compounds also were reported to show a decreasing effect in acrylamide contents (Zhu et al., 2009) with quercetin showing a 38% reduction. A possible explanation could be the presence of relatively higher average amounts of asparagine in white wine comparing with beer (11.96 mg/L in white wine, average value for 14 samples, Gomez-Alonso et al. (2007); to 2.43 mg/L in beer, average value of 4 samples, Kutlan and Molnar-Perl, 2003). White wine asparagine can act as precursor in acrylamide formation in grilled meat since asparagine content in muscle beef is low (13 mg/kg of meat) but glucose has mean levels of 1250 mg/kg of meat and fructose levels of 120 mg/kg of meat (Koutsidis et al., 2008).

During the four hours of marinade, asparagine from wine could be absorbed by the muscle and during grilling contribute to acrylamide formation.

The addition of herbs to the marinade presented unexpected results. The marinade prepared with white wine and herbs apparently shows an increase in acrylamide formation. This may happen due to the presence of ginger in the marinade, a similar effect to the ones reported to gingerbread and ginger cookies that usually present more than 500 μg/kg of acrylamide (Amrein et al., 2004; Kornbrust et al., 2010; Marková et al., 2012). Ginger is known as a natural antioxidant having in its composition several gingerol related compounds and diarylheptanoids (Yanishlieva, et al., 2006). Among these, ginger has curcumin, a potent antioxidant with a strong action against free radicals. Interestingly, curcumin was described by Hamzahoglu et al. (2013) as a potentially contributor to acrylamide formation due to its ability to convert asparagine into acrylamide. According to these authors, the carbonyl groups present in curcumin (Figure 2) can react directly with asparagine in low sugars systems, during the Maillard reaction promoting the formation of acrylamide during heating.

However, one cannot disregard the interesting mitigation effect of water marinated. Here, leaching of precursors is probably the most probable explanation, in particular free aminoacids and reducing sugar.

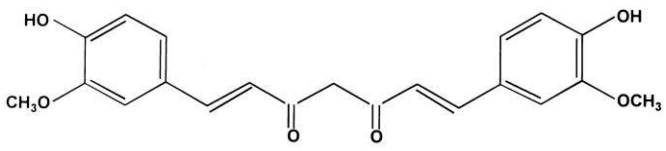

Figure 1- Structure of curcumin

Green tea marinades (Table 4) also show a mitigation potential of around 40 % in meat samples, with low influence from time of marination. The ability of green tea to reduce acrylamide formation maybe related to the natural flavonoids present in tea like catechin and gallocatechingallate (Zhang et al., 2008; Zhu et al., 2009), but the effect of water, as explained before, by a dilution and bleaching effect on acrylamide precursors, could also be of great importance.

Table 2- Mitigation effects of green tea marinades herbs in acrylamide formation in grilled beef

Marinade	AA levels µg/kg	Effect on AA %
Control	511 ±	
Tea2h	300±	- 41
Tea 4 h	277 ±	- 46
Tea 6 h	342 ±	- 33

Overall, marinating protein-rich raw food samples seems to be an interesting mitigation strategy for acrylamide formation in home-prepared meat foods.

Potato mitigation results

In this work, five common potato varieties were collected in local markets and fried in controlled conditions in 1 liter of peanut oil at 180ºC during different time periods. Reducing sugars, fructose + glucose and sucrose were determined using the neocuproine assay. Asparagine was determined using HPLC-FLD with FMOC-Cl as derivatization reagent. The results obtained are presented in Table 1.

Table 3- Concentrations of sugars and free asparagine in different potato varieties in mg/kg per fresh weight

Potato varieties	Glucose + frutose	Sucrose	Asparagine
Agria	840 ±	510	1390
Monalisa	1720	2560	1450
Marabel	995	1920	1150
Asterix	1978	2089	2090
Rodeo	1130	720	1520

The results obtained for the analysis of asparagine and reducing sugars are somewhat comparable with the ones reported by Amrein et al. (2003) that analyzed 17 potato varieties during 2 growing seasons, having found free asparagine at concentrations between 1400 and 5170 mg/kg per fresh weight, being generally more abundant than sugars. Glucose concentrations ranged from 40 to 2700 mg/kg and fructose values usually were lower than glucose. Sucrose concentrations ranged from 160 to 1800 mg/kg. Total reducing sugars and total free amino acids can vary considerably between different seasons, and storage temperature has a strong impact on the sugar content as reported by several authors (Amrein et al., 2003; Zhu et al., 2010; Vivanti et al., 2006).

Potatoes fried at constant temperature and different time periods were tested for acrylamide. The results for acrylamide levels are presented in Table 2 and Figure 1.

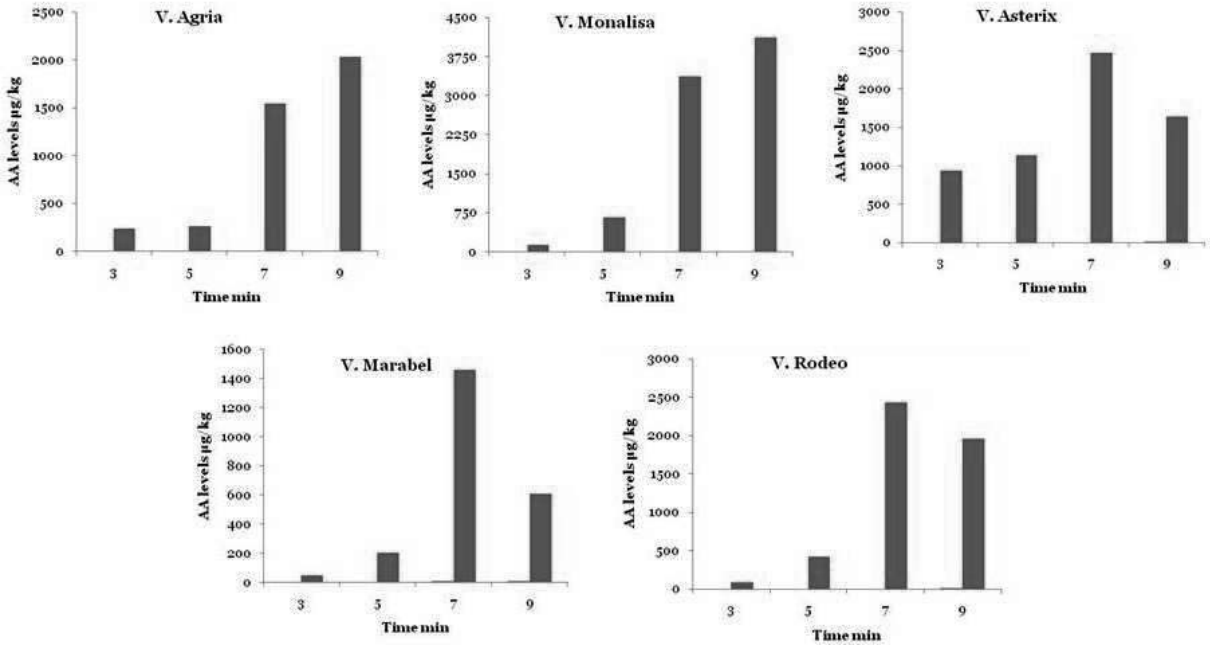

Figure 2- Acrylamide formation in different potato varieties at 180 ºC during 3, 5, 7 and 9 min.

Monalisa and Asterix varieties fried potatoes presented a brown coloration rather sharply after the first 5 min of frying, being still raw and hard in the inside. The other varieties presented adequate color and evolution during frying. Agria and Marabel were the varieties that presented the lowest quantities of acrylamide in the first minutes of processing. For longer periods the differences were reduced, except

for Marabel that presented the lowest acrylamide content, and Monalisa, with the highest.

Reducing sugars are known to be the limiting factor in acrylamide formation in potato products. Thus the choice for a variety with low concentrations of these precursors is important to reduce the formation of acrylamide in fried potatoes (Shojaee-Aliabadi et al., 2013). The correlation between reducing sugars / acrylamide and asparagine / acrylamide after 5 min of frying are illustrated in Figure 2. As expected a higher correlation (r= 0.983) was found between the amount of reducing sugars and acrylamide. There is also a good correlation between asparagine content *vs* acrylamide and total sugars *vs* acrylamide in our samples, with r=0.878 and r= 0.870, respectively. Sucrose alone *vs* acrylamide showed the least correlation, which was also expected taking into account the non-reducing properties of sucrose.

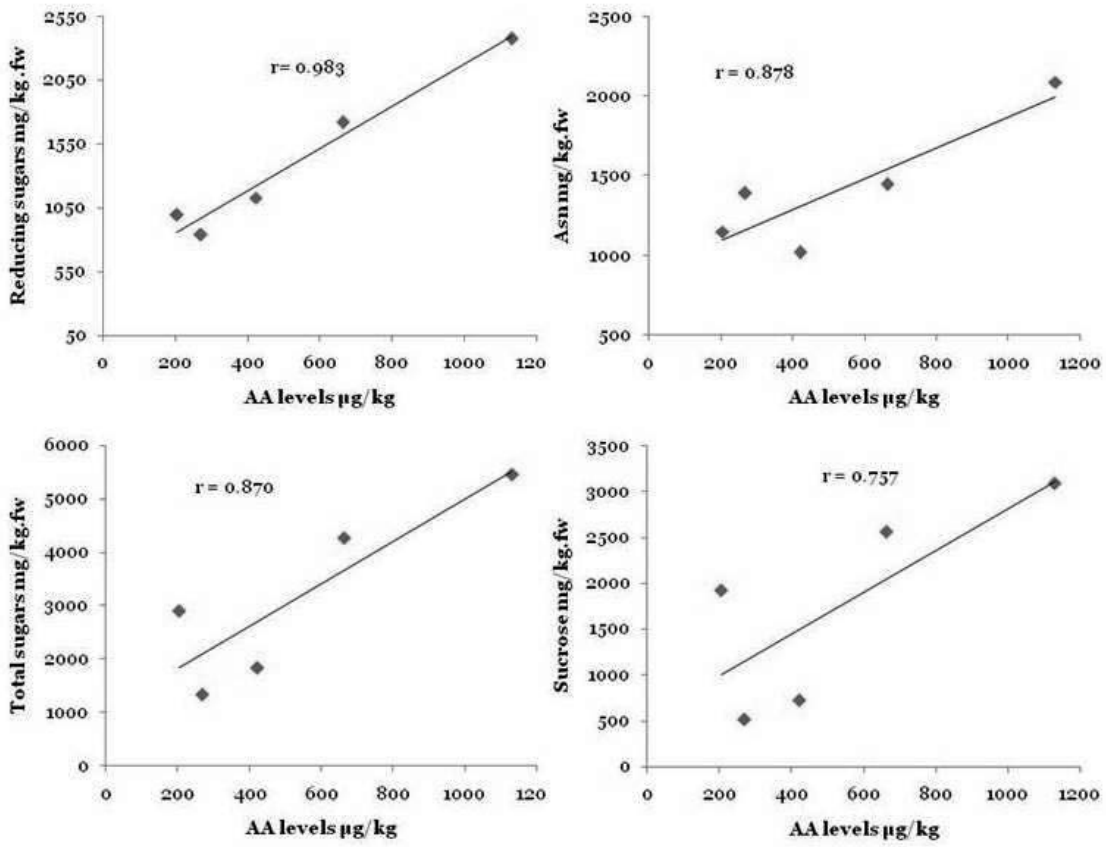

Figure 3- Correlation between acrylamide and precursors: asparagine, reducing sugars and sucrose.

The results obtained are in agreement with earlier findings. De Wilde et al. (2006), e.g., reported that acrylamide levels in fried potatoes derived from 16 different varieties correlated to reducing sugar content of the potatoes (R^2 = 0.82, n = 96). Zhu et al. (2010) also found a positive correlation between reducing sugars and acrylamide formation during heatingin 16 different commercial varieties of potato from 8 different countries. The same authors also described a great variation of reducing sugars and asparagine in the same potato variety acquired in different places and time periods. Shojaee-Aliabadi et al. (2013) tested 3 potato varieties for precursor's content and concluded that the varieties with lower reducing sugars content originated lower acrylamide levels in processed potatoes.

The results obtained herein, as well as the cited reports suggest that to minimize acrylamide content in fried potatoes, the consumer should select from all the commercially available potato varieties those which have lower reducing sugar content or the producers should indicated more often the varieties that are more adequate for frying. Agria, and Marabel varieties studied had lower levels of both acrylamide precursors and could be a good choice to prepare meals at home.

Concerning pre-fried potatoes, the results obtained for acrylamide presence are presented in Figure 1.

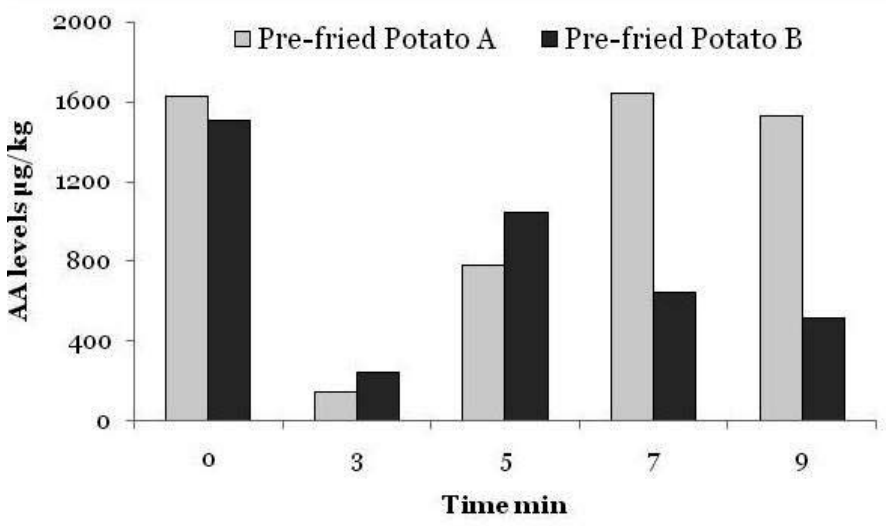

Figure 4- Acrylamide levels in pre-fried potatoes

Acrylamide analysis before frying shows that there is already a big quantity of acrylamide in the pre-fried samples. During the first minutes of frying a huge reduction of

the compound is noticed probably due to the evaporation of the water present in the frozen potatoes or drainage into de oil. Gokmen and Palazoglu (2009) verified the same behavior and suggested that a small part of acrylamide is lost during water evaporation under frying conditions. In case of frozen pre-fried potatoes probably this effect is higher because of all the frozen water present in the samples evaporate in the first minutes of heating. The potatoes were ready after 5 min of frying presenting a pale yellow coloration but still with a lot of residual water. The time range of 5 to 7 min seems to be the best compromise between crispy fried potatoes and lower acrylamide contents, but the results might be highly variable between samples, indicating again that the variety of potato used and pre-frying conditions, might have an important influence on acrylamide formation.

Conclusions

Acrylamide levels in home prepared food can be effectively reduced by adopting simple actions like marinating protein-rich food with beer and beer with herbs and spices. Green tea also shows a mitigation effect on acrylamide. Some care must be taken however when choosing the herbs and potential antioxidants to add flavor to food. As shown by the results, the effect of natural antioxidants maybe sometimes contradictory. Regarding the selection of potatoes for frying, varieties with low reducing sugars levels must be chosen in order to have lower acrylamide contents when frying potatoes at home. From the five varieties studied, Agria, and Marabel shown to be the best choices. The combined effect of selection of the most suitable potato varieties to fry with the effect of marinate meat before roasting, can significantly contribute to reducing acrylamide intake from home cooked food.

References

Agroportal, 2015. Available at: http://www.agroportal.pt/a/2013/tpetulante.htm

Ahn JS, Castle L, Clarke DB, Lloyd AS, Philo MR, Speck DR. Verification of the findings of acrylamide in heated foods. Food Addit Contam 2002; 19 (12): 1116-1124.

Amrein TM, Bachmann S, Noti A, Biedermann M, Barbosa MF, Biedermann-Brem S, et al. Potential of acrylamide formation, sugars, and free asparagine in potatoes: a comparison of cultivars and farming systems. J Agr Food Chem 2003; 51: 5556–5560.

Amrein TM, Schonbachler B, Escher F, Amado R. Acrylamide in Gingerbread: Critical Factors for Formation and Possible Ways for Reduction. J Agric Food Chem 2004; 52: 4282-4288.

Andersen ML, Outtrup H, Skibsted LH. Potential antioxidants in beer assessed by ESR spin trapping. J Agr Food Chem 2000; 48 (8): 3106-3111.

Bassama J, Brat P, Bohuon P, Boulanger R, Günata Z. Study of acrylamide mitigation in model system: Effect of pure phenolic compounds. Food Chem 2010; 123 (2): 558-562.

Becalski A, Lau B, Lewis D, Seaman S, Hayward S, Sahagian M, et al. Acrylamide in French fries: influence of free amino acids and sugars. J Agr Food Chem 2004; 52 (12): 3801-3806.

Becalski A, Lau BPY, Lewis D, Seaman SW. Acrylamide in foods: Occurrence, sources, and modeling. J Agr Food Chem2003; 51: 802–808.

Becalski A, Stadler R, Hayward S, Kotello S, Krakalovich T, Lau BP-Y, et al. Antioxidant capacity of potato chips and snapshot trends in acrylamide content in potato chips and cereals on the Canadian market. Food Addit Contam 2010; 27(9): 1193-1198.

Capuano E, Oliviero T, Açar O, Gökmen V, Fogliano V. Lipid oxidation promotes acrylamide formation in fat-rich model systems. Food Res Int 2010; 43 (4): 1021-1026.

Claeys WL, De Vleeschouwer Kl, Hendrickx ME. Effect of amino acids on acrylamide formation and elimination kinetics. Biotechnol Progr 2005; 21 (5): 1525-1530.

Cook DJ, Taylor AJ. On-line MS/MS monitoring of acrylamide generation in potato- and cereal-based systems. J Agr Food Chem 2005; 53: 8926–8933.

De Wilde T, De Meulenaer B, Mestdagh F, Govaert Y, Ooghe W, Fraselle S, et al. Selection criteria for potato tubers to minimize acrylamide formation during frying. J Agr Food Chem2006; 54(6): 2199-2205.

Dygert S, Li LH, Florida D, Thoma JA. Determination of reducing sugar with improved precision. Anal Biochem 1965; 13(3):367-374.

Ehling S, Shibamoto T. Correlation of acrylamide generation in thermally processed model systems of asparagine and glucose with color formation, amounts of pyrazines formed, and antioxidative properties of extracts. J Agr Food Chem 2005; 53 (12): 4813-4819.

FDE (2011). Acrylamide toolbox 2011. http://www.fooddrinkeurope.eu/uploads/publications_documents/Toolboxfinal260911.pdf.

Fernandes JO, Soares C. Application of matrix solid-phase dispersion in the determination of acrylamide in potato chips. J Chromatogr A 2007; 1175:1-6.

Friedman M. Chemistry, biochemistry, and safety of acrylamide. A review. J Agr Food Chem 2003; 51 (16): 4504-4526.

Gökmen V, Palazoğlu TK. Measurement of evaporated acrylamide during frying of potatoes: Effect of frying conditions and surface area-to-volume ratio. J Food Eng 2009; 93 (2): 172-176.

Gökmen V, Senyuva HZ. Acrylamide formation is prevented by divalent cations during the Maillard reaction. Food Chem 2007; 103: 196–203.

Gómez-Alonso S, Hermosín-Gutiérrez I, García-Romero E. Simultaneous HPLC analysis of biogenic amines, amino acids, and ammonium ion as aminoenone derivatives in wine and beer samples. J Agr Food Chem 2007; 55 (3): 608-613.

Hamzalıoğlu A, Mogol BA, LumagaRB, Fogliano V, Gökmen V. Role of curcumin in the conversion of asparagine into acrylamide during heating. Amino acids 2013; 44 (6): 1419-1426.

Hedegaard RV, Granby K, Frandsen H, Thygesen J, Skibsted LH. Acrylamide in bread. Effect of prooxidants and antioxidants. Eur Food Res Technol 2008; 227: 519–525.

Jin C, Wu X, Zhang Y. Relationship between antioxidants and acrylamide formation: A review. Food Res Int 2013; 51 (2): 611–620.

Kornbrust BA, Stringer MA, Lange NK, Hendriksen HV, Whitehurst RJ, van Oort M. Asparaginase-an enzyme for acrylamide reduction in food products. In: Whitehurst RJ, van Oort M.Enzymes in food technology 2nd Ed. Chichester: John Wiley and Sons; 2010. p. 59-87.

Kotsiou K, Tasioula-Margari M, Kukurová K, Ciesarová Z. Impact of oregano and virgin olive oil phenolic compounds on acrylamide content in a model system and fresh potatoes. Food Chem 2010; 123(4): 1149-1155.

Koutsidis G, Elmore JS, Oruna-Concha MJ, Campo MM, Wood JD, Mottram DS. Water-soluble precursors of beef flavour: I. Effect of diet and breed. Meat Sci 2008; 79 (1): 124-130.

Kutlán D, Molnár-Perl I. New aspects of the simultaneous analysis of amino acids and amines as their o-phthaldialdehyde derivatives by high-performance liquid chromatography: Analysis of wine, beer and vinegar. J Chromat A2003;987(1): 311-322.

Levine RA, Smith RE. Sources of variability of acrylamide levels in a cracker model. J Agr Food Chem 2005; 53 (11): 4410-4416.

Lopes C, Oliveira A, Santos AC, Ramos E, Gaio AR, Severo M, Barros H. Food Consumption in Porto. Faculdade de Medicina da Universidade do Porto 2006.

Marková L, Ciesarová Z, Kukurová K, Zieliński H, Przygodzka M, Bednáriková A, et al. Influence of various spices on acrylamide content in buckwheat ginger cakes. Chem Pap 2012; 66 (10): 949-954.

Mestdagh F, Maertens J, Cucu T, Delporte K, Van Peteghem C, De Meulenaer B. Impact of additives to lower the formation of acrylamide in a potato model system through pH reduction and other mechanisms. Food Chem 2008; 107: 26–31.

Mottram D, Wedzicha B, Dodson A. Food chemistry: Acrylamide is formed in the Maillard reaction. Nature 2002; 419: 448-449.

Quelhas I, Petisca C, Viegas O, Melo A, Pinho O, Ferreira IMPLVO. Effect of green tea marinades on the formation of heterocyclic aromatic amines and sensory quality of pan-fried beef. Food Chem 2010; 122: 98–104.

Rosén J, Hellenäs KE. Analysis of acrylamide in cooked foods by liquid chromatography tandem mass spectrometry. Analyst 2002; 127 (7): 880–882.

Rydberg P, Eriksson S, Tareke E, Karlsson P, Ehrenberg L, Törnqvist M. Factors that influence the acrylamide content of heated foods. In Chemistry and safety of acrylamide in food, pp. 317-328. Springer US, 2005.

Serpen A, Gökmen V. Evaluation of the Maillard reaction in potato crisps by acrylamide, antioxidant capacity and color. J Food Compos Anal 2009; 22 (6): 589-595.

Shojaee-Aliabadi S, Nikoopour H, Kobarfard F, Parsapour M, Moslehishad M, Hassanabadi H, et al. Acrylamide reduction in potato chips by selection of potato variety grown in Iran and processing conditions. J Sci Food Agr 2013; 93 (10): 2556-61.

Soares C, Fernandes J.O. MSPD Method to Determine Acrylamide in Food. Food Anal Method 2009; 2 (3): 197-203.

Soares C, Fernandes J.O. Screening of acrylamide in selected Portuguese foodstuffs and estimation of the dietary intake of the compound by the adult population of Porto, 2015.

Soares CM, Alves RC, Casal S, Oliveira MB, Fernandes JO. Validation of a Matrix Solid-Phase Dispersion method to determine acrylamide in coffee and coffee surrogates. J Food Sci 2010; 75 (3): T57-63.

Stockham K, Sheard A, Paimin R, Buddhadasa S, Duong S, Orbell JD, et al. Comparative studies on the antioxidant properties and polyphenolic content of wine from different growing regions and vintages, a pilot study to investigate chemical markers for climate change. Food Chem 2013; 140: 500-506.

Urbancic A, Kolar MH, Dimitrijevic D, Demsar L, Vidrih R. Stabilisation of sunflower oil and reduction of acrylamide formation of potato with rosemary extract during deep-fat frying. LWT - Food Sci Technol 2014; 57: 671–678.

Viegas O, Amaro LF, Ferreira IMPLVO, Pinho O. Inhibitory Effect of Antioxidant-Rich Marinades on the Formation of Heterocyclic Aromatic Amines in Pan-Fried Beef. J Agr Food Chem 2012; 60 (24): 6235-40.

Vivanti V, Finotti E, Friedman M. Level of acrylamide precursor's asparagine, fructose, glucose, and sucrose in potatoes sold at retail in Italy and the United States. Food Chem Toxicol 2006; 71: C81–C85.

Williams JSE. Influence of variety and processing conditions on acrylamide levels in fried potato crisps. Food Chem 2005; 90 (4): 875-881.

Yanishlieva NV, Marinova E, Pokorný J. Natural antioxidants from herbs and spices. Eur J Lip SciTechnol 2006; 108 (9): 776-793.

Zamora R, Delgado RM, Hidalgo FJ. Model reactions of acrylamide with selected amino compounds. J Agr Food Chem 2010; 58: 1708–1713.

Zamora R, Hidalgo FJ. Contribution of lipid oxidation products to acrylamide formation in model systems. J Agr Food Chem2008; 56:6075–6080.

Zeng X, Cheng KW, Jiang Y, Lin ZX, Shi JJ, Ou SY. Inhibition of acrylamide formation by vitamins in model reactions and fried potato strips. Food Chem 2009; 116: 34–39.

Zhang Y, Xu W, Wu X, Zhang X, Zhang Z. Addition of antioxidant from bamboo leaves as an effective way to reduce the formation of acrylamide in fried chicken wings. Food Addit Contam 2007; 24 (3): 242–251.

Zhang Y, Ying TJ, Zhang Y. Reduction of acrylamide and its kinetics by addition of antioxidant of bamboo leaves (AOB) and extract of green tea (EGT) in asparagine–glucose microwave heating system. J Food Sci 2008; 73 (2): 60–66.

Zhang Y, Zhang Y. (). Formation and reduction of acrylamide in Maillard reaction: A review based on the current state of knowledge. Crit Rev Food Sci 2007; 47: 521–542.

Zhang Y, Zhang Y. Effect of natural antioxidants on kinetic behavior of acrylamide formation and elimination in low-moisture asparagine–glucose model system. J Food Eng 2008; 85 (1): 105–115.

Zhu F, Cai Y-Z, Ke J, Corke H. Compositions of phenolic compounds, amino acids and reducing sugars in commercial potato varieties and their effects on acrylamide formation. J Sci Food Agric 2010; 90: 2254–2262.

Zhu F, Cai Y-Z, Ke J, Corke H. Evaluation of the effect of plant extracts and phenolic compounds on reduction of acrylamide in an asparagine/glucose model system by RP-HPLC-DAD. J Sci Food Agric 2009; 89: 1674–1681.

Zyzak, D. V., Sanders, R. A., Stojanovic, M., Tallmadge, D. H., Eberhart, B. L., Ewald, D. K., ... & Villagran, M. D. (2003). Acrylamide formation mechanism in heated foods. *Journal of agricultural and food chemistry*, *51*(16), 4782-4787.

Acknowledgements

The authors are thankful to FCT for financial support in the framework of the project POCI/AGR/61543/2004 and Cristina Soares PhD grant (SFRH/BD/39360/2007). This work has been supported by Fundação para a Ciência e a Tecnologia through PEst-C/EQB/LA0006/2011.

CHAPTER 5

Estimation of Acrylamide Dietary Intake by a Portuguese Population

5.1- Estimation of acrylamide dietary intake in a Portuguese adult sub-sample based on selected Portuguese foodstuffs

Abstract

Average intake of acrylamide was estimated in the adult population (n=2398; ages between 18 to 92 years old) of Porto, Portugal, based on a screening assay of acrylamide levels in targeted Portuguese foods, cooked at home or from industrial origin, and a published dietary questionnaire elaborated under the framework of EpiPorto project. Acrylamide content in the selected thermal processed foods was assessed by a matrix solid-phase dispersive (MSPD) extraction followed by Gas Chromatography-Mass Spectrometry (GC-MS) analysis. Average acrylamide content in foods ranged between 13 to 810 μg per kg, being the highest amounts present in fried potatoes and coffee surrogates. An estimated average daily intake for the adult sub-sample of Porto population of 41. 5 μg/day was calculated, ranging from 0.60 to 0.70 μg/kg of body weight for women and men, respectively. The main sources of acrylamide consumption were potato products (36%), meat (25%) and bread (12%), while coffee showed less importance than usually reported in similar studies, due to low levels of consumption reported.

Keywords: acrylamide, MSPD, GC/MS, foodstuffs, Portugal, Porto, dietary intake, screening, EpiPorto project

Estimation of acrylamide dietary intake in a Portuguese adult sub-sample based on selected Portuguese foodstuffs

Soares C.*, Fernandes J.O.

REQUIMTE, Laboratory of Bromatology and Hydrology, Faculty of Pharmacy, University of Porto, Rua Jorge de Viterbo Ferreira 228, 4050-313, Porto, Portugal

*Corresponding author e-mail: cristina.md.soares@gmail.com

Abstract

Average intake of acrylamide was estimated in the adult population (n=2398; ages between 18 to 92 years old) of Porto, Portugal, based on a screening assay of acrylamide levels in targeted Portuguese foods, cooked at home or from industrial origin, and a published dietary questionnaire elaborated under the framework of EpiPorto project. Acrylamide content in the selected thermal processed foods was assessed by a matrix solid-phase dispersive (MSPD) extraction followed by Gas Chromatography-Mass Spectrometry (GC-MS) analysis. Average acrylamide content in foods ranged between 13 to 810 μg per kg, being the highest amounts present in fried potatoes and coffee surrogates. An estimated average daily intake for the adult sub-sample of Porto population of 41. 5 μg/day was calculated, ranging from 0.60 to 0.70 μg/kg of body weight for women and men, respectively. The main sources of acrylamide consumption were potato products (36%), meat (25%) and bread (12%), while coffee showed less importance than usually reported in similar studies, due to low levels of consumption reported.

Keywords: acrylamide, MSPD, GC/MS, foodstuffs, Portugal, Porto, dietary intake, screening, EpiPorto project

Introduction

Acrylamide content in food has been the focus of many studies all over the world, after its discovery in foods, in 2002 (Tareke et al., 2002). Acrylamide is formed in foods subjected to thermal processing at temperatures above 120 °C in the presence of minute amounts of water, and its content tends to increase with temperature and heating time. In most foodstuffs, particularly potatoes and cereals processed at high temperatures, the main route for its formation is the Maillard reaction, taking as a starting point the reaction between asparagine and reducing sugars, and/or reactive carbonyls products. This proposal of formation pathway is consistent with the fact that most cooked contaminated foods present in the raw state high levels of free asparagine and reducing sugars (Stadler et al., 2002; Mottram et al., 2002).

Higher acrylamide levels are usually reported for carbohydrate-rich foods, such as fried/roasted potatoes, bakery cereal products (bread, crackers, biscuits, crispbread, breakfast cereals, etc.) and coffee and coffee surrogates (EFSA, 2012). Moderate amounts of acrylamide were also reported for protein-rich foods, such as meat, fish and seafood, particularly when roasted or grilled. In boiling foods usually a lacking of acrylamide is observed, which is due to the high moisture content that avoid reaction. Most of the acrylamide rich foods have a high consumption pattern worldwide and therefore acrylamide intake can be significant. In a recent European study aiming to describe the mean dietary intake in 27 centers of 10 countries in Europe, it was reported that the two major contributors to acrylamide intake were bread and coffee, which usually contribute with more than 50% of total dietary acrylamide intake (over 70 % in Denmark and Norway), followed by French fries and potato crisps (15-25%) (Freisling et al., 2013), results that weresimilar to those obtained in other European studies (EFSA, 2012).The average dietary intake varied from 15,3µg/day in Navarra, Spain, up to 41 µg/day in the Danish center of Aarhus,for women. For men, the distribution was consistent, with mean dietary intake from 15,5µg/day up to 48 µg/day in the same centers. These geographical differences are related to the dietary habits the population, highly different between Southern and Northern European countries.

Data concerning acrylamide levels in Portuguese foodstuffs are scarce. Our selves have published reports about the content of acrylamide in 17 commercial samples of French fries collected from local retailers (Fernandes and Soares, 2007), and 39 samples of a variety of food matrices namely processed cereal products (bread, toasts, breakfast cereals, snacks, cookies and biscuits), chocolates and baby-foods (Soares and Fernandes, 2009). In this study, a total of 250 samples of representative type of foods were analyzed for their

acrylamide contents, and mean levels of each food group were usedtogether with the results about consumption habits of Porto adult population (Lopes et al, 2006) to estimate the daily intake of acrylamide in the referred population and the corresponding contribution of each food item.

Experimental methodology

Material and Methods

Chemicals. Acrylamide (AA) of 99% purity grade was acquired from Aldrich (Steinheim, Germany). Acrylamide 1,2,3-C13 ($^{13}C_3$-AA, IS) 99% in methanol was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The ISOLUTE C_{18}/Multimode (1g/1g) layered SPE columns were from Biotage (Uppsala, Sweden). The bulk C_{18} sorbent 125 Å, 55-105 μm was from Waters (Milford, USA). Both SPE columns and bulk C_{18} were conditioned with methanol and water and used without drying. The n-hexane and ethyl-acetate were of pesticide residue analysis grade and acetonitrile was of ultrapure grade, all from Fluka. The potassium bromide for IR spectroscopy grade and the bromine analytical grade were from Merck (Darmstad, Germany). Analytical grade sodium chloride was from J.T. Baker (Deventer, Holland). Hydrobromic acid 48% and sodium tiosulphate volumetric solution 1 mol/L were from Riedel-de Häen (Seelze, Germany). *n*-Hexane and ethyl acetate (pesticide residue analysis grade) and acetonitrile (ultrapure grade), were from Fluka. Potassium bromide (IR spectroscopy grade) and bromine (analytical grade) were from Merck. Sodium chloride (analytical grade) was from J.T. Baker (Deventer, The Netherlands). Potassium hexacyanoferrate (II) trihydrate, zinc sulphate heptahydrate, sodium sulphate anhydrous and acetic acid (glacial) were obtained from Panreac (Barcelona, Spain). Hydrobromic acid 48% and sodium tiosulphate volumetric solution 1 mol/L were from Riedel-de Häen (Seelze, Germany). The MSPD empty columns were made of polyethylene with a reservoir of 25 ml, and a single frit with a pore size of 10 _m were acquired from Symta (Madrid, Spain)

Equipment. GC/MS analyses were performed in a gas chromatograph, model HP GC-6890, split-splitless injector, coupled to a Mass Selective Detector model Agilent MSD-5973N (Agilent, Palo Alto, CA, USA). The analytical separation was performed in a capillary column MDN-12 (30 mm x 0.25 mm x 0.25 μm DI) from Supelco (Steinheim, Alemanha). The centrifugations were made in a Heraeus Sepatek, model Labofuge Ae (Osterode, Germany) at 3000 g. The MSPD extractions were made in a vacuum manifold model Visiprep Solid Phase Extraction Manifold from Supelco (Taufkirchen, Germany) with capacity for 12

columns. Evaporation under a stream of nitrogen was carried out on a Pierce, model Reacti-therm 18790 (Rockford, IL, USA) with capacity for 9 vials.

GC/MS operating conditions. Gas chromatography. Carrier gas: helium (constant flow at 1 mL minute^{-1}). Sample injection volume: 1 μL (splitless, pulsed pressure 32 psi, 60 sec). Injector temperature: 280ºC. Oven temperature: 85ºC (1 minute), 15ºC minute^{-1} to 280ºC, hold 10 minutes (24 minutes), transfer line, 280 ºC. Mass-spectrometry. Electron energy, 70 eV (EI mode). Mode of acquisition: selected ion monitoring (SIM), ions m/z 106, 150 and 152 for 2,3-dibromopropionamide (2,3-DBPA) and ions m/z 110, 153, 155 for 2,3-$^{13}C_3$-dibromopropionamide (2,3-$^{13}C_3$-DBPA). The ions m/z 150 for 2,3-DBPA, m/z 155 for 2,3-DBPA($^{13}C_3$) were used for quantification and the others for confirmation. AA was determined with the internal standard, using the ratio of peak area of 2,3-DBPA to 2,3-$^{13}C_3$-DBPA. The identity of the peak was confirmed by retention time and by comparing the relative abundance ratios of the confirmatory ions to those of the standard solution.

Samples

A total of 250 food samples were acquired in local supermarkets between 2008 and 2011 (Table 1). Homemade dishes were prepared with commercially available products and based on traditional recipes (Table 1) using typical household setting. All samples were grinded and stored at -20 ºC until acrylamide analysis.

Acrylamide analysis

Acrylamide quantification was performed according to previous reports based on Matrix Solid Phase Extraction (MSPD) and GC-MS. Briefly, for potato products, 0.5 g of sample were mixed with 2 g of $C_{18}$and internal standard (I.S.) $^3C_{13}$-AA.Fats were removed with n-hexane and acrylamide extracted with water after maceration and centrifugation (Fernandes and Soares, 2007). For products derived from cereals and chocolate, sample and C_{18} amounts were doubled, following the same extraction procedure (Soares and Fernandes, 2009) Coffee and coffee substitutes samples were prepared using 0.5 g or 2.5 mL for ground coffee or coffee brew, respectively, being dispersed in 2 g of C_{18} with I.S. and the mixture placed on the top of SPE columns prepared with Multi Mode and ISOLUTE C_{18} where acrylamide was further extracted with water (Soares et al. 2010).

All aqueous extracts were derivatized with bromine and acrylamide derivative was extracted with ethyl acetate/n-hexane (4:1). The solvent was evaporated under a gentle nitrogen

stream (40ºC) and the resulting sample extracts were reconstituted in 0,5 ml of the same solvent mixture and analyzed by GC-MS in SIM Mode.

Meat and fish samples were prepared using a liquid extraction method also described previously (Soares and Fernandes, 2007). Briefly, 2 g of meat or fish samples were placed in a centrifuge tube with 20 mL of water and homogenized by vortexing. Internal standardwas added ($^3C_{13}$-AA solution) and placed in a water bath at 65 °C for 20 minutes. Upon cooling, 0.15 mL of CH_3COOH were added and 1 mL of each Carrez I and Carrez II solutions. Samples were placed at -20 ° C for 20 min and centrifuged at 9000 rpm for 15 min at 4 ° C. The supernatant was filtered through polyethylene reservoirs of 25 ml, and a single frit with a pore size of 10 μm acquired from Symta (Madrid, Spain) and the residue was further extracted with 10 mL of water. After homogenization and centrifugation (conditions above) supernatants were combined, filtered, and reduced to about 5 mL on the vacuum evaporator at 55ºC and the resulting solutions derivatized with bromine. After extracting the derivative with ethyl acetate/n-hexane and evaporation under a nitrogen stream, samples were analyzed by GC-MS in SIM mode as described above.

Food consumption data

Food consumption data was taken from a published food consumption report (Lopes et al, 2006), developed within the EpiPorto project, a community study with the overall aim to asses determinants of health in the adult population of Porto developed by the Department of Hygiene and Epidemiology, Faculty of Medicine from the University of Porto. Under the framework of this project,information on food consumption were collected in a report, using a semi-quantitative food frequency questionnaire previously validated, for the periodof 12 months prior to the interview and available at: http://higiene.med.up.pt/freq.php. The published report aimed to describe food consumption habits and nutritional intake of individuals of Portuguese nationality, residents in Porto city, with age over 18 years, and included 2398 subjects, 1477 women (61.6%) and 921 men (38.4%), grouped in 4 different clusters according to the age. Evaluation of questionnaires were performed between January 1999 and December 2003. Food frequency intakes are available for several food groups, sex, and group ages.

Dietary exposure calculations

To estimate the acrylamide exposure of the total adult population of Porto, for each age group and gender, the reported average value of food consumption for each item was

multiplied by a calculated value of acrylamide mean level obtained in the present study (Eq. (1)). Intakes from all sources of exposure were then summed and taken as the average intake per day.

$$AAintake(\mu g/day) = \frac{AAlevels(\mu g/kg\ fooditem) * fooditemconsumption\ (g/day)}{1000}$$

Eq.(1)- Calculation for the acrylamide average intake per day

To express the acrylamide daily intake in μg/kg of body weight per daythe results obtained from Eq.(1) were divided by the average body weight for the Portuguese population, 69 kg, obtained from the Special Eurobarometer for Health and Food nº 246 (EC, 2006). Dietary intakes were reported as meanand at 25th, 50th, 90th and 95thpercentiles of AA intake per kg of body weight per day. Food categories that contributed most to acrylamide exposure were identified and the distribution for each one of these foods was assessed by estimating the acrylamide intake by gender and age group for the analyzed population. Excel was used for data analysis.

Results and Discussion

Acrylamide levels in foodstuffs. Acrylamide levels in foodstuffs were obtained using validated methodologies previously reported (Fernandes and Soares, 2007; Soares and Fernandes, 2009 and Soares et al. 2010). Food groups evaluated in our study were chosen taking into account the expectations regarding acrylamide presence, based on published data from other countries. Potato products, bread, breakfast cereals, chocolates, cookies, coffee and coffee surrogates were collected in supermarkets in the Porto city and some homemade protein rich meals like grilled beef, pork and fish were also prepared and analyzed in this study. The results obtained for the screening of acrylamide levels present in selected Portuguese foodstuff are presented in Table 1.

Table 4- Acrylamide levels in selected Portuguese foodstuff in μg/kg (n=250)

Food groups	n	Acrylamide concentration μg/kg

		Range	Median	Mean
		Commercial foods		
		Potatoes		
Potato chips	28	124.9 - 1452.3	440.6	501.0
Frenchfries	9	203.9 - 937.4	531.5	542.0
Friedpotato snacks	6	188.0 - 1170.0	480.8	601.5
		Cereals		
Bread	8	n.d.- 85.9	40.0	41.8
Fried cereal snacks	12	27.0 - 283.0	97.5	109.7
Pastry	27	25.8 - 115.4	59.8	65.6
Breakfeast cereals	13	n.d.- 539.0	63.0	122.4
Cookies	8	49.0 - 996.0	320.0	393.1
		Chocolates		
Dark chocolate	11	44.0 - 100.0	105.0	111.8
Milk chocolate	17	14.0 - 86.0	39.0	42.4
White chocolate	2	12.0 - 20.0	16.0	16.0
Soluble chocolate	2	37.0 - 94.2	65.6	65.6
		Baby food		
Baby cereals	5	27.0 - 205.9	49.0	92.4
Baby porridge	2	25.0 - 46.0	35.5	35.5
Baby crackers	4	299.0 - 529.1	429.0	421.5
		Coffee and coffe esubstitutes		
Arabicacoffee	6	73.3 - 131.8	84.3	94.0
Robusta coffee	2	147.0 - 210.0	178.5	178.5
Robusta and arábica coffee	17	81.3 - 246.0	157.7	155.5
Decaffeinated coffee	5	60.0 - 237.0	98.0	121.1
Torrefacto and natural coffee	7	64.3 - 215.0	139.5	138.6
Instant coffee	6	118.0 - 363.1	295.5	274.2
Decaffeinated instant coffee	2	382.3 - 475.8	429.0	429.0
Roasted cereals with coffee	6	327.1 - 1256.9	841.0	810.5
Roasted cereals	4	237.0 - 685.2	376.5	418.8
Cappuccino	2	32.0 - 40.0	36.0	36.0

		Meat and fish samples		
Smoked chorizo	2	102.0 - 862.0	482.0	482.0
Bacon	1	n.d.	n.d.	n.d.
Smoked fish	1	13.0	13.0	13.0
Blood sausage	2	26.0 - 31.0	28.5	28.5
		Miscellaneous		
Instant soup	2	45.0 - 46.0	45.5	45.5
Ground cereals	2	n.d.	n.d.	n.d.
Popcorns	3	109.5 - 445.9	329.5	303.6
Cereal bar	1	29.0	29.0	29.0
		Home-madefood		
Roasted pork meat*	2	62.0 - 70.0	66.0	66.0
Baked potatoes*	2	59.0 - 63.0	61.0	61.0
Roast gravy*	1	111.0	111.0	111.0
Grilled steak	10	55.0 -213.2	104.5	108.1
Barbecue chicken	2	80.8 - 122,2	101.5	101.5
Grilled salmon	4	47.3 - 85.4	59.5	62.8
Grilled pork belly	1	396.0	396.0	396.0
Boiled beef	1	55.0	55.0	55.0
Patties	2	47.0 - 54.2	50.6	50.6

n.d.- not detected; *The roast marinade was prepared with red wine, salt, garlic, pepper, cayenne pepper, onion, olive oil, clove and rosemary. The meat was covered with this marinade during one hour and then roasted in a oven at 200 ºC during 2 hours along with the marinade (gravy) and potatoes cut in quarters added to the broiler 1 hour later.

Acrylamide mean levels for potato products ranged from 501-602 μg/kg; these values are in agreement to those reported in literature, with values ranging from 338 to 1053 μg/kg (EFSA, 2012; Lee et al., 2013; Normandin et al. 2013).

In cereal products, the higher acrylamide value was found on cookies (mean 393 μg/kg), and the lowest found in white bread (41.8 μg/kg). Similar results have been reported by Senyuva and Gokmen (2005), Mattys et al. (2005) and EFSA (2012), with level ranging from, 108-112 μg/kg, 30-135 and 30-138μg/kg, respectively. Being acrylamide formed by reaction between asparagine and reducing sugars, it is expected to be present in higher

amounts in carbohydrate rich foods like potato and cereal based foodstuff submitted to high temperature processing (Arvanitoyannis and Dionisopoulou, 2014).

Among the chocolates group analyzed, dark chocolates showed higher acrylamide value (mean 119 μg/kg). In general, the values reported were similar to those found by Senyuva and Gokmen (2005) and Mattys et al. (2005) with 57 μg/kg and 108 μg/kg, respectively.

Coffee is known to have considerable amounts of the acrylamide due to the high temperatures achieved during roasting (Arvanitoyannis and Dionisopoulou, 2014). Acrylamide values found in the samples analyzed were widely differing (ranging from 32 to 1257 μg/kg) as usually reported in literature. EFSA (2012) reported average values of 256 μg/kg in roasted coffee and 1350 μg/kg in coffee substitutes. Mattys et al. (2005) found 114 μg/kg in coffee and Arisseto et al. (2007) found 582 μg/kg in instant coffee and 174 μg/kg in roasted coffee from Brazil.

The large variation between individual foods items within each group, particularly potato chips (125 - 1452 μg/kg), cookies (49 - 996 μg/kg), and coffee substitutes (327 - 1257 μg/kg) might be explained by the different techniques of industrial preparation of the products (Freisling et al., 2013).

Data about acrylamide presence in protein rich foods is scarce and only a few authors have tested beef and fish samples. Some authors reported fairly high amounts of acrylamide in beef samples subjects to frying and grilling (Kaplan et al., 2009) showing that acrylamide can also be formed in food matrices with low concentrations of asparagine and reducing sugars (Arvanitoyannis and Dionisopoulou, 2014). Kaplan et al. (2009) analyzed several foods grilled in charcoal, reporting acrylamide values in grilled beef between 49 - 250 μg/kg, and 23 - 28 μg/kgin grilled chicken. Olmez et al. (2008) found acrylamide in meat patty in the range<10 - 203μg/kg, and also in bread-coated fried chicken: 34 μg/kg. Chen et al. (2008) analyzed typical Chinese foods and found important acrylamide levels in beef jerky: 121-376 μg/kg. Zhang et al. (2007) found 214.3 μg/kg of acrylamide in deep fried chicken wings. Svensson et al. (2003) reported the presence of acrylamide in fish products like fish fingers with 30 μg/kg and deep fried fish with 39 μg/kg. Leung et al. (2003) reported 93 μg/kg of AA in grilled fish slices.

In this study we obtained values between 55 and 213 μg/kg in grilled beef, from 80 to 122 μg/kg in grilled chicken, and from 47 to 85 μg/kg in grilled salmon, which is in line with the values reported in literature.

Average daily intake of acrylamide by the adult Population of Porto.

Given the limited information on food consumption in Portugal, the use of nutritional information obtained from specific country regions, although not representative of thewhole nation, can be the starting point for dietary intake evaluation of food contaminants. Under the framework of the EpiPorto project, a community study developed by the Department of Hygiene and Epidemiology, Faculty of Medicine from the University of Porto with the objective to assess health determinants in the adult population of Porto, information on food consumption was collected, using a semi-quantitative food frequency questionnaire previously validated for the period of 12 months prior to the interview, which are the basis of the calculations presented in this work (Lopes et al., 2006, available at: http://higiene.med.up.pt/freq.php). This report, carried out with the support of the Portuguese Food Safety Authority, aimed to describe the food consumption and nutritional intake of individuals of Portuguese nationality, residing in Porto, aged over 18 years and is freely available for consultation. The final sample consisted of 2398 subjects, 1477 women (61.6%) and 921 men (38.4%). Quantities in g/day can be obtained for each food items in a database available at:

http://higiene.med.up.pt/consumoalimentarporto/formconsumosg.php.

The average daily consumption of selected food items in which acrylamide was determined was used to determine the acrylamide exposure of the adult population of Porto (ages between 18 to 92 years old). The results are presented in Table 2.

Table 5- Acrylamide levels in selected food items (μg/kg), food consumption of selected food items (g/day), daily average intake of acrylamide (μg/day), dietary intake of acrylamide (μg/ kg body weight day) and the percentiles of exposure for the population of the city of Porto

Food item	Acrylamide levels μg/kg	Food consumption g/day	Average intake μg/day	Dietary intake μg/ kg body weight day	P25th	P50th	P90th	P95th
Chicken	101.5	29.3	2.974	0.043	0.039	0.043	0.050	0.051
Beef and pork	118.9	61.7	7.333	0.106	0.066	0.089	0.164	0.248
Coldmeats	173.2	6.1	1.056	0.015	0.001	0.003	0.043	0.059
Oilyfish (salmon)	62.8	17.9	1.123	0.016	0.014	0.015	0.020	0.021
Bread*	41.8	122.2	5.109	0.074	0.041	0.071	0.142	0.147
Fried Potatoes	591.8	18.3	10.830	0.157	0.085	0.119	0.277	0.400
Roasted or boiled potatoes	61.0	69.2	4.221	0.061	0.602	0.612	0.628	0.630
Breakfast cereals	122.4	5.3	0.649	0.009	0.003	0.005	0.023	0.032
Milk crackers	462.3	7.1	3.283	0.048	0.044	0.051	0.054	0.054
Cookies	393.1	3.5	1.376	0.020	0.007	0.016	0.041	0.046
Pastry	65.6	15.4	1.010	0.015	0.011	0.013	0.022	0.022
Chocolates	66.0	3.0	0.198	0.003	0.001	0.002	0.006	0.006
Coffee	138.9	10.0	1.395	0.020	0.017	0.025	0.047	0.053
Coffee surrogates	526.1	0.514	0.271	0.004	0.004	0.006	0.009	0.009
Roasted cereals	418.8	0.220	0.092	0.001	0.001	0.001	0.002	0.002
Patties	50.5	11.5	0.581	0.008	0.008	0.008	0.009	0.009
Total			**41.5**	**0.600**	**0.944**			**1.790**

*The food group Bread includes: white bread, toasted bread, whole wheat bread,rye bread, brown bread and corn bread

Based on the data of the daily consumption of food items, bread was consumed in the highest amount by the adult population of Porto with a total consumption of 122.2 g per person per day, followed by roasted or boiled potatoes with 69.2 g per day and meat 61.7 g per day. Mean acrylamide levels in these foodstuff corresponds to 41.8 μg/kg, 61.0 μg/kg and 118.9 μg/kg respectively, being therefore considered as foods with low acrylamide content. On the opposite, some food with lower consumption patterns but high acrylamide levels may have a major contributionfor acrylamide daily intake, as fried potatoes,meat, cookies, and coffee.

In 2005, JECFA estimated an average acrylamide exposure for the general population of 1 μg/kg bw day and up to 4 μg/kg bw day for consumers with high dietary

exposure (JECFA, 2010). In our study, 0.60 μg/kg bw day were assessed for the general population, achieving 1.79 μg/kg bw day for the consumers who eat more than the average or consume foods with higher amounts of acrylamide, which is in the expected range (Hargin, 2013). Our average intake of acrylamide per day corresponds to 41.5 μg/day, higher than most average intakes reported by other European countries like Poland (23 μg/day), Spain (15 μg/day), Greece (21 μg/day), Italy (15 μg/day) or France (20 μg/day) but similar to UK (35 μg/day) and Denmark (35 μg/day) (Freisling et al., 2013; Mojska et al., 2010). A direct comparison between acrylamide intake estimated with other studies is difficult because acrylamide content in foods depends greatly on the regional preferences of the consumers and typical preparations and also on the method used to estimate the exposure to the compound (Freisling et al., 2013). In our case, the contribution of meat sample (25%) to the intake of acrylamide was much higher than contributions reported elsewhere (0.1 to 2.8 %) because of the higher acrylamide content in meat samples found in our study, and the fact that meat is highly consumed by the population of Porto, corresponding to 15% of the total energy intake per day per individual (Lopes et al., 2006).

Foods that contribute the most to acrylamide intake were calculated for both men and women, separated by age groups as in the food questionnaire used. In Figure 1 and Figure 2 the contribution of each food group to the mean intake of acrylamide per day are detailed.

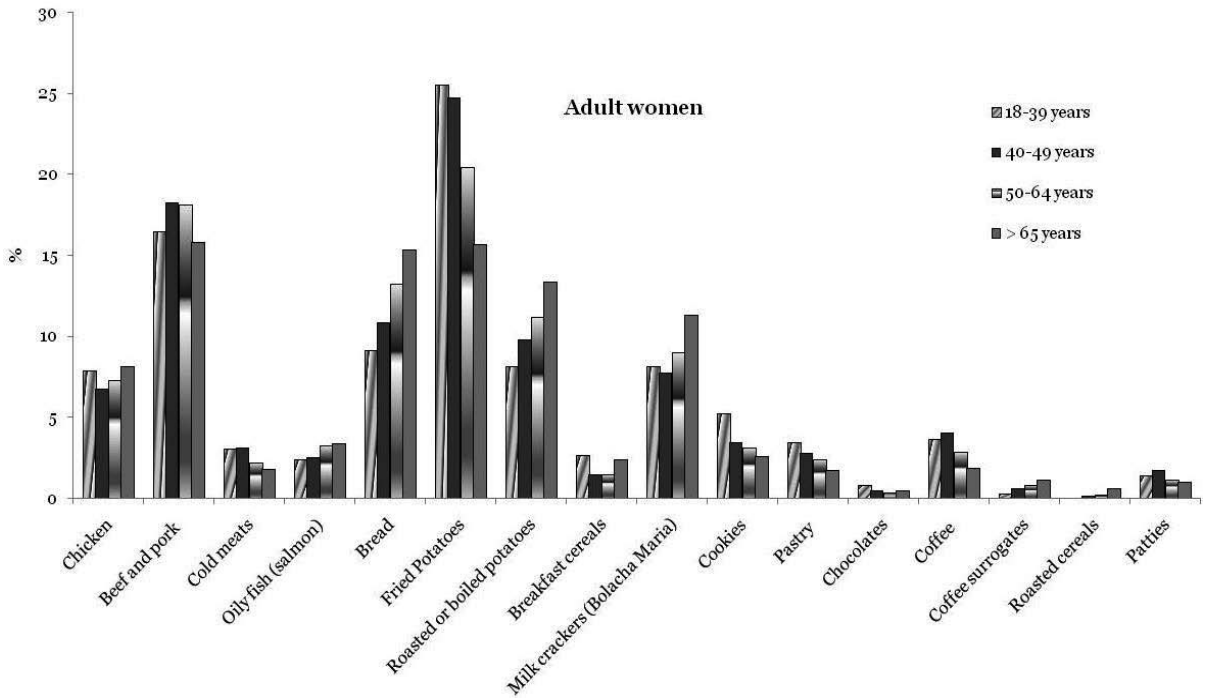

Figure 1- Contribution to each food group to the mean dietary intake of acrylamide for adult women.

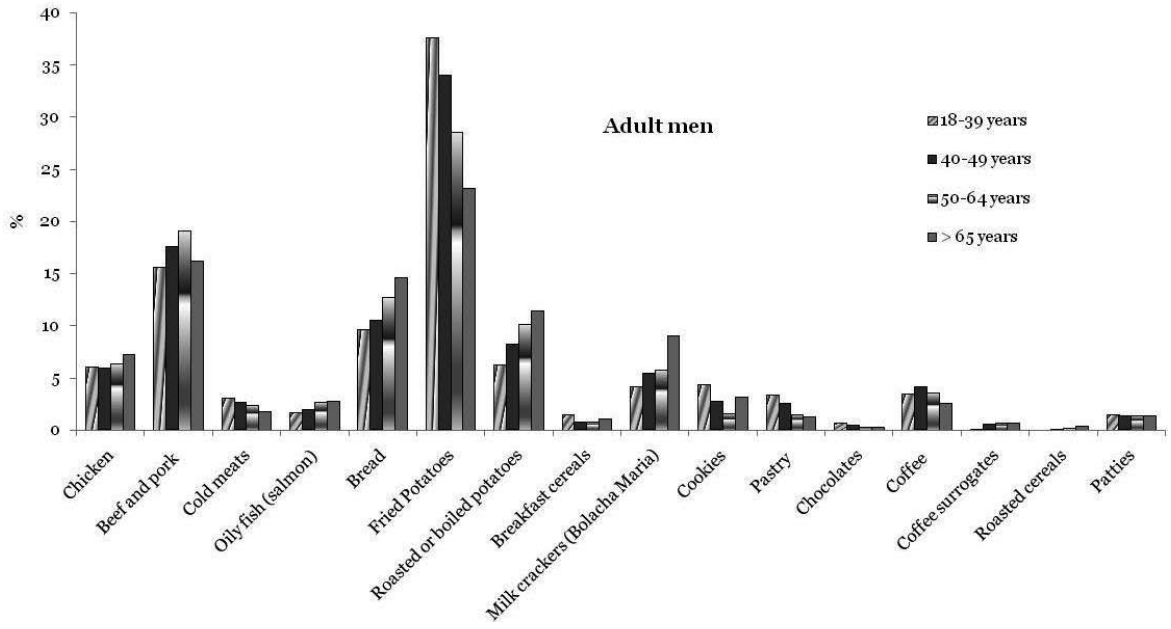

Figure 2- Contribution to each food group to the mean dietary intake of acrylamide for adult men

Individually, fried potato contributes the most to acrylamide intake in both men and women, followed by meat and bread. Coffee contributes with less than 5% to acrylamide intake in both genders, which is due to the lower consumption reported, taking into account that mean acrylamide levels found in our screening assay were in line with other reports about acrylamide levels in coffees. It is also possible to verify that food habits change with

age, reducing the consumption of fried potato and increasing the consumption of bread and boiled potatoes. Milk crackers are an important contributor to the intake in population over 65 years old.

As reported by other studies (Freisling et al., 2013; Hargin, 2013), the results show that men ingest more acrylamide per day than women (Figure 3), and the intake of acrylamide decreases with age.

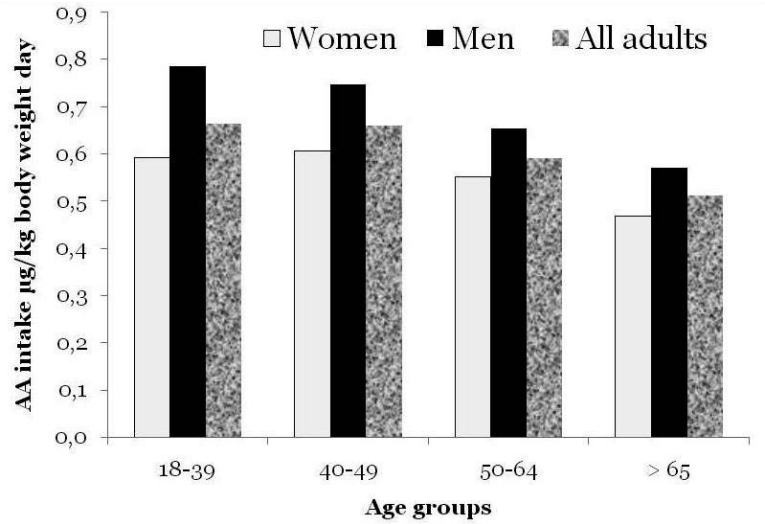

Figure 3- Average daily intake of acrylamide (µg/kg bw day) among women, men and all adult population of different age groups in Porto, Portugal.

Freisling et al. (2013) also reported a small correlation between drinking habits and acrylamide consumption, specially for women. In our study a good correlation between acrylamide intake and energy intake (kcal) per day, and a moderate correlation between acrylamide intake and caffeine intake (mg/day) were found, while no correlation was observed between acrylamide intake and alcohol consumption, as can be seen in Figure 4. Data about energy, caffeine, and alcohol consumption were obtained from Lopes et al. (2006).

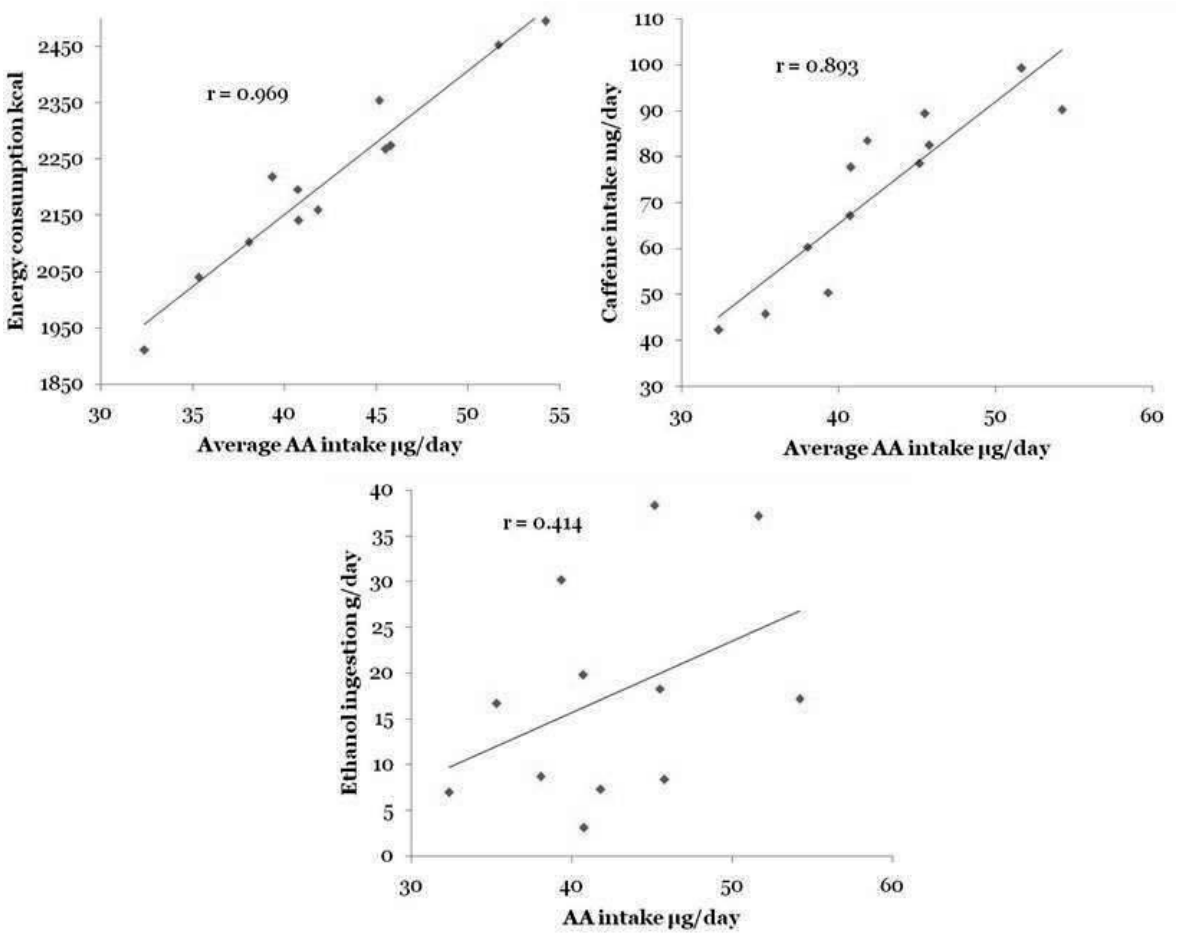

Figure 4- Correlation between acrylamide intake and ethanol, caffeine and energy intake per day by the total adult population of Porto.

Conclusions

A large screening of acrylamide levels in different kind of foods largely consumed in Portugal, from commercial source or cooked at home, showed results somewhat similar to those reported from other countries, but probably higher than those expected in the Mediterranean region, taking into account the specificity of eating habits. By using food consumption data taken from a published food consumption report focused on the population of the city of Porto (Lopes et al., 2006), an estimated average daily intake for the adult population of Porto of 41,5 µg/day was calculated, ranging from 0.60 to 0.70 µg/kg of body weight/day for women and men, respectively. These results are shown in the upper level of the scale when compared with the results obtained in other European countries or regions. The main food contributors to acrylamide ingestion are fried potatoes, bread, and

meat. Due to the low consumption patterns, coffee shown to be not an important source of acrylamide in this population.

References

Arisseto AP, Toledo MC, Govaert Y, Van Loco J, Fraselle S, Weverbergh E, et al. Determination of acrylamide levels in selected foods in Brazil. Food Addit Contam 2007; 24 (3): 236-241.

Arvanitoyannis IS, Dionisopoulou N. Acrylamide: Formation, Occurrence in Food Products, Detection Methods, and Legislation. Crit Rev Food Sci 2014, 54 (6): 708-733.

Can NO, Arli G. Analysis of acrylamide in traditional and nontraditional foods in Turkey using HPLC-DAD with SPE cleanup. J Liq Chromatogr R T 2014; 37 (6): 850-863.

Chen F, Yuan Y, Liu J, Zhao G, Hu X, Survey of acrylamide levels in Chinese foods. Food Addit Contam B 2008; 1: 85–92.

EFSA. European Food Safety Authority. Update on acrylamide levels in food from monitoring years 2007 to 2010. EFSA Journal 2012; 10 (10): 2938-2976. Available online at: www.efsa.europa.eu/efsajournal.

European Commission. Special Eurobarometer nº246. Health and Food 2006. Available at: http://ec.europa.eu/health/eurobarometers/index_en.htm?Page=14

Fernandes JO, Soares C. Application of matrix solid-phase dispersion in the determination of acrylamide in potato chips. J Chromatogr A 2007; 1175:1-6.

Freisling H, Moskal A, Ferrari P, Nicolas G, Knaze V, Clavel-Chapelon F, et al. Dietary intake of adults in the European Prospective Investigationinto Cancer and Nutrition differs greatly according to geographical region. Eur J Nutr 2013; 52: 1369-1380.

Hargin KD. Using total diet studies to assess acrylamide exposure. In: Moy GG, Vannoort RW, editors. Total Diet Studies. London: Springer; 2013. p. 489-500.

JECFA. FAO/WHO Joint FAO/WHO Expert Committee on Food Additives, Seventy Second Meeting, Summary and Conclusions. 2010.

Kaplan O, Kaya G, Ozcan C, Ince M, Yaman M. Acrylamide concentrations in grilled foodstuffs of Turkish kitchen by high performance liquid chromatography-mass spectrometry, Microchem J 2009; 93: 173–179.

Lee S, Yoo M, Koo M, Kim HJ, Kim M, Park S-K, et al. In-house–validated liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for survey of acrylamide in various processed foods from Korean market. Food Sci Nut 2013; 1 (5): 402–407.

Leung KS, Lin A, Tsang CK, Yeung STK. Acrylamide in Asian foods in Hong Kong. Food Addit Contam 2003; 20 (12): 1105-1113.

Lopes C, Oliveira A, Santos AC, Ramos E, Gaio AR, Severo M, Barros H. Food Consumption in Porto. Faculdade de Medicina da Universidade do Porto 2006. Available at:www.consumoalimentarporto.med.up.pt. (In Portuguese).

Matthys C, Bilau M, Govaert Y, Moons E, De Henauw S, Willems JL. Risk assessment of dietary acrylamide intake in Flemish adolescents. Food Chem Toxicol 2005; 43 (2): 271–278.

Mojska H, Gielecinska I, Szponar L, Oltarzewski M. Estimation of the dietary acrylamide exposure of the Polish population. Food Chem Toxicol 2010; 48: 2090-2096.

Mottram D, Wedzicha B, Dodson A. Food chemistry: Acrylamide is formed in the Maillard reaction. Nature 2002; 419: 448-449.

Normandin L, Bouchard M, Ayotte P, Blanchet C, Becalski A, Bonvalot Y, et al. Dietary exposure to acrylamide in adolescents from a Canadian urban center Food Chem Toxicol 2013; 57: 75-83.

Ölmez H, Tuncay F, Özcan N, Demirel S. A survey of acrylamide levels in foods from the Turkish Market. J Food Comp Anal 2008; 21: 564–568.

Senyuva HZ, Gokmen V. Survey of acrylamide in Turkish foods by an in-house validated LC-MS method, Food Addit Contam 2005; 22: 204-209.

Soares C, Fernandes JO. MSPD Method to Determine Acrylamide in Food. Food Anal Method 2009; 2 (3): 197-203.

Soares CM, Alves RC, Casal S, Oliveira MB, Fernandes JO. Validation of a Matrix Solid-Phase Dispersion method to determine acrylamide in coffee and coffee surrogates. J Food Sci 2010 Apr; 75 (3): T57-63.

Stadler RH, Blank I, Varga N, Robert F, Hau J, Guy PA, et al. Acrylamide from Maillard reaction products. Nature 2002; 419: 449-450.

Tareke E, Rydberg P, Karlsson P, Eriksson S, Tornqvist M. Analysis of Acrylamide, a Carcinogen Formed in Heated Foodstuffs. J Agr Food Chem 2002; 50: 4998-5006.

Zhang Y, Xu W, Wu X, Zhang X, Zhang Z. Addition of antioxidant from bamboo leaves as an effective way to reduce the formation of acrylamide in fried chicken wings. Food Addit Contam 2007; 24 (3): 242–251.

Acknowledgements

The authors are thankful to FCT for financial support in the framework of the project POCI/AGR/61543/2004 and Cristina Soares PhD grant (SFRH/BD/39360/2007).

CHAPTER 6

Conclusions

Conclusions

Experimental work conducted for the preparation of this dissertation allowed significant advances in knowledge on acrylamide in thermally processed foods, at different levels: analytical methods, mitigation, and human exposure.

A. Analytical methods for acrylamide quantification

A new methodology for the quantification of acrylamide in food based on Matrix Solid-Phase Dispersion (MSPD) and Gas Chromatography - Mass Spectrometry (GC-MS) was developed and validated. It must be emphasized that MSPD was for the first time applied to the extraction of acrylamide from different foodstuff namely fried and roasted potatoes, processed cereal products (bread, toasts, breakfast cereals, snacks, cookies and biscuits), chocolates and baby-foods. The method exhibit noteworthy analytical features, namely the carrying out of the analysis in an easier, simpler and more expeditious manner, showing advantages over the generally employed liquid extraction methods, which makes it an advantageous alternative for the routine analysis of acrylamide.

An optimized similar method (using specifically designed custom made bi-layered Multimode/C_{18} cartridges for the MSPD purification step) was developed and validated for the determination of acrylamide in more complex matrices such as coffee ground, coffee brews and related beverages. This extraction method compared favourably with more traditional multi-step procedures involving much manual handling, larger amounts of sample, sorbents, and organic solvents, and longer analytical times, usually employed for this kind of matrices.

B. Mitigation of acrylamide

Concerning the reduction of acrylamide formation in thermally processed foods, and consequently decreasing of acrylamide ingestion by consumers, some different strategies must be proposed for different food items, taking into account the need to maintain the sensorial properties of the finished products.

Home made fried potatoes

Experiments performed have confirmed previous findings about the importance of chosen varietal potatoes with low reducing sugars levels to have lower acrylamide contents in fried potatoes at home. From 5 potato cultivars most used in Portugal, two of them, Agria and Marabel, shown to be the most adequate to obtain a final product with lower acrylamide levels.

Home made roasted meat

The effect of a previous marinade of the meat with beer (alone or added with herbs and spices), white wine, or green tea, showed to be a very effective approach to decrease acrylamide formation during meat roasting, with reductions of 40% or higher.

The combined effect of selecting adequate potato varieties for frying with the effect of marinades before meat cooking can contribute significantly to reduced acrylamide ingestion from home-made food cooking.

Coffee

Experiments performed have confirmed previous findings that options to reduce the amount of acrylamide ingested trough coffee are the selection of blends with higher arabica percentages and darker degrees of roasting. In the specific case of "expresso" brew, shorter brews instead of long ones, are preferable because contains low amounts of acrylamide, but this will obviously depend on the consumers' preferences.

At industrial level, the developed work showed that operations of peeling of unripe coffee and pulping of immature coffee grains contributes to decreased asparagine levels, and consequently acrylamide levels in the finished product. Changes in roasting conditions, namely a careful control of the hot air flow velocity, showed to be a potential contributor to the preparation of roasted coffee with less acrylamide.

C. Human exposure to acrylamide

Taking into account a large screening of acrylamide levels in different kind of foods largely consumed in Portugal, from commercial source or cooked at home, carried out within this thesis, and food consumption data taken from a published food consumption report focused on the population of the city of Porto, an estimated average daily intake for the adult population of Porto of 41,5 μg/day was calculated, ranging from 0.60 to 0.70 μg/kg of body weight/day for women and men, respectively. The main food contributors to acrylamide ingestion are fried potatoes, bread, and meat, while coffee showed less importance than usually reported in similar studies, due to low levels of consumption reported.