**Catarina Isabel Bento Petisca**

# Furanic compounds in food products: assessment and mitigation strategies

Thesis presented to obtain the PhD degree in Pharmaceutical Sciences - Nutrition and Food Science Specialty

Faculty of Pharmacy of University of Porto

Under the supervision of:
Prof. Isabel Maria Pinto Leite Viegas Oliveira Ferreira
and the co-supervision of:
Prof. Olívia Maria de Castro Pinho

Porto,

February, 2013

**Legend of the figures presented on the cover (from left to right):**

**Figure 1:** Food products containing furanic compounds: coffee, deep-fried fish and bakery products.

**Figure 2:** Chemical structures of some furanic compounds

**Figure 3:** HPLC-DAD instrument

**Figure 4:** Chromatogram of a coffee sample

**Figure 5:** GC-MS instrument

Agradeço à Fundação para a Ciência e Tecnologia (FCT) pelo suporte financeiro referente à bolsa de Doutoramento com referência SFRH / BD / 48745 / 2008 no âmbito do QREN-POPH-Tipologia 4.1 – Formação Avançada, comparticipado pelo Fundo Social Europeu e por fundos nacionais do MCTES. Agradeço, também, à Faculdade de Farmácia da Universidade do Porto por ter facilitado o suporte logístico.





***Aos meus pais, avós e Hugo***

## Aknowledgements

Chega ao fim mais uma etapa da minha vida e, como tal, não poderia deixar de agradecer a todas as pessoas que de alguma forma contribuíram para esta tese fosse concluída.

Em primeiro lugar quero agradecer à Professora Isabel por ter aceitado ser minha orientadora, por todo o trabalho, ajuda, dedicação e amizade que sempre demonstrou. Sem a sua ajuda, dificilmente teria chegado até aqui e é do fundo do coração que digo que será sempre uma pessoa inesquecível.

À Professora Olívia por há muitos anos atrás me ter convidado para fazer investigação e por me ter aberto todas as portas possíveis. Sem a sua ajuda, amizade, dedicação e co-orientação, seguramente hoje não estaria aqui.

À Professora Susana pelas conversas, discussões de trabalho e transmissão de conhecimento que me ajudaram a ver as coisas muitas vezes de outro prisma e por, nesta reta final ter sido uma óptima companhia no café matinal.

À Dra. Eulália e à Engenheira Elisa por terem sido incríveis e quase segundas mães! Não há palavras para descrever o quão grata estou e vou estar eternamente por tudo o que fizeram (e continuam a fazer) por mim. Vão estar sempre no meu coração.

Ao Professor Zé Fernandes e à Sara por me terem sempre disponibilizado o ambiente necessário para que tanto o trabalho laboratorial como a escrita da tese pudesse ser realizado, pela amizade e por terem sempre uma palavra amiga.

To Professor Michael, for the availability to receive me in the TU Graz, for the conversations, knowledge and friendship.

Ao Armindo por ser o amigo que é, e a quem devo tudo o que sou profissionalmente. Obrigada pela amizade, pelo conhecimento, pelas conversas, pela (sempre) boa disposição, pela sempre palavra sábia a dizer.

À Zita, pelo companheirismo, pela amizade, pelos jantares e noitadas a trabalhar em que simplesmente fazia companhia. À Rebeca por também estar sempre presente, pela sempre ajuda ao próximo, pela sempre disponibilidade a qualquer hora do dia (ou da noite), pelas conversas e troca de conhecimento. À Carina, por ser a pessoa fantástica e maravilhosa, inteligente e trabalhadora que é e pela amizade e pelas “críticas construtivas” que fazem dela, umas das melhores pessoas para pedir uma opinião. À Trini por ter entrado na minha vida! Por ser uma pessoa fantástica com quem tive o privilégio de trabalhar, de ser amiga e que mesmo longe ser a amiga maravilhosa que é.

À Rita por ter sido a “minha menina” que, apesar das dores de cabeça, é uma amiga inesquecível. Obrigada pelas noitadas de trabalho pela disponibilidade e acima de tudo, pela amizade. À Ana Salgueiro, pelo apoio, amizade e sinceridade, e por todas as aventuras inesquecíveis durante 3 meses. Não podia ter ido para a Áustria com melhor pessoa. À Carla e Vânia pela amizade e por terem sido incansáveis na busca dos pormenores escondidos. À Bruna e à Catarina que, apesar de estarem há menos tempo no laboratório não deixam de ser amigas fantásticas e companheiras de sala maravilhosas. À Tânia por ter sido sempre muito prestável e por ser uma óptima companheira de sala.

Ao David, Edgar e Christian, pela amizade, pelos almoços onde se falava de tudo, pelo companheirismo e por sempre me terem “adoptado” para o grupo.

To Tatjana for everything! For the friendship, for the availability, for the kindness in receive me in her home; for showed me a different Austria and for being the special person that she is. I would like to thank to Thomas, Andrej, Reni, Raji, Willi, Sandra, Lisa, Carmen Julia and more recently, Barbara for being so special friends!

Ao sr. Rui, d. Clara e Tiago por terem sido a segunda família e por me terem feito sentir um bocadinho menos falta de casa. Obrigada por todo o amor, amizade, carinho e todo o apoio dado, especialmente nesta fase final.

Aos meus pais e avós por sempre me terem apoiado em todas as decisões da minha vida e por estarem sempre presentes. Esta tese é o reflexo da pessoa em que me tornei e que não seria a mesma se não fossem todos os sacrifícios que fizeram por mim e que estarei eternamente grata.

Ao Hugo, por ser o companheiro fantástico e inigualável que é. Contigo passámos muitas provas, mas sempre as superámos, mesmo estando à distância! Obrigada pelo apoio, pelo, carinho, pelo amor, pela amizade, pelo companheirismo e pela compreensão. Sei que nesta fase final não fui a pessoa mais paciente do mundo, mas tu estiveste sempre lá, sempre com um sorriso e uma palavra amiga.

Finalmente agradeço a todas as pessoas que não estando aqui mencionadas contribuíram de alguma forma para que este trabalho pudesse ser realizado.

A todos, o meu MUITO OBRIGADA!

*“What we do in life echoes in eternity.” [...]*

## Author's Declaration

Under the terms of the "Decreto-lei nº 216/92, de 13 de Outubro" is hereby declared the following original articles were prepared in the scope of this thesis.

Under the terms of the referred "Decreto-lei", the author declares that she afforded a major contribution to the conceptual design and technical execution of the work, interpretation of the results and manuscript preparation of the published articles included in the thesis.

### Publications in Internationals Peer-Reviewed Journals

**Petisca, C.**; Henriques, A.R.; Pérez-Palacios, T.; Pinho, O.; Ferreira, I.M.P.L.V.O. Assessment of HMF and furfural in commercial bakery products. Submitted

**Petisca, C.**; Henriques, A.R.; Pérez-Palacios, T.; Pinho, O.; Ferreira, I.M.P.L.V.O. Effect of baking process in Hydroxymethylfurfural and Furfural content of model cakes: Mitigation strategies and its impact on volatile profile. Submitted.

Pérez-Palacios T., **Petisca C.**, Melo A., Ferreira I.M.P.L.V.O. 2013. Quantification of 5-hydroxymethylfurfural in coated deep fried products: optimisation of the extraction procedure by using statistical design. Food Analytical Methods, 6 (1): 10 -16.

Pérez-Palacios, T.; **Petisca, C.**; Henriques, A. R.; Ferreira, I.M.P.L.V.O. 2013. Impact of cooking and handling conditions on furanic compounds in breaded fish products. Food and Chemical Toxicology, 55C: 222-228.

Pérez-Palacios T., **Petisca C**., Melo A., Ferreira I.M.P.L.V.O. 2012. Quantification of furanic compounds in coated deep fried products simulating normal preparation and consumption: optimisation of HS-SPME analytical conditions by Response Surface Methodology. Food Chemistry, 135 (3): 1337–1343.

**Petisca C**.; Pérez-Palacios T.; Pinho O.; Ferreira I.M.P.L.V.O. Validation and application of a HS-SPME-GC-MS methodology for quantification of furanic compounds in espresso coffee. Food Analytical Methods, Submitted revised version.

**Petisca C**.; Pérez-Palacios T.; Farah A.; Pinho O.; Ferreira I.M.P.L.V.O. 2012. Furans and other volatile compounds in ground roasted and espresso coffee using headspace solid-phase microextraction: effect of roasting speed. Food and Bioproducts Processing. In press. DOI: http://dx.doi.org/10.1016/j.fbp.2012.10.003.

**Invited Oral communications**

**Petisca C**. Furans in Food. II Jornadas de Ciência Alimentar. UTAD – Vila Real, 17th – 18th October 2011.

**Petisca C**. Analysis of HMF and related furans in foods – Exposure of the Portuguese Population. Special Topics in Food Chemistry. Graz University of Technology – Biochemistry Institute, 23rd March, 2010.

**Other Oral communications**

Moreira, P.*, Henriques, A.R., **Petisca, C.**, Viegas, O., Henriques, C., Ferreira, I.M.P.L.V.O., Pinho, O. Quantification of hydroxymethylfurfural and furfural in bread by high performance liquid chromatography, in IJUP - Sixth Meeting of Young Researchers of University of Porto, 13$^{th}$ – 15$^{th}$ February, 2013.

Pérez-Palacios, M.T., **Petisca, C.**, Pinho, O., Ferreira, I.M.P.L.V.O*. Headspace solid-phase microextraction of volatile and furanic compounds in coated fish sticks: effect of the extraction temperature, in WASET - World Academy of Science, Engineering and Technology, Singapore, 12th – 13th September, 2012.

**Petisca C**.*, Perez Palacios M.T., Martins Z.E., Pinho O., Ferreira I.M.P.L.V.O., Espresso coffee: influence of roasted degree in furan composition, in XVII Encontro Galego-Portugués de Química, Pontevedra, 9th to 11th November, 2011

**Petisca C**.*, Perez Palacios M.T., Pinho O., Ferreira I.M.P.L.V.O. Quantification of furanic compounds present in espresso and aromatised espresso coffee samples using SPME-GC/MS. RAFA, in Recent Advances in Food Analysis, Prague Czech Republic, 1st to 4th November, 2011.

**Petisca C**.*, Pinho O., Ferreira I.M.P.L.V.O., Murkovic M. Uv/Vis spectrometry for evaluation of chemical interaction between HMF and lysine / BSA. VI Congresso Ibérico/XXII Reunión Nacional Espectroscopía (VI CIE - XXII RNE), 8th to 10th, 2010.

**Petisca C**.*, Farah A., Pinho O., Ferreira I.M.P.L.V.O. Optimisation of a HE-SPME-GC/MS method for screening of volatile compounds in espresso coffee. IJUP10, Third Meeting of Young Researchers of U.Porto, Porto, 17th to 19th February, 2010.

**Posters in conferences**

**Petisca, C.**, Moreira, P., Silva, R., Pinho, O., Ferreira, I.M.P.L.V.O*. Hydroxymethylfurfural and furfural in model cakes: Effect of baking process and mitigation strategies, in EuroFoodChem XVII, Istanbul, 7$^{th}$ – 10$^{th}$ May, 2013.

Henriques, A.R.*, **Petisca, C**., Pinho, O., Ferreira, I.M.P.L.V.O. Furfural stability in model systems containing ascorbic acid and citric acids: effect of pH, time and temperature, in IJUP - Fifth Meeting of Young Researchers of University of Porto, 22$^{nd}$ – 24$^{th}$ February, 2012.

**Petisca, C.,** Martins, Z.E.*, Pinho, O., Ferreira, I.M.P.L.V.O. Furanic compounds in espresso coffee capsules: validation of a HS-SPME-GC-MS methodology, in IJUP - Fifth Meeting of Young Researchers of University of Porto, 22$^{nd}$ – 24$^{th}$ February, 2012.

**Petisca, C.;** Perez Palacios M.T., Fernandes, J.*, Pinho O., Ferreira I.M.P.L.V.O. Furanic compounds presente in Arabica coffee with and without defective coffee beans, in 11º Encontro de Química dos Alimentos, Bragança, 16th – 19th September, 2012.

**Petisca C**.*, Farah A., Pinho O., Ferreira I.M.P.L.V.O. Analysis of volatile compounds of Espresso coffee and powdered samples by HE-SPME-GC/MS. In Österreichische Lebensmittel-chemikertage, May, 2010, Leibnitz, Austria.

**Petisca C**.*, Pinho O., Ferreira I.M.P.L.V.O. Screening of furans in aromatised coffee samples by SPME-GC-MS: comparison with conventional coffee. In Recent Advances in Food Analysis - RAFA, Prague - Czech Republic, 4-6 November, 2009.

*Presenting author

## Resumo

Os compostos furânicos resultam do processamento térmico e contribuem para as propriedades sensoriais dos alimentos cozinhados, em geral, a sua presença é apreciada, no entanto, devido aos seus potenciais efeitos nocivos na saúde humana, estudos relacionados com a sua formação, conteúdo numa variedade de alimentos e estratégias de mitigação continuam a ser necessários. Além disso, estas estratégias para reduzir o teor de compostos furânicos devem manter as características de aroma e sabor. A presente tese proporciona novos resultados sobre a composição em compostos furânicos de café, produtos panados fritos e produtos de padaria, simulando as condições usuais de preparação e de consumo. A avaliação de teores vestigiais de compostos resultantes do processamento térmico requer cuidados processos extrativos e de purificação para a quantificação fiável destes compostos em matrizes tão complexas como os alimentos selecionados.

Metodologias de HS-SPME-GC-MS foram aplicadas, inicialmente, para o *screening* e monitorização, do teor de compostos voláteis, especialmente, furanos em café moído e expresso, produtos panados fritos e em bolos. Posteriormente, estas metodologias foram otimizadas e validadas para a quantificação de determinados furanos voláteis, utilizando $d_4$-furano como padrão interno. Adicionalmente, selecionaram-se as condições adequadas para a quantificação de compostos furânicos menos voláteis por HPLC/DAD, particularmente em panados e em produtos de panificação e pastelaria. Para garantir a sensibilidade, fiabilidade e precisão dos métodos analíticos desenvolvidos, realizou-se uma otimização cuidadosa e validação em cada matriz. Para este efeito, a maioria das metodologias anteriores foram otimizadas com recurso a métodos multivariados utilizando curvas de superfície de resposta, a fim de selecionar as condições mais favoráveis para a extração dos compostos em estudo.

A monitorização de furanos e outros compostos voláteis em café 100% Arábica moído, torrado a diferentes velocidades até ao mesmo grau de torra média, bem como nos respetivos cafés expresso indicou que as percentagens relativas dos furanos e pirroles foram superiores nas amostras de café expresso do que no café moído. Em particular, o furfural e o 5-methilfurfural revelaram um aumento progressivo com velocidade de torra, deste modo, a avaliação destes compostos é relevante. O café expresso preparado a partir de diferentes tipos de cápsulas hermeticamente fechadas, disponíveis comercialmente, para consumo doméstico, foi estudado para a determinação de furano e compostos furânicos mais abundantes, nomeadamente furfural, furfuril álcool, 5-metillfurfural e furfuril acetato. O furfuril álcool foi o principal derivado furânico volátil

(31,2-55,7 mg $mL^{-1}$), enquanto que o furano apresentou o menor teor (cerca de 0,23 mg $mL^{-1}$), sendo este valor concordante com o que está descrito na literatura para amostras de café expresso.

Os produtos panados fritos apresentam condições favoráveis para a formação de compostos furânicos, devido às altas temperaturas atingidas pelo revestimento de pão durante a fritura. Curiosamente, em paralelo com as amostras de café, o furfuril álcool foi o composto furânico volátil presente em maiores quantidades (10,48 mg $g^{-1}$) nestes produtos alimentares, o furano estava presente em menor quantidade (5,59 mg $g^{-1}$), embora em níveis suficientemente altos para ser incluído pela EFSA na classe de alimentos com alto conteúdo em furano.

Os produtos comerciais de padaria e pastelaria, tais como pão, biscoitos e bolos / pastéis são amplamente consumidos. Deste modo, realizou-se uma pesquisa sobre a presença de hidroximetilfurfural (HMF) e furfural nestes alimentos para avaliar o efeito do tipo de produto e da sua composição nutricional no teor destes compostos. Os biscoitos apresentaram o maior conteúdo em HMF (7,84 mg $kg^{-1}$) e em hidratos de carbono, juntamente com o teor mínimo de humidade, por outro lado, as amostras de pão apresentaram as quantidades mais elevadas de furfural (5,27 mg $kg^{-1}$), fibras, proteínas e humidade. No entanto, as amostras que continham chocolate na superfície revelaram quantidades extraordinariamente elevadas de ambos os compostos furânicos (13.09.mg $kg^{-1}$ de HMF, e 116,8 mg $kg^{-1}$ para o furfural).

No que respeita a estratégias de mitigação para reduzir os compostos furânicos voláteis e HMF em panados fritos, o ajuste do método e das condições de cocção, tais como a utilização de um forno elétrico, fritar no óleo de girassol a 160 º C durante 4 min, ou esperar 10 min após a cocção, reduz significativamente o teor destes compostos, mas influencia o perfil de compostos do aroma. Além disso, o equipamento usado na cocção também influencia a formação de furanos, assim como, o perfil de voláteis em bolos modelo. Por exemplo, cozer em micro-ondas, por períodos curtos, levou à formação de quantidades insignificantes de HMF e furfural. No entanto, outros compostos voláteis responsáveis pelo aroma e sabor foram igualmente diminuídos, o que pode afetar a apreciação por parte dos consumidores. Ao comparar a composição de bolos modelo cozidos em forno com libertação de vapor e em forno de convecção, o primeiro provou ser uma estratégia de mitigação apropriada, uma vez que revelou menor teor de HMF mesmo quando cozido durante longos períodos, sem modificar o perfil de aroma e sabor dos compostos.

## Abstract

Furanic compounds arise from heat-treatment processing and contribute to the sensory properties of cooked foods, in general their presence is appreciated; however, due to its potential harmful effects on human health studies related with its formation, content in a variety of foods and mitigation strategies are still needed. Moreover, those strategies to reduce furanic compounds content should maintain the overall aroma and flavour. This thesis offers new insights on the furanic compounds composition of coffee, coated deep-fried and bakery products, simulating the usual preparation and consumption conditions. The assessment of trace amounts of heat generated compounds mainly requires careful and accurate extraction and clean-up procedures for screening and reliable quantification of these compounds in the complex matrices selected.

HS-SPME-GC-MS methodologies were applied, first, for screening and monitoring on total ion content mode of volatile compounds, especially furans in ground and *espresso* coffee, in coated deep-fried products and in model cakes. Secondly, these methodologies were optimized and validated for quantification of specific volatile furans, using $d_4$-furan as internal standard. Additionally, HPLC-DAD conditions were chosen for the quantification of less volatile furanic compounds, particularly in coated deep-fried and in bakery products. To ensure the sensitivity, reliability and accuracy of analytical methods developed, a careful optimisation and validation was performed in each matrix. To this end, most of the former methodologies were optimised by multivariate optimisation using the response surface curves, in order to select the most favourable conditions for the extraction of compounds under study.

The determination of furans and other volatile compounds in ground *Arabica* coffee, roasted at different speeds to the same medium roast degree, as well as in the respective *espresso* brews indicated that the relative percentages of furans and pyrroles were higher in *espresso* samples than in the ground ones. In particular, furfural and 5-methylfurfural showed a progressive increment with roasting speed, thus their quantification becomes of greater relevance. *Espresso* coffee from different types of hermetically closed capsules, commercially available for home consumption, were studied for the determination of furan and major furanic compounds, namely furfural, furfuryl alcohol, 5-methylfurfural and furfuryl acetate. Furfuryl alcohol turned out to be the main volatile furanic derivative (31.2 – 55.7 µg $mL^{-1}$), while furan presented the lowest content (around 0.23 µg $mL^{-1}$), this amount was within that reported in literature for *espresso* coffee samples.

Coated deep-fried food matrices showed a predisposition for the formation of furanic compounds due to the high temperatures reached by the bread coat during frying.

Interestingly, in parallel to coffee samples, furfuryl alcohol revealed the highest amounts (10.48 μg $g^{-1}$) in these foodstuffs, while furan presented lower amount (5.59 μg $g^{-1}$), though still high enough to be included in EFSA's class for high furan content foods.

Commercial bakery products, such as bread, biscuits and cakes/pastry are widely consumed. Thus, a survey on the presence of hydroxymethylfurfural (HMF) and furfural in the former foodstuffs was conducted to investigate the effect of the bakery type and nutritional composition in their amounts. Biscuits presented the highest HMF (7.84 mg $kg^{-1}$) and carbohydrate contents, together with minimum moisture values, while bread samples showed the highest amounts of furfural (5.27 mg $kg^{-1}$), protein, fibre and moisture. Nonetheless, samples containing chocolate on their surface constitute a more confined group that revealed outstandingly high amounts of both of these furanic compounds (13.09.mg $kg^{-1}$ for HMF, and 116.8 mg $kg^{-1}$ for furfural).

Concerning mitigation strategies to reduce volatile and less volatile furanic compounds in coated deep-fried products, the adjustment of cooking method and conditions, such as using an electric oven or deep-frying in sunflower oil at 160 ºC during 4 min, or waiting 10 min after cooking, led to a significant reduction of those compounds, but also influenced global volatile profile. Furthermore, the baking equipment also influenced furanic compounds formation and volatile profile of model cakes. For instance, microwave baking for short periods led to the formation of insignificant amounts of HMF and furfural. However, other volatile compounds responsible for aroma and flavour of baked products were similarly altered, which may affect consumers' satisfactoriness. By comparing composition of model cakes baked in steam and convection ovens, the former proved to be an appropriate mitigation strategy, since it revealed lower HMF contents even when baked for long periods, without modifying the profile of aroma and flavour compounds.

# Table of Contents

**PART II. Furanic compounds in Coffee**

## List of Figures

## List of Tables

## List of Abbreviations

| Abbreviations | Names |
|---|---|
| ADI | Acepted Daily Intake |
| ANOVA | Analysis of Variance |
| bw | Body weight |
| CAR/PDMS | Carboxen/Polydimethylsiloxane |
| CCD | Central Composite Design |
| CYP | Cytocrome P450 |
| DAD | Diode Array Detection |
| DNA | Deoxyribonucleic acid |
| DNPH | 2,4-Dinitrophenylhydrazine |
| EFSA | European Food Safety Authority |
| FDA | Food and Drug Administration |
| fw | Fresh weight |
| GC-MS | Gas chromatography-Mass spectrometry |
| HMF | Hydroxymethylfurfural |
| HPLC | High Performance Liquid Chromatography |
| HS | Headspace |
| IARC | International Agency for Research on Cancer |
| IS | Internal standard |
| JECFA | Joint FAO/WHO Expert Committee on Food Additives |
| LC | Liquid chromatography |
| LOD | Limits of detection |
| LOQ | Limits of quantification |
| PA | Polyacrylate |
| PCA | Principal Component Analysis |
| PDMS | Polydimethylsiloxane |
| PUFAs | Polyunsaturated fatty acids |
| RP | Reversed-phase |
| RSD | Relative Standard Deviation |
| RSM | Response Surface Methodology |
| S | Static |
| SIM | Single Ion Monitoring |
| SMF | Sulfoxymethylfurfural |
| SPE | Solid Phase Extraction |
| SPME | Solid Phase Microextraction |

| | |
|---|---|
| SULTs | Sulfotransferases |
| TBA | 2-Thiobarbituric acid |
| TCA | Trichloroacetic acid |
| TIC | Total ion current |
| UV | Ultraviolet |
| WHO | World Health Organisation |

## General Scope and Objectives

Food heat-treatment serves three main purposes: i) firstly, the enhancement of desirable texture, flavour and colour, in order to make food products more palatable and appetizing for consumption; ii) secondly, the reduction of microbial content to eliminate potential food poisoning agents and finally, iii) achieve the preservation effects for an extended shelf life (storability) of the products.

The impact of high temperatures provokes coagulation and denaturation of proteins as well as structural and chemical changes of fats and carbohydrates, which makes foods even tastier and improves absorption of nutrients in the digestive tract. However, undesirable changes can also occur, such as loss of nutrients and/or the formation of harmful heat-generated compounds and off-flavours.

Food processing induces positive and negative factors with strong impact on food quality. The positive aspects are mostly associated to food safety, nutritional value, sensory quality and functional health benefits. On the other hand, negative aspects are related with loss of thermo labile compounds (i.e., vitamins and essential amino acids) and formation of undesired tastes and off-flavours. Moreover, the arising of neoformed contaminants, like furan and furanic compounds, is a well-established phenomena resulting in the loss of nutritional value, sensorial quality of heated foods and health concern.

Furan and furanic compounds represent a wide class of heterocyclic, low molecular weight molecules that are formed as products or intermediates in heat-induced reactions and can significantly contribute to the sensory properties of heated foods. For this reason, they are being considered of primary economic importance to the flavour industry. Since in 1995, furan was considered a possible carcinogenic compound (Group 2B) by the International Agency for Research on Cancer (IARC), several researchers start to develop new methodologies for the assessment of this compound in food. In 2004, the Food and Drug Administration (FDA) reported a headspace-gas chromatography coupled to mass spectrometry (HS-GC-MS) methodology to analyse furan in food with possible changes from other researchers. Indeed, because of the high temperatures applied in the FDA's methodology, other researchers found that it could generate additional furan amounts and the method was revised years after. Up till now, there are numerous methods for detection and quantification of furan in food, although none of them is an official methodology, and several drawbacks have been pointed out, since the complexity of cooked food matrices requires optimised and validated extraction procedures for each type of product.

Furanic compounds such as hydroxymethylfurfural (HMF) and furfural are considered chemical markers of severe heat treatment or inadequate storage conditions. The furanic ring and the possible liver and gut toxicity show the importance of analysing these compounds in food products. Since HMF and furfural are less volatile than the parent furan their monitoring in food products with a prolonged shelf-life are of great importance. Different analytical techniques have been applied for the analysis of HMF and furfural in foods. Among the different available techniques, colorimetric procedures are commonly used. However, due to the instability of the colour formed, the time required and the use of hazardous chemicals, these procedures are being substituted by more advanced techniques such as chromatographic techniques. The use of liquid chromatography (LC) revealed to be a more sensitive and reliable methodology, however the need of different extraction techniques (depending on matrix complexity) make difficult to choose an accurate methodology for the analysis of furanic compounds in foods with different characteristics. Thus, the natural composition variability of basic ingredients and type of processing requires the use of proper sample pre-treatment techniques. Different extraction and separation approaches should be used to isolate compounds with distinct physicochemical features (polarity, solubility, volatility, among others). Using the appropriate sample preparation methodology, analytical errors are reduced.

So far, no conclusive results are available in the literature in order to reduce furanic compounds in foods and readably applicable on industrial level. Most of the research available deals with model systems, while information on cooking conditions that can mitigate furan and furanic compounds formation in foods as they are usually consumed is very limited. Therefore, much caution is needed when extrapolating figures from simple model systems to real food products in order to avoid incorrect conclusions. Up today, only a few number of mitigation strategies have been demonstrated to effectively reduce furanic compounds levels in food, probably due to the great variety of precursors and formation pathways. Furanic compounds occur simultaneously with formation of colour, flavour and texture of heated foods. Figure I show that, excepting fresh raw products, furanic compounds are present in every step, namely, drying/roasting, retailing/processing and food preparation. Therefore, it is very difficult to mitigate their formation without affecting the food sensory acceptability.

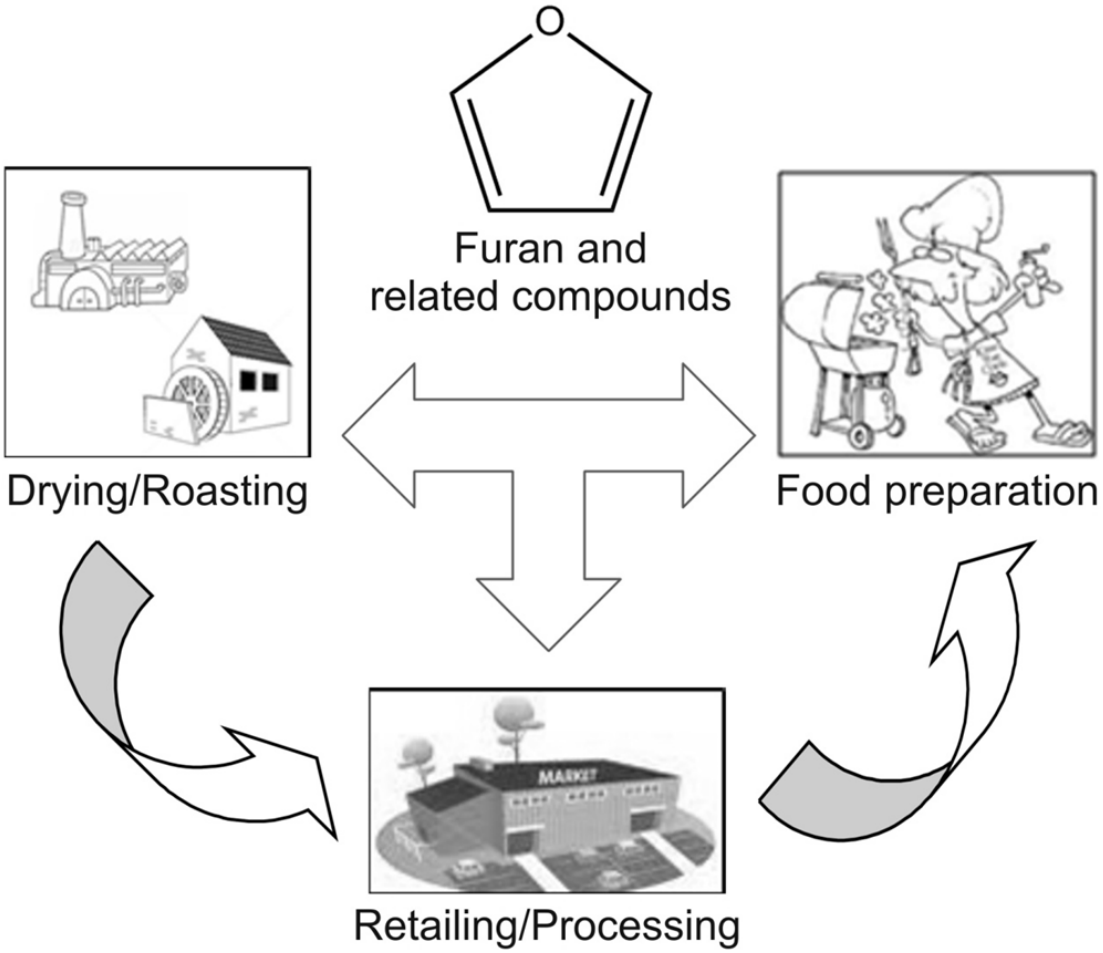



**Figure I:** Furanic compounds present in food chain.

The work described in this thesis was conducted to provide new insights about composition of furanic compounds in food products that are described as potential contributors to daily intake, namely, coffee, coated deep-fried and bakery products as well as the pursue for mitigation strategies that will reduce its formation keeping aroma and flavour characteristics. To achieve these major goals, different analytical methodologies were optimised and validated for screening and quantification of volatile and less volatile furanic compounds in the selected matrices, simulating usual preparation and consumption. Several specific goals were established and organised in three main topics:

*i)* Optimisation and validation of extraction procedures and chromatographic methodologies for analyses of furanic compounds, namely:

- Optimisation of solid-phase microextraction (SPME) sampling parameters for GC-MS analysis of volatile compounds, especially furan and furanic compounds presented in ground roasted and *espresso* coffee;

- Validation of a HS-SPME-GC-MS methodology for quantification of major furanic compounds and furan in *espresso* coffee;

- Validation of a HS-SPME-GC-MS methodology for quantification of volatile furanic compounds in coated deep-fried fish products simulating the eating process, performing the preparation as usually cooked and consumed using Response Surface Methodology (RSM) for selection of the most efficient extraction conditions;

- Validation of an extraction procedure and a high performance liquid chromatography coupled to diode array detection (HPLC-DAD) methodology for the analysis of HMF in coated-deep fried products, also simulating consumption conditions and using RSM approach;

- Validation of an extraction procedure and a HPLC-DAD methodology for the analysis of HMF and furfural in bakery products using different procedures presented in literature and choosing the best methodologies, according matrix complexity.

*ii)* Application of the validated methodologies for the monitoring:

- of furan and furanic compounds profile of ground roasted and *espresso* coffee subjected to different roast speeds;

- of furan and furanic compounds content in different types of *espresso* coffee from hermetically closed capsules including 100 % Coffee *Arabica*, blends of *Coffea Arabica* and *Coffea Robusta* and artificially aromatised coffee;

- of furan and furanic compounds content in coated deep-fried products according usual cooking and handling conditions;

- of the effect of time and temperatures, the use of different oils or alternatively replace the processing by dry-oven baking, reheating in the microwave oven or wait a period of time after cooking on furanic compounds;

- of HMF and furfural content in model cakes subject to different heating times and temperatures and using different oven types;

- of HMF and furfural content in commercial bakery products, investigating the effect of type of bakery product on the amount of HMF and furfural. The relationship between HMF and furfural content and formulation information provided by the manufacturer was also checked.

*iii*) Search for cooking/baking mitigation strategies to reduce furanic compounds content while keeping aroma and flavour characteristics in homemade processed products, namely, coated deep-fried and baked products:

- experiments related with search of mitigation strategies for reduction of furanic compounds in homemade processed products included analyses of volatile compounds with major relevance on aroma and flavour.

**Thesis organisation**

This thesis is divided in six parts and includes nine chapters (see page xxxix). Chapters are closely related to each other, and the approach chosen in each one was typically dependent on the conclusions attained in previous ones.

**Part I** include **Chapter 1** and present a brief overview of furan and other less volatile furanic compounds, namely, HMF and furfural, in order to answer six main questions: “How are these compounds formed?”; “Which is their distribution in foods?”; “Is their daily intake high?”; “Are these compounds genotoxic and /or mutagenic?”; “Which are the analytical methodologies more appropriate for their quantification?” and “What are the adequate mitigation strategies?”.

**Parts II, III and IV** include chapters 2 to 7 and are related with optimisation and validation of chromatographic analytical methodologies for the analyses of furan and/or furanic compounds in different foodstuffs**. Part II** deals with furanic compounds in coffee: **Chapter 2** describes optimisation of a HS-SPME-GC-MS methodology for the analysis of furan and other volatile compounds in ground and *espresso* coffee subject to different roasting

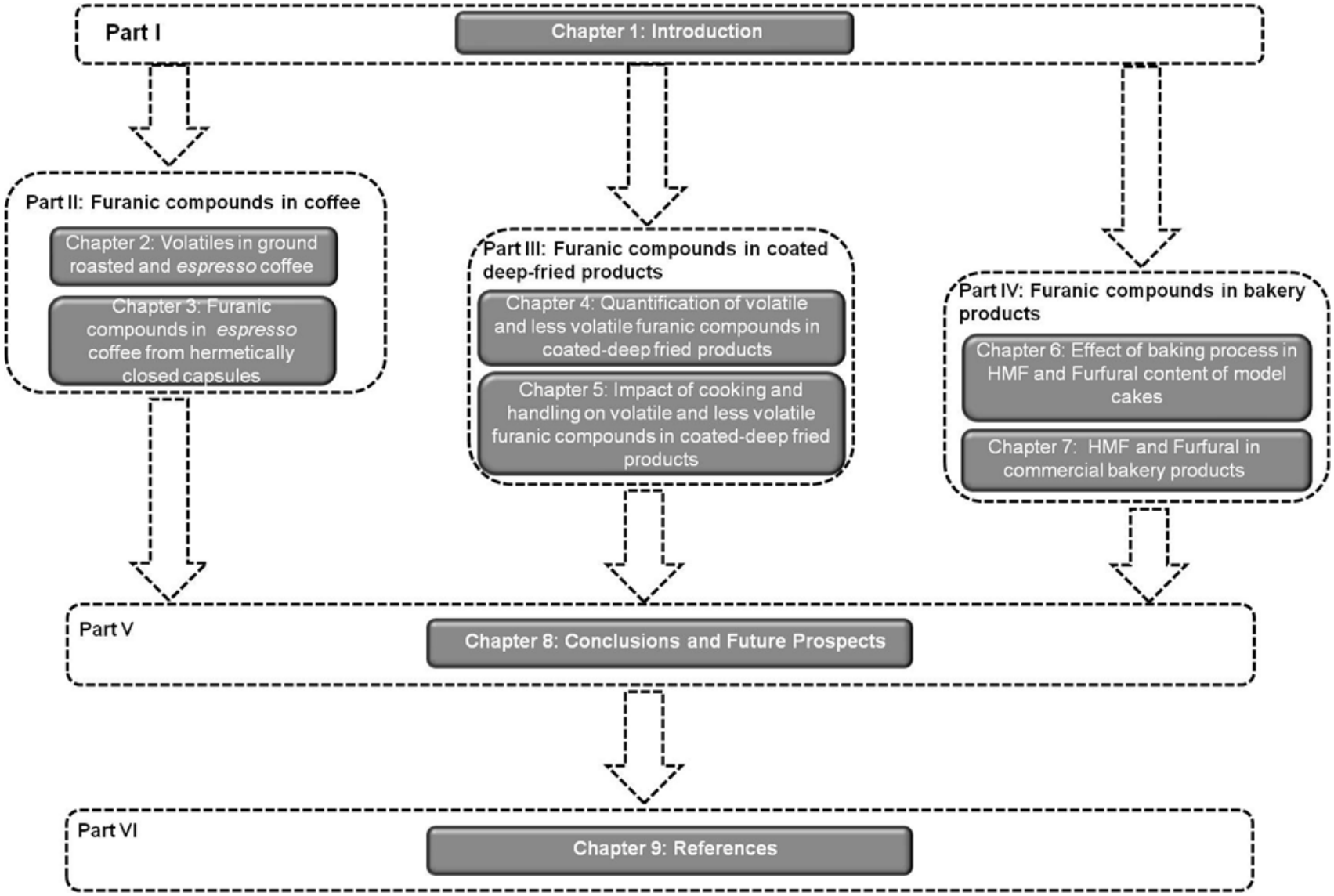
speed. **Chapter 3** describes the validation of the HS-SPME-GC-MS method previously optimised for evaluation of volatile profile in espresso coffee for the simultaneous quantification of major furanic compounds and furan in *espresso* coffee from hermetically closed capsules, and its application in the analyses of 100% Coffee *Arabica*, bends of C. *Arabica* and C. *Robusta* and artificially aromatized coffee to understand the real intake amount of furan and major furanic compounds in this type of *espresso* coffee widely consumed nowadays.

**Part IV** deals with furanic compounds in coated deep-fried fish products simulating normal preparation and consumption: **Chapter 4** presents the validation of two analytical methodologies for quantification of volatile and less volatile furanic compounds in coated-deep fried products performing its preparation as usually cooked and consumed and simulating the eating process. Volatile furanic compounds were quantified by HS-SPME-GC/MS and HMF was quantified by HPLC/DAD. Optimisation of the extraction conditions was performed in both methods by response surface methodology. **Chapter 5** describes the impact of cooking and handling conditions on volatile and less volatile furanic compounds in coated deep-fried products.

**Part IV includes Chapter 6** and **Chapter 7** that are related with HMF and furfural in bakery products. **Chapter 6** describes the effect of baking process in HMF and furfural content of model cakes, and mitigation strategies to reduce its formation. For this purpose a methodology was optimised and validated for efficient extraction of HMF and furfural from model cakes by HPLC. This methodology was applied to study the influence of different heating times and oven types in HMF content. Additionally, the influence of mitigation strategies on cake volatile profile was evaluated by SPME-GC/MS. In **Chapter 7**, the validated HPLC-DAD methodology previously described in Chapter 6 was extended to the analysis of different commercial bakery products and was an important tool to monitoring HMF and furfural amounts in this type of products.

**Part V** includes **Chapter 8** that presents the overall conclusions from this thesis as well as the future prospects.

**Part VI** includes **Chapter 9** with all the references cited throughout the thesis.


Part I
Chapter 1: Introduction
Part II: Furanic compounds in coffee
Chapter 2: Volatiles in ground roasted and *espresso* coffee
Chapter 3: Furanic compounds in *espresso* coffee from hermetically closed capsules
Part III: Furanic compounds in coated deep-fried products
Chapter 4: Quantification of volatile and less volatile furanic compounds in coated-deep fried products
Chapter 5: Impact of cooking and handling on volatile and less volatile furanic compounds in coated-deep fried products
Part IV: Furanic compounds in bakery products
Chapter 6: Effect of baking process in HMF and Furfural content of model cakes
Chapter 7: HMF and Furfural in commercial bakery products
Part V
Chapter 8: Conclusions and Future Prospects
Part VI
Chapter 9: References

# PART I

# CHAPTER 1

## Introduction

## 1.1. Introduction

Food heat-treatment is used since ancient times and is associated with the increase and preservation of organoleptic and nutritional properties. Continuous improvement of advanced thermal processes had impact on both social and economic development of Humanity. Since the late 19th century, the focus began to change from home cooking to more industrialized processes. It was only in the 20's that microbial safety and quality issues, especially nutritional quality issues started to be a health concern. Consequently, the technology is constantly improving and new technologies became available every day, such as steam, irradiation and microwave treatments with the objective of improving home-cooking while maintaining all the sensorial and quality properties. Nevertheless, food quality properties depends on several variables from "farm to fork", including the quality of the raw material, processing techniques, packaging and cooking (Van Boekel *et al.*, 2010).

Several factors on food processing have impact food quality; the most relevant positive aspects as well as negative aspects are summarized in Table 1.1.

**Table 1.1:** Positive and negatives aspects of food processing. (Adapted from Van Boekel *et al.*, 2010)

| Positive Aspects | Negative Aspects |
|---|---|
| **Food Safety** | |
| i) *Pathogens*: The main benefit of food processing is inactivation of food-borne pathogens, as is normally required by Food Safety Legislation. | i) Losses of certain (essential) nutrients due to chemical reactions (e.g. vitamin C, available lysine). |
| ii) *Other aspects*: inactivation of natural toxins and enzymes, prolongation of shelf-life. | |
| **Nutritional Value** | ii) Formation of undesired compounds, e.g. acrylamide, furanic compounds, heterocyclic amines, polycyclic aromatic hydrocarbons |
| iii) Improved digestibility, bioavailability of nutrients. | |
| **Sensory Quality** | iii) Formation of compounds that have a negative effect on flavour perception (off-flavours) |
| iv) Taste, texture and flavour. | |
| **Enhancement of health benefits** | iv) Loss of texture, discolouration among others. |
| v) Effects of processing on health promoting compounds (i.e. melanoidins) | |

The positive aspects of food processing are mostly related with four main issues: i) Food safety, namely the destruction and inactivation of food- borne pathogens, natural toxins and enzymes, this is the most beneficial effect; ii) Nutritional value, since heat-process enhance digestibility of food and nutrient bioavailability, for example, denatured proteins can be hydrolysed more easier than not denatured ones and the destruction of plant cell walls can improve the bioavailability of certain compounds; iii) Sensory quality, because thermal processes lead to the formation of desirable flavour compounds. Many neo-formed compounds presenting antioxidant, antimicrobial and antiallergenic effects as well as modulating activity *in vitro* have been detected in heated foods (Van Boekel *et al.*, 2010; Wang *et al.*, 2011; Echavarría *et al.*, 2012; Vhangani & Van Wyk, 2013); iv) Enhancement of health benefits - related to specific effects of processing on health promoting compounds (i.e. melanoidins) (Van Boekel *et al.*, 2010).

Detrimental effects of thermal processes are also relevant. The loss of thermo labile compounds such as vitamins, essential amino acids (lysine, tryptophan) and/or the formation of undesired tastes and off-flavours cause loss in the nutritional value and sensorial quality of heated foods (Languerre *et al.*, 2011; Van Lancker *et al.*; 2011; Capuano & Fogliano, 2011; Mesías *et al.*, 2012).

Heat-generated compounds may create a risk to human health. For some years, adverse effects from neo-formed contaminants have been the subject to increased attention, particularly acrylamide, nitrosamines, heterocyclic amines, polycyclic aromatic hydrocarbons, furanic compounds (Furan, HMF) and advanced glycation endproducts (Soares *et al.*, 2006; The HEATOX project, 2007; Lineback & Stadler, 2009; Costa *et al.,* 2009; Quelhas *et al.*, 2010; Viegas *et al.,* 2012b).

*Maillard* reaction is one of the most important sources of heat-generated compounds (Parker, 2013). Intensive studies have been carried out around this complex reaction that involves reducing sugars and amino acids creating characteristic flavours, aromas, and colours (browning) in heated foods. The emphasis has been given to the identification of the compounds involved in these attributes and in understanding the chemical pathways involved (Nursten, 2005).

### *1.1.1. Maillard Reaction*

The French chemist Louis Maillard was the first to describe the reaction, thus the name *Maillard* reaction, but it was afterwards, in 1953, that the first comprehensible scheme was put forward by Hodge in 1953 and later reviewed by Martins *et al.*, (2000) (Figure 1.1).

The term *Maillard* reaction is used for a multifaceted network of reactions involving carbonyl compounds, specifically reducing sugars, and amino compounds, such as amino acids or peptides. These reactions are also called nonenzymatic browning, as they are accompanied by the formation of brown pigments (melanoidins). Nevertheless, other reactions, such as caramelization, can also cause nonenzymatic browning of food. Therefore, the *Maillard* reaction should be considered as a special case of nonenzymatic browning (Davidek & Davidek, 2004).

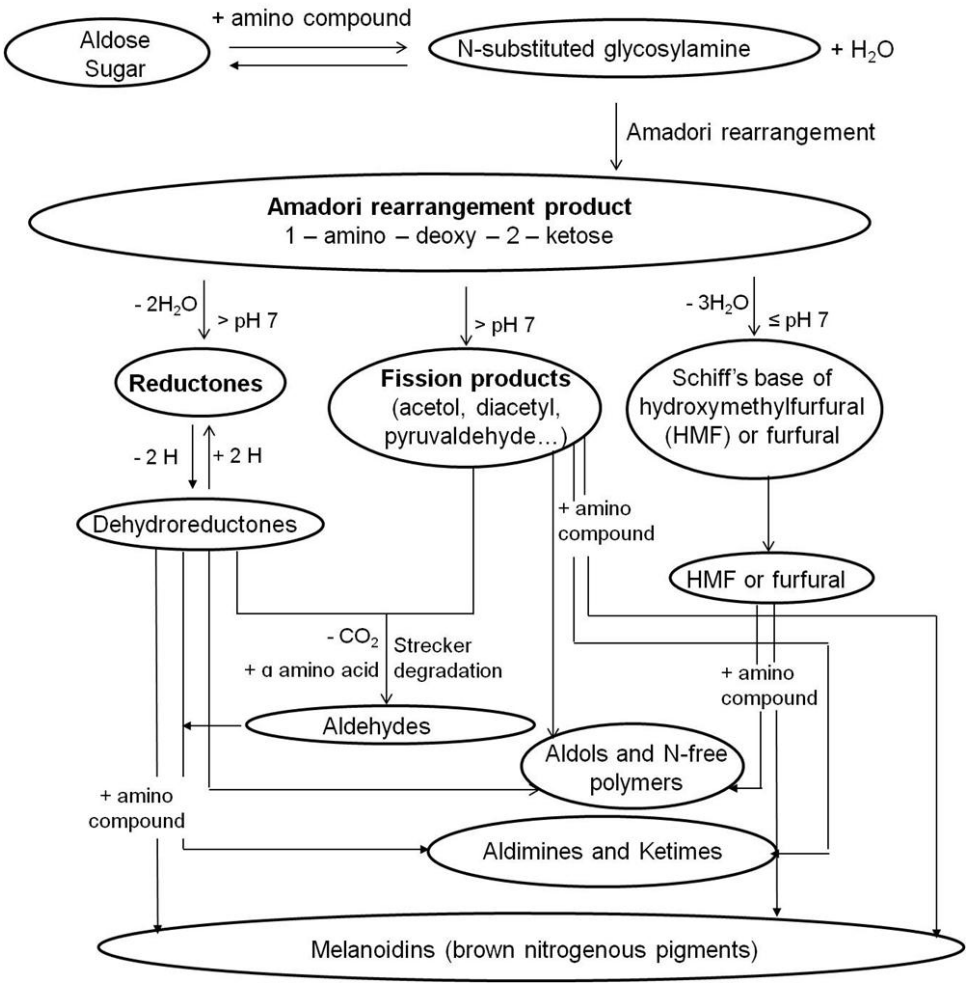



**Figure 1.1:** Scheme of the *Maillard* reaction (Adapted from Martins *et al.*, 2000).

The *Maillard* reaction occurs in almost every product namely when processing at elevated temperatures (> 150 ºC) or during storage for prolonged periods. As an example, it is of importance in the flavours formation in coffee and chocolate. The reaction begins with a condensation between a reducing sugar (e.g. glucose) and a compound having a free amino group of an amino acid or mainly the ε-amino group of lysine presented in proteins. The condensation product (N-substituted glycosylamine) is afterwards rearranged to form the *Amadori* product 1-amino-1-deoxy-2-ketose which is then degraded into different compounds depending on the pH of the system (pH < or > than 7) and ending in melanoidins formation (Figure 1.1). (Martins *et al.*, 2000; Nursten, 2005).

Nowadays, determining the origin of volatile compounds responsible for flavour is still a challenge due to their numerous origins. Once the technique of GC-MS was developed for the separation and identification of relatively volatile substances, the search for compounds with specific odours was greatly intensified. For example, Baltes & Bochmann, 1987 studied heat model systems of serine, threonine and sucrose and identified about 350 furans with different polarity and volatility. Its quantity and quality depend on the precursors, thermal processing parameters, pH, and quantitative ratio of amino nitrogen to reducing sugar, showing that the *Maillard* reaction is actually a complex network of various reactions involving reactants and products with high reactivity. Its mechanism is still a controversial issue; therefore the reaction is difficult to control and its study is still a challenge (Martins *et al.*, 2000; Nursten, 2005)

### *1.1.2. Furan and furanic compounds*

Furan and furanic compounds represent a wide class of heterocyclic, low molecular weight molecules that are formed as products or intermediates in heat-induced reactions and can significantly contribute to the sensory properties of heated foods (Maga, 1979). For this reason, they are being considered of primary economic importance to the flavour industry. Indeed, flavour-patent literature mainly regards the use of furan and related compounds as food flavourings and flavour enhancers. The common feature of furanic molecules is the furan ring that can present one or more substitutes. The number and nature of the functional groups greatly affect the chemical and physical properties of these molecules, such as their solubility and volatility (Budavari *et al.*, 2001). Among them, the most studied are Furan, HMF and Furfural (Crews & Castle, 2007; Abraham *et al.*, 2011; EFSA, 2004b). Figure 1.2 represents the furan ring and the possible R substitutions that origin furan derivatives.

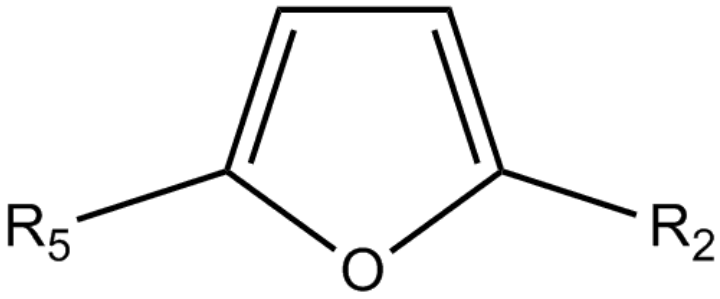



**Figure 1.2:** Structural formulas of the investigated compounds. Substituents $R_2$ and $R_5$ are H, unless specified otherwise (Furan = $C_4H_4O$). HMF ($R_2$ = CHO, $R_5$ = $CH_2OH$); Furfural ($R_2$ = CHO).

*1.1.2.1. Formation of furan and furanic compounds in food*

1.1.2.1.1. Furan

Furan is a colourless chemical ($C_4H_4O$) having a low molecular weight of 68.08 mg $mol^{-1}$ and a high volatility with the boiling point of 31 °C (Vranová & Ciesarová, 2009).

Heat-induced formation of furan in food can occur through a variety of pathways. Model reactions with individual compounds or mixtures of precursors provided important insight on mecanisms of furan formation during heating processes (Figure 1.3). It seems that the most important food constituents serving as precursors for furan production are ascorbic acid, unsaturated fatty acids, sugars, and amino acids (Crews & Castle, 2007).

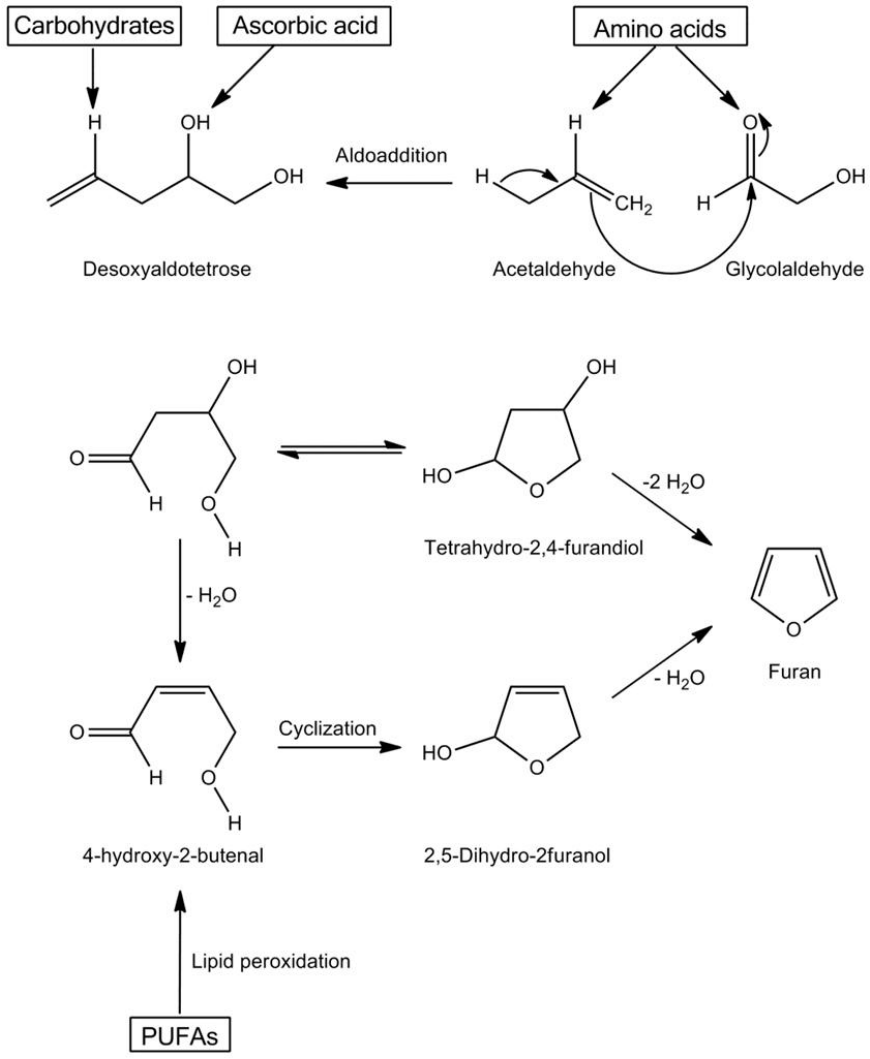



**Figure 1.3:** Potential routes of furan formation from different percurors present in food. PUFAs: polyunsaturated fatty acids (Adapted from Moro *et al.*, 2012).

Perez Locas & Yaylayan, (2004) made some experiments with furan precursors in order to determine the origin and mechanistic pathways of formation of this compound. These experiments showed that the most efficient precursor for the formation of furan is L-ascorbic acid followed by polyunsaturated fatty acids (PUFAs). The mechanism from PUFAs is initiated by their oxidative degradation through reactive oxygen species, resulting in the generation of a range of lipid peroxidation products (Figure 1.3). The resulting product 4-Hydroxy-2-butenal can form furan through cyclization and dehydration. The formation of furan through thermal degradation of sugars appears to involve formation of the reactive intermediates 1-deoxyosone and 3-deoxyosone from degradation of hexoses and pentoses, which further react to aldotetrose derivatives, such as aldotetrose itself, 2-deoxyaldotetrose, and 2-deoxy-3-ketoaldotetrose (Figure 1.3). Aldotetrose derivatives are also involved in the formation of furan from the degradation of ascorbic acid and dehydroascorbic acid. Finally, the mechanism of furan formation from amino acids involves the production of two key molecules acetaldehyde and glycolaldehyde. Both aldehydes occur as important intermediates in the thermal degradation of amino acids and are able to undergo aldol addition. The resulting 2-deoxyaldotetrose can then further react to yield furan (Figure 1.3) (Perez Locas & Yaylayan, 2004). Additionally, reaction conditions such as temperature, time, and pH can also significantly affect furan formation (Fan *et al.*, 2008).

At low-medium pH (4–7), other substituted furans can arise from the *Maillard* reaction, namely HMF represented in Figure 1.4 or furfural represented in Figure 1.5 (when hexoses or pentoses are involved, respectively). These furanic compounds are formed via enolization, and are highly reactive compounds that take part in further reactions.

1.1.2.1.2. HMF

HMF is considered a final product of the Maillard reaction and is considered a chemical marker of drastic thermal conditions during food processing. In theory, HMF can react further by decarboxylation, oxidation, dehydration, and polycondensation reactions giving origin to other furanic compounds, such as 5 - methyfurfural, and furfural (Luijkx *et al.*, 1993; Morales, 2009; Arribas-Lorenzo & Morales, 2010; Nikolov & Yaylayan, 2011).

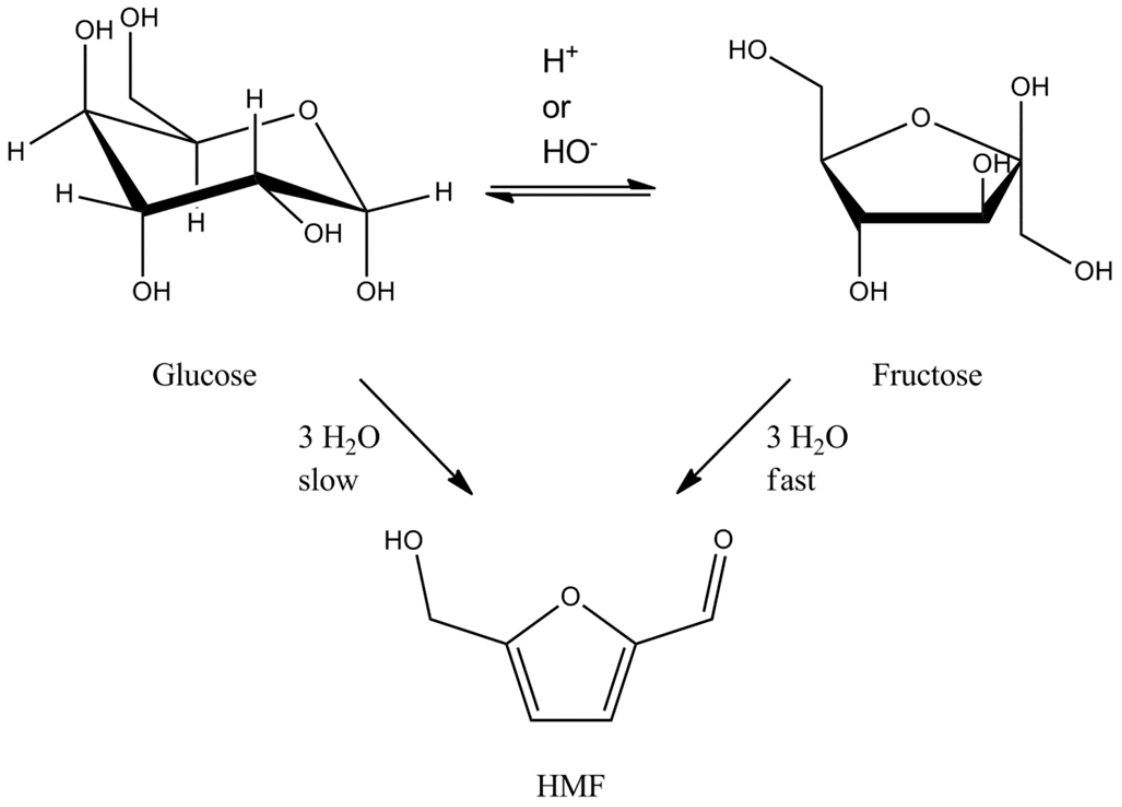



**Figure 1.4**: Scheme of the main routes for HMF formation during heat processing of foods (Adapted from Verendel *et al.*, 2011).

1.1.2.1.3. Furfural

Furfural is present in many fruits and in tea, coffee and cocoa. It is produced during the acid hydrolysis or heating of polysaccharides containing hexose or pentose fragments (Paine *et al.*, 2008).

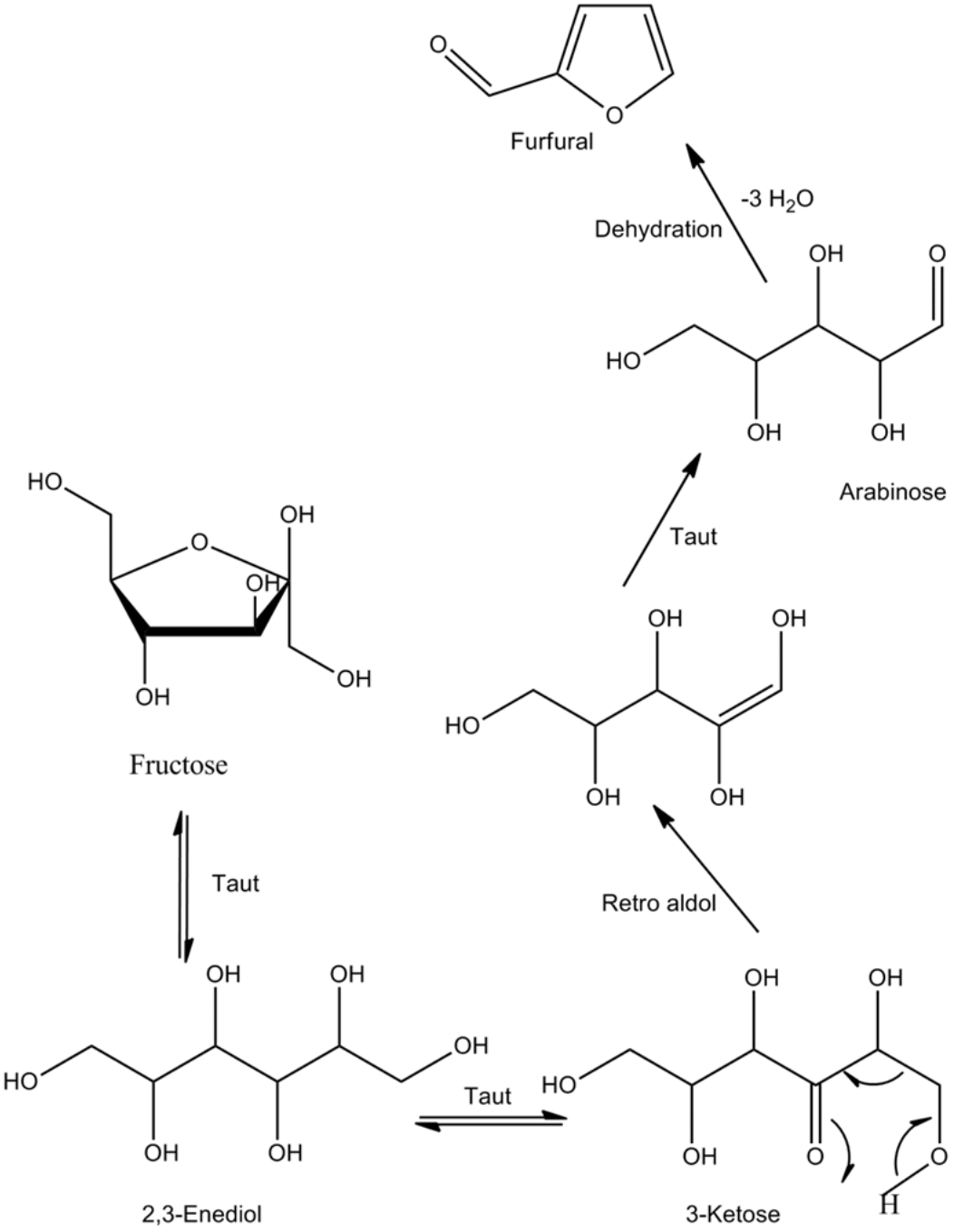



**Figure 1.5:** Estimated reaction mechanism to furfural from d-glucose. **Taut:** Tautomerization (Adapted from: Aida *et al.*, 2007).

The interconversion of furfural in other furfuryl compounds provided the basis for the JECFA allocating a Group Acceptable Daily Intake (ADI) of 0-0.5 mg $kg^{-1}$ body weight (bw) for furfural, furfuryl alcohol, furfuryl acetate, among others (JECFA, 2002). Additionally, the possibility of furfural being an intermediate in furan formation should be taken into account and more studies should be assessed (Figure 1.6) (Becalski & Seaman, 2005).

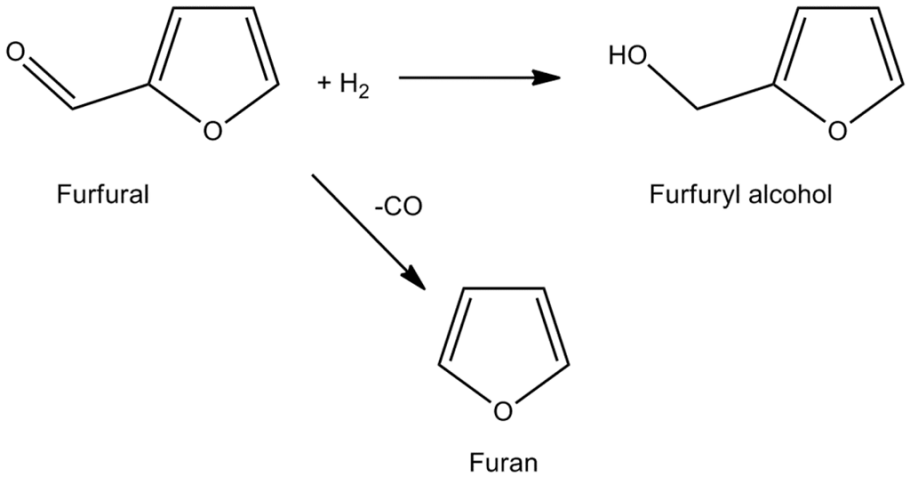



**Figure 1.6**: Possible hydrogenation products of furfural, arising from the reduction of the C=O group and/or the furan ring (Adapted from Merlo *et al.*, 2009).

In conclusion, HMF and furfural can respond by several reactions to form different intermediates, achieving the final *Maillard* reaction products. Figure 1.7 summarised the possible routes.

.

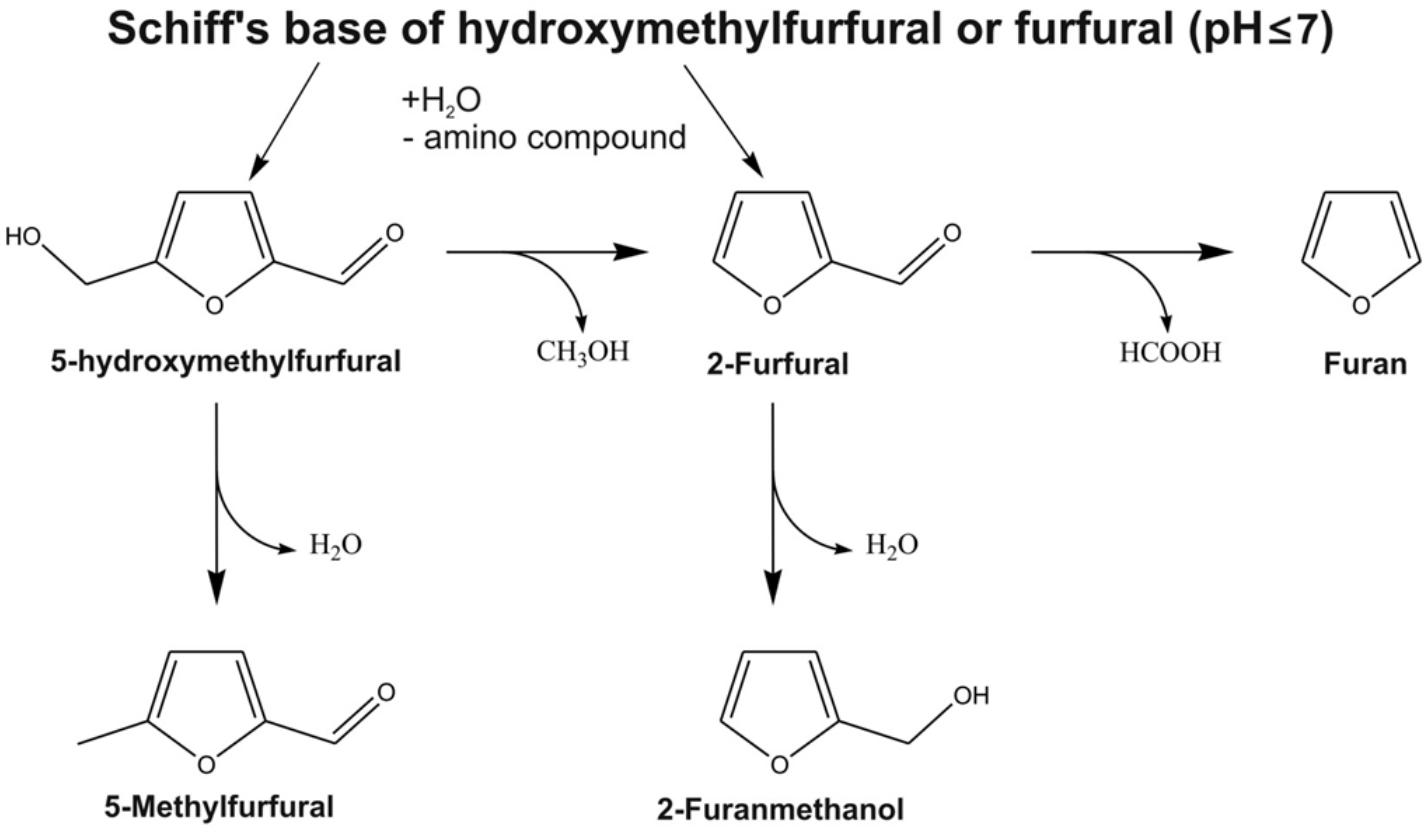



**Figure 1.7:** Summary of possible furanic compounds routes.

*1.1.2.2. Assessment of furan and furanic compounds in food*

In 2004, FDA announced the findings of its exploratory surveys of furan in selected foods. Since then, the discover of analytical methodologies, risk assessment and mitigation strategies of furan and its derivatives has largely increased, being an important issue of health concern.

1.1.2.2.1. Furan

Since FDA scientists first identified furan in a number of heat-treated food items (FDA, 2004), a large number of samples from a wide variety of food categories that undergo heat processing have been analysed worldwide. Results from these analyses have been published as individual reports or assembled in online databases on furan content in food (EFSA, 2004a; FDA, 2004; Kuballa *et al.*, 2005; EFSA, 2011a). Representative data on furan in various food categories reported by the European Food Safety Authority (EFSA) (EFSA, 2011a) are shown in Table 1.2.

Between 2004 and 2010, a total of 5 050 results were reported for foods samples. Data was distributed into 21 different food categories (some of them with subcategories). By far the highest contents are found in solid coffee category, especially for roasted bean and roasted ground coffee. However, it can be observed that the furan content is considerably lower for the coffee brewed samples. Several publications reported substantial reductions (up to 50 %) of the furan content after brewing depending on the method used (Hasnip *et al.*, 2006; Kuballa *et al.*, 2005; La Pera *et al.*, 2009; Zoller *et al.*, 2007). Automatic coffee machines produce brews with higher levels of furan, because a higher ratio of coffee powder to water is often used giving a lower dilution factor and because of the closed system favouring retention of furan (Kuballa *et al.*, 2005; Zoller *et al.*, 2007; Crews *et al.*, 2009; Altaki *et al.*, 2011). Standard home coffee-making machines produced much lower levels. Other food group with particular interest in furan content is baby food since they may form the sole diet for many infants. The lowest value found was for baby food containing fruits and vegetables and the highest content was found in baby food containing only vegetables. Additionally, furan contents of more than 100 g furan $kg^{-1}$ food were found in certain processed soups, cereals products, meat products, and sauces, all prepared by temperature treatments (EFSA, 2010; 2011a).

High concentrations of furan or contradicting levels of contamination were also reported for certain food categories such as fruit and vegetables juices, nutrition drinks, and bakery

products (EFSA, 2009a, 2010; 2011a). Thus, recent HS-GC analyses were conducted and focused on the analysis of furan in these food categories. High furan levels were found in sterilized baby carrot juices, in prune juices, and in different flavours of a particular type of pharmaceutical nutrition drink. Moreover, bakery products, especially rye and whole grain-based products, showed high furan levels (Wegener *et al.*, 2010; Moro *et al.*, 2012).

For future works, EFSA (2011a) advertises the need of reducing uncertainty associated with the exposure estimates. It would be beneficial if future testing of furan comprises analyses of samples as purchased followed by analyses of the same samples as consumed, indicating the exact cooking preparation with time, temperature and handling information.

**Table 1.2:** Representative furan data presented in several food categories (Adapted from EFSA, 2011a).

| Product category | Furan (µg kg$^{-1}$) | | |
|---|---|---|---|
| **Solid coffee** | **N** | **P25** | **Max** |
| Coffee, roasted bean | 30 | 2160 | 11000 |
| Coffee, roasted ground | 110 | 476 | 6900 |
| Coffee, not specified | 596 | 1040 | 6588 |
| Coffee instant | 109 | 58 | 2200 |
| **Coffee brew** | | | |
| Coffee, roasted bean | 10 | 63 | 360 |
| Coffee, not specified | 13 | 0 - 9 | 248 |
| Coffee, roasted ground | 51 | 0 - 5.8 | 228 |
| Coffee instant | 15 | 0 - 5 | 0 - 10 |
| **Baby food** | | | |
| Vegetables only | 281 | 22 | 233 |
| Non specified | 303 | 4.7 - 8.4 | 215 |
| Meat and vegetables | 550 | 24 | 169 |
| Cereal based | 163 | 1.0 - 10 | 96 |
| Fruits and vegetables | 70 | 0 - 3.4 | 66 |
| Fruits only | 250 | 0 - 2.2 | 58 |
| Infant formula | 11 | 0 - 2.5 | 2.2 - 10 |
| Soups | 270 | 2.5 - 5 | 225 |
| Sauces | 271 | 0 - 4 | 175 |
| Fish | 47 | 0 - 1.2 | 172 |
| Cereal product | 190 | 0 - 3.3 | 168 |
| Meat products | 174 | 0 - 4 | 160 |
| Fruit juice | 250 | 0 - 1.5 | 90 |
| Baked beans | 57 | 2.7 - 10 | 80 |
| Milk products | 64 | 0 - 0.3 | 80 |
| Soy sauce | 94 | 14 | 78 |
| Vegetables | 192 | 0 - 3 | 74 |
| Vegetable juice | 80 | 0.3 - 6 | 60 |
| Fruits | 142 | 0 - 3 | 36 |
| Beer | 102 | 0 -2 | 28 |
| **Others** | | | |
| Snacks and crisps | 133 | 4.7 - 5.1 | 47 |
| Cocoa | 14 | 2.6 - 3.7 | 40 |
| Sweets | 61 | 0 - 2 | 34 |
| Soy products | 15 | 0.5 | 28 |
| Vegetable fats | 13 | 0 - 0.32 | 10 |
| Wine and liquors | 20 | 0.38 | 6.5 |
| Soft drinks | 18 | 0.2 - 0.6 | 4.5 |
| Tea | 22 | 0 - 1 | 3.7 |

**N** – Number of samples analysed.

**P25** – 25$^{th}$ Percentile

**Max** – Maximum content found.

#### 1.1.2.2.2. HMF

HMF is well known as a marker of food quality, it is generated as a result of excessive heating or inadequate storage conditions in a wide range of foods containing carbohydrates. The HMF amount formed in carbohydrate-rich foods is related with the heat load applied during processing. Another sources of HMF are the ingredients used in the formulations, such as, caramel or honey. HMF concentrations in foods are widely variable, exceeding sometimes 1 g $kg^{-1}$ in dried fruits and caramel products (Murkovic & Pichler, 2006; Gaspar & Lucena, 2009; Cocchi *et al.*, 2011). HMF is also found in bakery products, malt, fruit juices, coffee, and vinegar. A summary of HMF content in foods is shown in Table 1.3. Generally, HMF is used as a quality marker of food products such as processed fruits (Rada-Mendoza *et al.*, 2002, 2004; Louarme & Billaud, 2012; Teixidó *et al.*, 2011), coffee (Murkovic & Bornik, 2007; Del Campo *et al.*, 2010) honey (Tosi *et al.*, 2002; Fallico *et al.*, 2004; Estevinho *et al.*, 2012) and milk (Van Boekel, 1998; Morales & Jiménez-Pérez, 2001; Le *et al.*, 2011). HMF is also used for monitoring the heating processes applied to cereal products such as pasta drying (Doxastakis *et al.*, 2007), bread baking (Ramírez-Jiménez *et al.*, 2000a; Ramírez-Jiménez *et al.*, 2000b), bread slices toasting (Ramírez-Jiménez *et al.*, 2001; Kirit *et al.*, 2012) as well as extrusion of baby cereals (Fernández-Artigas *et al.*, 1999; Ramírez-Jiménez *et al.*, 2003) and breakfast cereals (Rufián-Henares *et al.*, 2006a; Delgado-Andrade *et al.*, 2008). Although the concentrations in some food products such as dried fruits, caramel and vinegar are extremely high, bread and coffee are the most important contributors to dietary HMF intake (Murkovic & Pichler, 2006).

**Table 1.3:** HMF content in a wide range of food products.

| Product category | HMF (mg kg$^{-1}$) | Reference |
|---|---|---|
| **Coffee products** | | |
| Coffee | 24 - 2186 | Murkovic & Pichler, 2006; Arribas-Lorenzo & Morales, 2010 |
| Coffee instant | 400 - 4100 | Murkovic & Pichler, 2006; Arribas-Lorenzo & Morales, 2010 |
| Coffee decaffeinated | 430 - 983.2 | Kanjahn *et al.*, 1996; Arribas-Lorenzo & Morales, 2010 |
| Honey | 0.0 - 1145 | Abraham *et al.*, 2011; Rizelio *et al.*, 2012; Foo Wong *et al.*, 2012 |
| Beer | 3.3 - 9.2* | Lo Coco *et al.*, 1995; Yuan & Chen, 1998 |
| Jam | 2.7 - 410.9 | Vorlová *et al.*, 2006; Teixidó *et al.*, 2011; Abraham *et al.*, 2011 |
| Fruit juices | 0.2 - 707.7 | Vorlová *et al.*, 2006; Gaspar & Lucena, 2009; Teixidó *et al.*, 2011; Abraham *et al.*, 2011; Zhang *et al.*, 2012 |
| Cookies | 0.49 - 74.6 | Ait-Ameur *et al.*, 2006 |
| Biscuits | 0 - 182.5 | Delgado-Andrade *et al.*, 2009; Teixidó *et al.*, 2011 |
| **Bread products** | | |
| White | 3.4 - 68.8 | Ramírez-Jimenéz *et al.*, 2000a |
| Bread (crumb) | 0.6 - 2.2 | Ramírez-Jimenéz *et al.*, 2000a |
| Bread (crust) | 18.3 - 176.1 | Ramírez-Jimenéz *et al.*, 2000a |
| Toasts | 11.8 - 590.7 | Ramírez-Jimenéz *et al.*, 2000a; Teixidó *et al.*, 2011 |
| Snacks | 2.2 - 10 | Ramírez-Jimenéz *et al.*, 2000a |
| French mini bread | 1.87 - 2.05 | Fang *et al.*, 2012 |
| French soft bread | 1.38 - 1.41 | Fang *et al.*, 2012 |
| Rye-wheat bread | 44.5 | Abraham *et al.*, 2011 |
| Bakery products | 4.1 - 151.2 | Ramírez-Jimenéz *et al.*, 2000b |
| **Cereal products** | | |
| Breakfast cereals | 6.9 - 240.5 | Rufián-Henares *et al.*, 2006b; Teixidó *et al.*, 2011 |
| Infant cereals | 0.4 - 65.5 | Ramírez-Jimenéz *et al.*, 2003 |
| Cereal bar | 43.2 | Abraham *et al.*, 2011 |
| **Baby food** | | |
| Milk-based | 0.18 - 0.25 | Gökmen & Şenyuva, 2006 |
| Cereal-based | 0 - 57.18 | Gökmen & Şenyuva, 2006 |
| Fruit | 0 - 28.9 | Vorlová *et al.*, 2006; Čížková *et al.*, 2009; Mesías-García *et al.*, 2010 |
| Vegetable | 0.58 - 2.87 | Mesías-García *et al.*, 2010 |
| Dried fruits | 61.6 - 2900 | De La Iglesia *et al.*, 1997; Murkovic & Pichler, 2006; Abraham *et al.*, 2011 |
| Roasted almonds | 0.04 - 155.5 | Agila & Barringer, 2012; Abraham *et al.*, 2011 |
| **Vinegars** | | |
| Wine | 0 - 21.5* | Theobald *et al.*, 1998 |
| Balsamic | 4859 - 6550 | Cocchi *et al.*, 2011 |
| **Wines and spirits** | | |
| Red wine | 1.0 - 1.3* | Yuan & Chen, 1998 |
| Mulled wine | 13.7 | Abraham *et al.*, 2011 |
| Whiskey | 1.4 - 13.1* | De La Iglesia *et al.*, 1997; Jaganathan & Dugar, 1999 |
| Brandy | 3.15 – 9.97* | Goldberg *et al.*, 1999; Canas *et al.*, 2003 |
| Rum | 1.7 – 14.9* | Goldberg *et al.*, 1999; Alcázar *et al.*, 2006 |
| Madeira wines | 4.80 - 491.9* | Pereira *et al.*, 2011 |
| **Chocolate** | | |
| Milk | 87.4 - 164.7 | Teixidó *et al.*, 2011 |
| Dark | 42.1 - 75.6 | Teixidó *et al.*, 2011 |
| White | 98.9 | Teixidó *et al.*, 2011 |
| Praline | 273.8 | Abraham *et al.*, 2011 |

**Table 1.3:** *(continued)*

| Product category | HMF (mg $kg^{-1}$) | Reference |
|---|---|---|
| **Beverage powder** | | |
| W/ coffee | 286.1 | Abraham *et al.*, 2011 |
| W/ cocoa | 503.8 | Abraham *et al.*, 2011 |
| **Soft beverages** | | |
| Carbonated | 0.17 - 8.26* | Zhang *et al.*, 2012a; Wang & Schnute, 2012 |
| Tea | 0.05 - 1.63* | Zhang *et al.*, 2012a |
| Caramel products | 1500 - 2528 | De La Iglesia *et al.*, 1997; Gaspar & Lucena, 2009 |
| **Others** | | |
| Wheat chicken nuggets | 0.77 - 0.95 | Fang *et al.*, 2012 |
| Coat-deep fried products | 1.25 | Pérez-Palacios *et al.*, 2013 |
| Potato chips | 1.82 - 2.05 | Fang *et al.*, 2012 |

*mg/L

The levels of HMF may vary considerably between the individual food groups and even in the same food depending mainly on the processing conditions applied. Thus, sufficient measurements for certain foods should be available. This aspect is important in order to be able to record the variability, as representatively as possible, as well as, detailed information on the processing and production processes, giving hints for the formation of HMF in food. The great variability in HMF contents leads to unquantifiable uncertainties with regard to intake estimates. Several studies (Murkovic & Pichler, 2006; Husøy *et al.*, 2008; Rufián-Henares *et al.*, 2008; Arribas-Lorenzo & Morales, 2010; Abraham *et al.*, 2011) have been carried out for quantification of HMF in different kinds of foods, however, the data on exposure are still incomplete; especially data on levels in coffee (beverage) and dairy products are scarce. Moreover, there is no indication of other foods that contain particularly high levels of HMF. It should be pointed out that, on the basis of the available data, there is a need for analysis of HMF content in foods (Abraham *et al.*, 2011).

1.1.2.2.3. Furfural

Furfural is used as a flavouring agent in a variety of food products and alcoholic and non-alcoholic beverages. Moreover, furfural and many of its derivatives occur widely as natural constituents of the food supply. It has been detected in a broad range of processed fruits and fruit juices, vinegars, spirits, caramel, among others. The highest concentrations of furfural in foods have been reported in cocoa and coffee (55-255 mg $kg^{-1}$), caramel (239.2 mg $kg^{-1}$), spirits (0.25-23.46 mg $L^{-1}$), and vinegars (0.10-34 mg $kg^{-1}$), as can be seen in Table 1.4 (WHO, 1999).

As observed for HMF, the furfural content in foods may vary significantly between food categories and also in the same food product. Thus, it is important to record the variability within each product as representatively as possible, as well as sufficient measurements should be available.

**Table 1.4:** Summary of furfural content in food products.

| Product category | Furfural (mg $kg^{-1}$)* | References |
|---|---|---|
| Coffee and cocoa | 55 - 255 | Maarse *et al.*, 1994 |
| Fruit juices | 0.3 – 9.4* | Lo Coco *et al.*, 1997; Yuan & Chen, 1998; Zang *et al.*, 2012a |
| Beer | 0.08 – 0.35* | Lo Coco *et al.*, 1995; Yuan & Chen, 1998 |
| Honey | 0.52 – 50.6 | Lo Coco *et al.*, 1996; Gaspar & Lucena, 2009; Foo Wong *et al.*, 2012 |
| **Infant formulas** | | |
| Liquid | 0.115 – 0.245* | Albalá-Hurtado *et al.*, 1997 |
| Powdered | 0.162 – 0.229* | Albalá-Hurtado *et al.*, 1997 |
| Cow | 0.043 – 0.101* | Albalá-Hurtado *et al.*, 1997 |
| Adapted and follow-up | 0.002 – 0.005* | Ferrer *et al.*, 2002 |
| **Vinegars** | | |
| Balsamic | 2.60 - 34 | Gaspar & Lopes, 2009; Cocci *et al.*, 2011 |
| Red and white wine | 0.10 – 1.35* | Giordano *et al.*, 2003 |
| Oils (nuts and seeds) | 1.4 – 8.7 | Durrmaz & Gökmen, 2010 |
| **Spirits** | | |
| Bourbon | 9.92 – 10.95* | Alcázar *et al.*, 2006 |
| Almond liquor | 2.22 – 2.39* | Alcázar *et al.*, 2006 |
| Brandies | 0.7 – 23.46* | Canas *et al.*, 2003; Goldberg *et al.*, 1999 |
| Whiskeys | 0.25 -19.2* | Goldberg *et al.*, 1999; Jaganathan & Dugar, 1999 |
| Rum | 0.25* | Goldberg *et al.*, 1999 |
| Madeira wines | n.d. – 9.77* | Pereira *et al.*, 2011 |
| Red and white wines | 0.1 – 0.3* | Yuan & Chen, 1998 |
| Fruit desserts | n.d. – 5.7 | Louarme & Billaud, 2012 |
| Caramel | 239.2 | Gaspar & Lucena, 2009 |
| Fruit baby food | 2.1 – 3.3 | Čížková *et al.*, 2009 |
| Cola | 0.2* | Yuan & Chen, 1998 |
| Carbonated beverages | n.d. – 0.39* | Zang *et al.*, 2012a |
| Tea | n.d. – 0.37 | Zang *et al.*, 2012a |

*mg $L^{-1}$

*1.1.2.3. Dietary intake*

1.1.2.3.1. Furan

In Europe and according to the EFSA (2011a), the mean upper bound furan intake across surveys for adults was estimated to range between 0.03 and 0.59 μg $kg^{-1}$ bw *per* day. Coffee category was identified as the major contributor to the overall adult furan exposure. Minor potential contributors to adult furan exposure include beer, ready-to-eat soups, sauces and fruit juices. Regarding adolescents, the mean furan intake was estimated to range between 0.02 and 0.13 μg $kg^{-1}$ bw *per* day. Coffee was still a major contributor, but to a lesser degree than for adult population. Other major contributors were cereal-based products, ready-to-eat soups, sauces, and fruit juice. For children mean intake was estimated to range from 0.04 to 0.22 μg $kg^{-1}$ bw *per* day and for toddlers mean intake was estimated to range between 0.05 to 0.31 μg $kg^{-1}$ bw *per* day. The major contributors to furan exposure in these two population groups were fruit juice, milk-based products and cereal-based products. Additionally for toddlers, jarred baby foods were also major contributors. For infants, there is limited available information however the mean exposure was estimated to range between 0.09 and 0.22 μg $kg^{-1}$ bw *per* day. The main contributors for furan exposure were jarred baby food and ready-to-eat soups (EFSA, 2011a).

Regarding United States exposure, estimates for furan are similar to those made in Europe for children, but inferior for adults. The lower intake estimated for adults may be related with the relatively low coffee consumption in the United States (WHO, 2011; Moro *et al.*, 2012).

1.1.2.3.2. HMF

To our knowledge, there are few studies regarding the dietary intake of HMF. The earlier estimate for the intake of this furanic compound ranged from 30 to 150 mg $person^{-1}$ and was reported by Janzowski, *et al.* (2000). In 2008, Rufián-Henares & De la Cueva, estimated the daily intake for Spanish population concerning three different scenarios: minimum (2.1 mg $day^{-1}$), median (9.7 mg $day^{-1}$) and maximum (23 mg $day^{-1}$) HMF content in food. They concluded that the estimated median intake was about 10 mg $person^{-1}$ $day^{-1}$, which is 20-fold above the threshold of concern stated by EFSA in 2005 (540 μg $person^{-1}$ $day^{-1}$). The results of these authors are in accordance with those reported by Delgado-Andrade *et al.* in 2007, who have calculated a daily HMF intake of 5.1 mg for Spanish adolescents, measured in the whole diet after a 24 h recall. Husøy *et al.* (2008) also

estimated the HMF daily intake by using 24 h dietary recalls from 53 Norwegian volunteers. They reported that the mean daily intake for HMF was 5.6 mg person$^{-1}$. Moreover, in a study conducted in Germany, Abraham *et al.* (2011) estimated the HMF intake between 67 and 215 µg kg$^{-1}$ bw day$^{-1}$. The HMF intake was calculated for mean concentrations combined with mean and high consumption figures. Additionally, due to missing occurrence data from Germany concerning coffee intake, it was not possible to include this type of food as a source of HMF exposure, however, based on data in coffee reported by Spanish and Norwegian studies described above, total daily exposure in Germany could be much higher. Nevertheless data on dietary exposure is still very limited. Additional studies are therefore needed to assess average, medium and maximum intake for different populations and segments of population. For an accurate estimate of dietary intake, data on the concentrations of HMF in several foods as they are eaten are necessary. As highlighted for other food toxicants, domestic storage and cooking conditions may strongly affect the actual exposure to HMF (Capuano & Fogliano, 2011).

#### 1.1.2.3.3. Furfural

Studies regarding furfural intake are scarce. Because it is used as a flavouring substance ingredient in many food categories, the estimated daily *per* capita furfural intake in Europe is approximately 10 µg kg$^{-1}$ bw day$^{-1}$ (IOFI, 1995) and the intake in the United States of America has been estimated to be about 2 µg kg$^{-1}$ bw day$^{-1}$ (Adams *et al.*, 1997). The theoretical maximum daily intake assuming that consumers consume at all times all flavoured foods with maximum concentrations, has been estimated to be 136 µg kg$^{-1}$ bw (CEFS, 1998). Thus, the intake of furfural from use as flavouring substance represents 1-7 % of the total intake (EFSA, 2004b; WHO, 1999).

*1.1.2.4. Genotoxicity and mutagenicity of furan and furanic compounds*

1.1.2.4.1. Furan

Furan has been classified as a possible carcinogen (Group 2B) by IARC. The Group 2B is assigned to compounds and exposure conditions for which there is limited evidence of carcinogenicity in humans and insufficient evidence of carcinogenicity in experimental animals, requiring therefore more studies (IARC, 1995).

Studies on furan toxicokinetics in rats show that furan is well absorbed in gastrointestinal tract, extensively metabolized, and eliminated via expired air, urine, and faeces. Furan is metabolized by cytochrome P450 (CYP) enzymes, mostly by CYP2E1, to its major metabolite cis-2-butene-1,4-dial, a highly reactive electrophile recognized as the key mediator of furan toxicity and carcinogenicity (Peterson *et al.*, 2000; Selmanoğlu *et al.*, 2012) (Figure 1.8). Due to the relatively high activity of CYP2E1 to bioactivate furan to its reactive intermediate, the liver is the most important target organ of furan toxicity (Selmanoğlu *et al.*, 2012; Moro *et al.*, 2012).

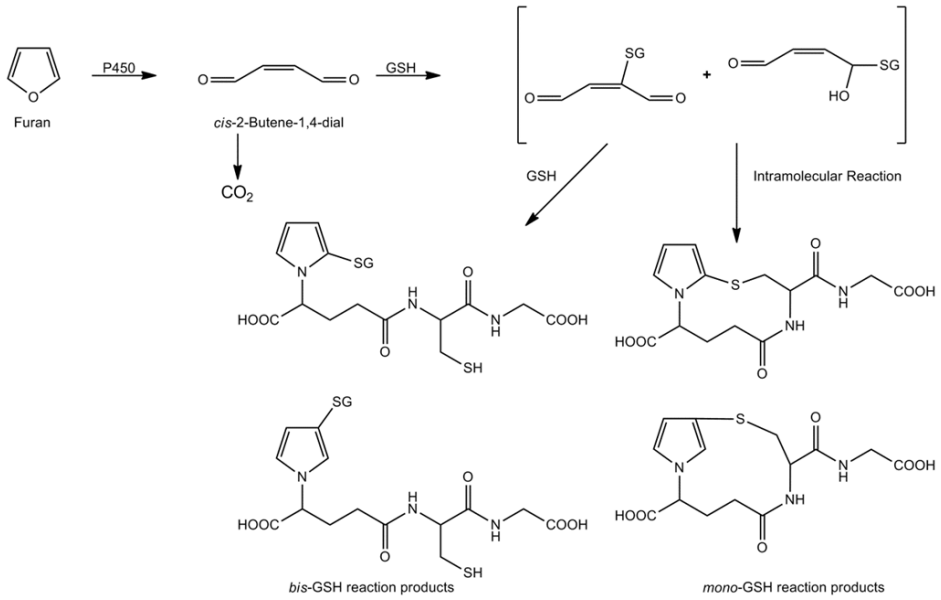



**Figure 1.8:** Cytochrome P450 mediated bioactivation of furan and reactivity of cis-2-butene-1,4-dial (Adapted from Peterson *et al.*, 2006).

The hepatocarcinogenicity and toxicity of furan requires metabolic activation to develop its toxic effects. Results from Peterson *et al.*, (2006) indicates that the overall metabolism of furan is initiated via CYP catalyzed oxidation to cis-2-butene-1,4-dial. This α,β-unsaturated dialdehyde reacts in vitro with protein and DNA nucleophiles.

1.1.2.4.2. HMF

Based on data reported in literature it is not clear whether human exposure to HMF represents a potential health risk. Janzowski *et al.,* (2000) concluded that HMF does not pose a serious health risk in cell systems, even though the highest concentrations in specific foods approach the biologically effective concentration range. The major concern for HMF is related to its conversion to sulphoxymethylfurfural (SMF) by sulphonation of the allylic hydroxyl function of HMF catalyzed by sulfotransferases (SULTs). The resulting sulphate ester can induce genotoxic and mutagenic effects through a highly electrophilic allyl carbocation (Figure1.9) (Abraham, 2011).

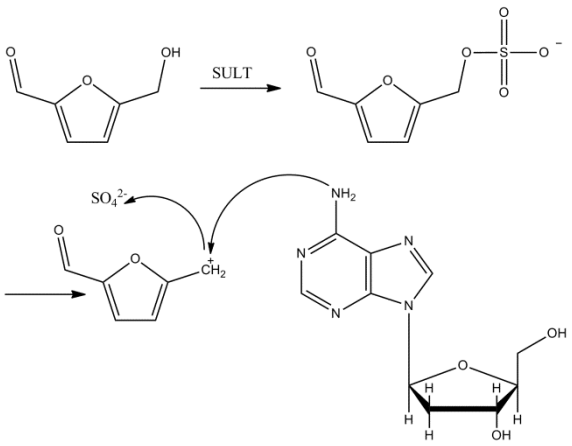



**Figure 1.9:** Metabolic conversion of HMF by SULTs and generation of DNA adducts (Adapted from Monien *et al.*, 2012)

HMF has been evaluated by EFSA when dealing with furfuryl and furan derivatives. The report concluded that based on available data provided mainly by the Industry and based on genotoxicity studies identified by EFSA, negative results in the carcinogenicity studies carried out in rats and mice indicate that under normal conditions HMF is of no concern to human health (EFSA, 2010). In 2009, Monien *et al.* detected SMF in the blood of FVB/N mice after HMF intravenous administration suggesting that sulfo conjugation may indeed effect HMF-related carcinogenicity observed in animal experiments. Moreover, since humans express SULTs in extrahepatic tissues more extensively than rodents do, humans can be more sensitive to HMF (Dunn & Klaassen, 1998; Teubner *et al.*, 2007). This suggests that the risk associated with HMF exposure from food may be higher for humans than that indicated by experiments in rodents. Indeed, since HMF intake is very high, even a limited conversion would expose humans to amount of SMF that can have negative effects on health. In the light of the new findings about HMF genotoxicity potential, the importance of lowering HMF content in heated foods and, in turn, dietary exposure should be carefully re-evaluated (Capuano & Fogliano, 2011).

### 1.1.2.4.3. Furfural

Furfural may enter the body by two ways: via respiratory track and /or percutaneous route (Flek & Sedivic, 1978). Furfural is excreted from the body by fast liver metabolism and excretion. The biotransformation of furfural takes place in two ways: the major part is oxidised and conjugated with glycine to furoylglycine, the smaller part condensate with acetic acid and also conjugate with glycine to 2-furanacryluric acid (Figure 1.10) (Flek & Sedivic, 1978). The major excretion route is via the urine, whereas exhalation by expired air and faecal excretion are minor routes (Nomeir *et al.*, 1992).

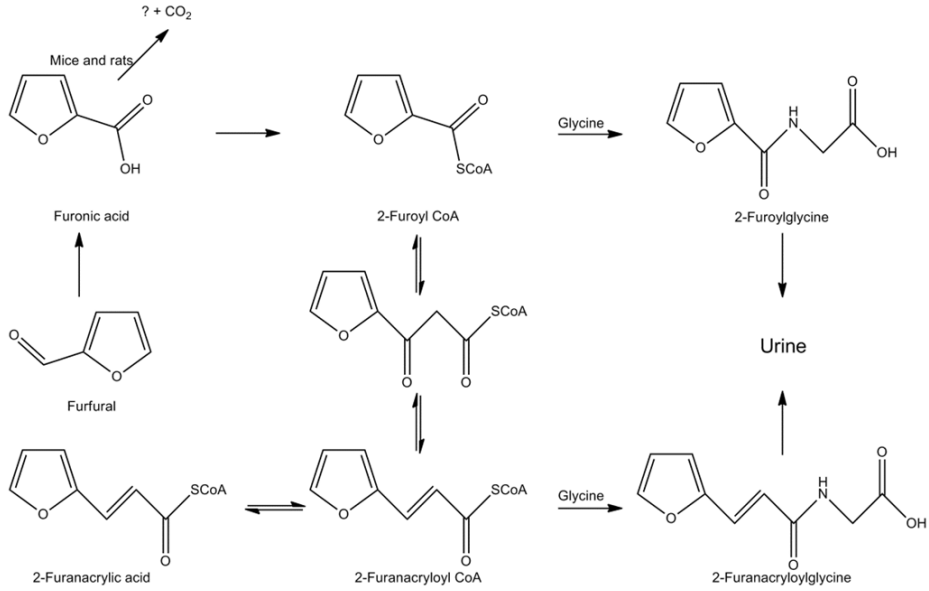



**Figure 1.10:** Metabolism of furfural in humans and in rats (Adapted from WHO Food Additives Series 42 – Furfural, 1999).

Di Pede *et al.*, 1991 studied the exposure of workers to furfural concentrations exceeding the threshold limit value of 8 mg $m^{-3}$ and concluded that this furanic compound produce irritation on respiratory tract and/or eyes (Arts *et al.*, 2004). Following this, the EFSA (2011b) published an opinion on Flavouring group regarding furfural derivatives. Based on the data available the Panel concluded that furfural is not of concern with respect to genotoxicity, including the structurally related furfuryl alcohol, 5-methylfurfural and other related substances. (EFSA, 2011b) Nevertheless, since there is a lack of the real intake of these compounds, future studies concerning the real food intake and toxicological effects should be taken.

*1.1.2.5. Analytical methodologies*

1.1.2.5.1. Furan

Furan is analysed using GC-MS method. Because furan is volatile, HS methods are employed. The FDA published the first quantitative method for furan in food (FDA, 2004). It used sample preparation under cold conditions and HS sampling following incubation at 80 ºC. Quantification was based on standard additions and used a deuterated IS ($d_4$-furan). As the interest in furan analyses grew, several variations of this procedure were tested and introduced by other laboratories (Crews & Castle, 2007; Zoller *et al.*, 2007; Vranová & Ciesarová, 2009). The HS method has the advantage in that no sample purification is required. The disadvantage is that the foodstuff is heated (according to FDA method at 80°C for a minimum of 30 min), meaning that furan might be formed during analysis. Senyuva & Goekmen (2005) recently described the formation of furan in unprocessed foods during HS-GC-MS analysis even under mild (40 °C) thermal conditions. Because of these considerations, several researchers developed and validated analytical methods using SPME in combination with GC-MS (Goldmann *et al.* 2005; Ho *et al.* 2005; Bianchi *et al.* 2006). SPME consists in a fibre coated with a polymeric material that is exposed to the HS vapours to adsorb volatiles that are thereafter desorbed thermally in the injection port of the GC to drive off the volatiles onto the GC column (Crews & Castle, 2007; Zoller *et al.*, 2007; Vranová & Ciesarová, 2009). Comparing both methods, in direct headspace analysis a portion of the HS gas is taken and injected directly into the GC-MS achieving, for most foods, limits of detection (LOD) between 0.3 and 1.0 μg $kg^{-1}$. Regarding liquid foods, such as coffee, the LOD is as low as 0.2 μg $kg^{-1}$. On the other hand, SPME allows sample concentration working at room temperature and affords higher sensitivity. LOD in the low ng $kg^{-1}$ range can be achieved for furan (Goldmann *et al.*, 2005; Bianchi *et al.*, 2006; Crews & Castle, 2007).

Recently, EFSA (2011a) published a list of LOD and limits of quantification (LOQ) reported in literature for furan analyses. Analytical methods used included HS-GC-MS and HS-SPME-GC-MS since there are no specific indications in the European Commission Recommendations concerning a specific analytical procedure that should be used. Results are summarized in Table 1.5.

**Table 1.5:** LOD and LOQ found from several works published (Adapted from EFSA, 2011a).

| | LOD (µg $kg^{-1}$) | | LOQ (µg $kg^{-1}$) | |
|---|---|---|---|---|
| **Food Group** | **Min** | **Max** | **Min** | **Max** |
| Coffee, roasted bean | 0.07 | 5 | 0.2 | 10 |
| Coffee, roasted ground | 0.05 | 40 | 0.1 | 100 |
| Coffee, not specified | 0.05 | 7 | 0.1 | 28 |
| Coffee instant | 0.07 | 7 | 0.2 | 28 |
| Coffee brew | 2 | 2 | 5 | 10 |
| Baby food | 0.03 | 5 | 0.04 | 10 |
| Infant formula | 0.5 | 5 | 1 | 10 |
| Vegetables | 0.07 | 5 | 0.2 | 10 |
| Fruits | 0.14 | 5 | 0.18 | 10 |
| Vegetable juice | 0.2 | 2.7 | 0.6 | 10 |
| Fruit juice | 0.05 | 5 | 0.1 | 10 |
| Fish | 0.5 | 5 | 0.32 | 10 |
| Cereal products | 0.1 | 16 | 0.3 | 40 |
| Meat products | 0.07 | 8 | 0.18 | 20 |
| Milk products | 0.5 | 0.5 | 0.18 | 10 |
| Beer | 0.5 | 2.7 | 0.18 | 9.1 |
| Soy sauce | 0.07 | 2 | 0.2 | 10 |
| Soups | 0.1 | 5 | 0.18 | 10p |
| Sauces | 0.07 | 5 | 0.18 | 10 |
| Baked beans | 0.07 | 5 | 0.2 | 10 |
| Other products | 0.1 | 8 | 0.18 | 20 |

It can be observed in that the minimum and maximum values reported for LOD and LOQ ranged from 0.03 to 40 µg $kg^{-1}$ and from 0.04 to 100 µg $kg^{-1}$, respectively being the highest LOD and LOQ reported for “ground roasted coffee".

1.1.2.5.2. HMF

Different analytical techniques have been applied for the analysis of HMF in foods. Among the different techniques available, colorimetric and chromatographic procedures are the most commonly used. Colorimetric methods are based on colour reactions that can ideally be measured in the visible range. The usual reactive agents for HMF colorimetric determination are aniline (Friedemann *et al.*, 1964), *p*-toluidine and 2-thiobarbituric acid (TBA) (Anam & Dart, 1995).

Colorimetric methods were used for HMF quantification in honey (Anam & Dart, 1995), milk (Morales *et al.*, 1992, 1996) and fruit puree (Garza *et al.*, 1999). However, these methods have some disadvantages such as the instability of the colour complex formed, the time required and the use of hazardous chemicals (Martysiak-Zurowska & Borowicz, 2009).

LC techniques are preferable for accurate and reliable measurement of less volatile furanic compounds in several food products. LC techniques enable specific determination of HMF, and the formation of a coloured derivative is not required because of the strong ultraviolet (UV) absorption of HMF at 284 nm. Reversed – phase (RP)- LC methods have been widely used to determine the contents of HMF in many food items, such as coffee (Murkovic & Bornik, 2007; Arribas-Lorenzo & Morales, 2010), milk (Chávez-Servín *et al.*, 2005), infant formulas (Ferrer *et al.*, 2002), breakfast cereals (Rufián-Henares *et al.*, 2006b), among others. Additionally, some authors reported a precolumn derivatization with 2,4-dinitrophenylhydrazine (DNPH), which gives a better selectivity due to the absorption maximum at 400 nm of the DNPH (Lo Coco *et al.*, 1996; Murkovic & Pichler, 2006). Nevertheless, when using traditional LC coupled to UV detection, the most important is the recommendation of the HMF peak purity evaluation (Rufián-Henares *et al.*, 2006b).

Many compounds present in food matrices absorb at 280 nm, consequently, cleanup steps are required depending on the food matrix complexity. For simple matrices, such as alcoholic beverages, coffee, and fruit juices, extraction with water and subsequent filtration are commonly applied. However, the use of solid-phase extraction (SPE) cartridges is also appropriate to clarify some food extracts, such as honey (Driffield *et al.*, 2005). In milk - based products, the extract can be heated during several minutes after the addition of oxalic acid (Chávez-Servín *et al.*, 2005). In other cases, extraction of HMF is improved by use of methanol or ethyl acetate, an subsequent evaporation and reconstitution of the extract with water (or mobile phase) prior to analysis (Fallico *et al.*, 2003). The use of *Carrez* I (15% $K_4Fe(CN)_6$) and *Carrez* II (30% $ZnSO_4$ ) solutions as

clarifying agents instead of classical acids such as trichloroacetic (TCA), metaphosphoric, or sulfosalicylic, is recommended for cereal and tomato products, due to the possible *in situ* production of HMF from glucose present in the food matrix at low pH (Ramírez-Jiménez *et al.* 2000b, Cárdenas-Ruiz *et al.*, 2004).

The use of different analytical methods for HMF quantification and the use of inaccurate analytical methods or inadequate extraction procedures are a drawback to establish a reliable database for HMF content in processed foodstuffs, pivotal for estimation of exposure and consequently a reliable risk assessment. Table 1.6 shows a summary of several LOD and LOQ found for HMF using different food products.

**Table 1.6:** Summary of LOD and LOD found for HMF in food.

| Food Group | LOD (mg $kg^{-1}$) | | LOQ (mg $kg^{-1}$) | | References |
|---|---|---|---|---|---|
| | Min | Max | Min | Max | |
| Roasted coffee | 0.035 | 0.7 | 0.120 | 4 | Murkovic & Pichler, 2006; Arribas-Lorenzo *et al.*, 2010; Teixidó *et al.*, 2011 |
| Honey | 0.008 | 0.7 | 2.5 | | Lo Coco *et al.*, 1996; Rizelio *et al.*, 2012; |
| Jam | 0.03 | 0.7 | 2.5 | | Vorlová *et al.*, 2006; Teixidó *et al.*, 2011 |
| Fruit juices | 0.03 | 0.7 | 2.5 | | Vorlová *et al.*, 2006; Teixidó *et al.*, 2011 |
| Cookies/Biscuits | 0.05 | 0.36 | 2.5 | | Ait-Ameur *et al.*, 2006; Delgado-Andrade *et al.*, 2009; Teixidó *et al.*, 2011 |
| Cereal-based products | 0.01 | 0.7 | 0.05 | 2.5 | Rufián-Henares *et al.*, 2006a; Teixidó *et al.*, 2011 |
| Baby-food | 0.005 | 0.008 | 0.027 | | Gökmen *et al.*, 2006; Mesías-García *et al.*, 2010 |
| Vinegars* | 0.00006 | 0.012 | 0.00019 | 0.00414 | Giordano *et al.*, 2003; Gaspar & Lucena, 2009; Gaspar & Lopes, 2009 |
| Chocolate | 0.7 | | 2.5 | | Teixidó *et al.*, 2011 |
| Caramel products | 0.00006 | | 0.00019 | | Gaspar & Lucena, 2009 |
| Madeira wines | 1.22 | | 3.68 | | Pereira *et al.*, 2011 |
| Coat-deep fried products | 0.0076 | | 0.025 | | Pérez-Palacios *et al.*, 2013 |
| Spirits | 0.0085 | | 0.028 | | Albalá-Hurtado *et al.*, 1997 |

*mg $L^{-1}$

It can be observed that the minimum and maximum values reported for LOD and LOQ ranged from 0.00006 to 1.22 mg $kg^{-1}$ and from 0.00019 to 4 mg $kg^{-1}$, respectively. The highest LOD was reported for Madeira wines and the highest LOQ was reported for roasted coffee.

1.1.2.5.3. Furfural

Several methods used to determine furfural in foods are based on the method of Keeney & Bassette (1959), which used a colorimetric reaction with TBA. However, a strict control of time and temperature reaction is required due to the instability of the reaction product that gives high variability of results (Martysiak-Zurowska & Borowicz, 2009). To prevent interference problems and to increase the speediness of analysis, chromatographic procedures such as GC (López *et al.*, 2002; Pérez *et al.*, 2002; Adahchour *et al.*, 2003; Giordano *et al.*, 2003; Gaspar & Lopes, 2009; Tsai & Kao, 2012;) and LC (Albalá-Hurtado *et al.*, 1997; Yuan & Chen, 1998; Yuan & Chen, 1999; Es-Safi *et al.*, 2000; Canas *et al.*, 2003; Rufián-Henares *et al.*, 2006a; Gaspar & Lucena, 2009; Mesías-García *et al.*, 2010) has been developed.

When using chromatographic methodologies, the maximum absorbance of furfural is 277 nm, which is common to many other compounds presented in food matrices. This fact shows the importance of applying a previous step of cleanup procedures. For liquid samples, it can be used an extraction with water alone or mixed with an organic solvent, such as acetonitrile and hexane (Alcázar *et al.*, 2006; Gökhan & Gökmen, 2010) and extraction using SPE cartridges (López *et al.*, 2002). For semi-solid and solid matrices the use of *Carrez* I and II solutions as clarifying agents is commonly used (Rufián-Henares *et al.*, 2006a; Mesías-García *et al.*, 2010).

As mentioned for HMF, the use of different analytical methods for furfural determination as well as inadequate extraction procedures is a disadvantage to establish a reliable database for furfural content in foods and consequently for estimation of exposure.

Table 1.7 present the LOD and LOQ found for several authors using different analytical methods. It can be observed that the minimum LOD was described by Gaspar & Lucena (2009) for honey, caramel, balsamic vinegar and fruit juices, and maximum LOD by Pereira *et al.* (2011) for Madeira wines. Regarding LOQs values, the minimum found was 0.0007 mg $L^{-1}$ also for Gaspar & Lucena (2009) and the maximum LOQ found was 3.69 mg $L^{-1}$ also for Pereira *et al.* (2011).

**Table 1.7:** Summary of LOD and LOQ found for furfural in food.

| Food Group | LOD (mg kg$^{-1}$) | | LOQ (mg kg$^{-1}$) | | References |
|---|---|---|---|---|---|
| | Min | Max | Min | Max | |
| Infant formulas* | 0.01 | 0.01 | 0.02 | 0.05 | Albála-Hurtado *et al.*, 1997; Rufián-Henares *et al.*, 2001; Ferrer *et al.*, 2002 |
| Spirits* | 0.0048 | 0.4 | 0.0159 | 0.14 | Jaganathan *et al.*, 1999; Canas *et al.*, 2003; Alcázar *et al.*, 2006 |
| Breakfast cereals | 0.01 | | 0.05 | | Rufián-Henares *et al.*, 2006a |
| Baby food | 0.0035 | | 0.0116 | | Mesías-García *et al.*, 2010 |
| Balsamic vinegars* | 0.00023 | 0.00137 | 0.0007 | 0.00414 | Gaspar & Lopes, 2009; Gaspar & Lucena, 2009 |
| Honey* | 0.00023 | 0.00137 | 0.0007 | 0.00414 | Gaspar & Lopes, 2009; Gaspar & Lucena, 2009 |
| Fruit juices* | 0.00023 | 0.004 | 0.0007 | | Yuan & Chen, 1998; Gaspar & Lucena, 2009 |
| Caramel* | 0.00023 | | 0.0007 | | Gaspar & Lucena, 2009 |
| Madeira wines* | 1.22 | | 3.69 | | Pereira *et al.*, 2011 |

*mg L$^{-1}$

*1.1.2.6. Mitigation Strategies*

Reduction of furan, HMF, furfural and related furanic compounds is likely to be more challenging when comparing with other contaminants resulting from food processing for two main reasons. The first reason is related with heating time and temperatures, since lower these parameters can affect pasteurisation and sterilisation processes and consequently, the microbiological food safety. The second reason is about the widespread occurrence of these compounds in many food types, especially heat-processed foods. Furanic compounds are correlated with different reactions following multiple routes and involving different precursors and intermediates. Up to now, very little information is available about the possible routes to mitigate furan, HMF, furfural and related furanic compounds in foods and consequently, the consumers' intake.

The technological measures suggested reducing furanic compounds content from formulation until post-processing part. Thus, mitigation strategies can be separated in two distinct points: 1) preventive interventions and 2) removal interventions. The first approach aimed to keep furanic compounds formation as low as possible during the heating process and the second approach aimed to remove or decompose the already formed furanic compounds from the final product.

Unfortunately, furanic compounds formation follows the same pathways that lead to brown and flavour compounds. For instance, Capuano *et al.*, 2008, 2009 reported, a high correlation between HMF content and browning development so that modeling the time – temperature profile by reducing heating times and/or temperatures is likely to reduce HMF concentrations in the same time resulting in a reduction of browning development which can potentially compromise the quality and acceptability of final products. The same happens when mitigation strategies based on changes in recipes are applied, for example by replacing reducing sugars with non reducing sugars or polyalchols. However, it has been also reported that replacing glucose or fructose with sucrose in biscuits results in higher level of HMF when baked at temperature > 250 ºC (Ait-Ameur *et al.*, 2007). A highly reactive fructofuranosyl cation has been postulated to form from sucrose at high temperature which yields HMF very quickly (Perez-Locas & Yaylayan, 2004). The effect of dough pH on HMF formation has been reported. Generally in bakery products increasing the pH of the dough results in decreased levels of HMF (Gökmen *et al.*, 2007). In fact, low pH as well as the presence of acidic catalyst promotes heat-induced decomposition of furan derivatives (Lee & Nagy, 1990).

Furan presents high volatility, thus cooking in open vessels will allow furan loss and reduction of its content. However, this may be of limited applicability due to microbiological reasons. Canned and jarred foods have to be sealed hermetically and for coffee it would be technically difficult to purge coffee of furan whilst retaining all the flavour and aroma substances that the consumer demands. Thus, the best approaches appear to involve intervention in the reaction mechanisms. Substitution of atmospheric oxygen by nitrogen reduces the autoxidation of unsaturated fatty acids and also reduces furan formation from several precursors, particularly ascorbic acid (Märk *et al.*, 2006). Thus, modification of the atmosphere within heating system might be effective in reducing furan in foods.

So far, no conclusive results, readably applicable at industrial level, are available in the literature to reduce furanic compounds in foods. Most of the research available in the literature deals with model systems, information on the effect of several ingredients on furan, HMF or furfural formation/mitigation in foods is very limited. Therefore, much caution is needed when extrapolating results from simple model systems to food products in order to avoid incorrect conclusions. From a technological point of view, further research is warranted to better understand the role of ingredients in favouring or inhibiting furan, HMF, furfural and related substances in food since studies on the effects of formulation-based preventive properties are almost totally lacking. (Capuano & Fogliano, 2011; Anese & Suman, 2013)

# PART II

## Furanic Compounds in Coffee

# CHAPTER 2

## Furans and other volatile compounds in ground roasted and *espresso* coffee using headspace solid-phase microextraction: Effect of roasting speed

## Abstract

The profile of major classes of furanic compounds, as well as other volatile compounds, was evaluated by HS-SPME-GC/MS in *Arabica* ground coffee roasted at three flow speeds, and in their corresponding *espresso* coffees. A total of 113 and 105 volatile compounds were respectively identified in ground coffee and *espresso* coffee. They were clustered in the following chemical classes: furans, pyrroles, pyridines, pyrazines, ketones, hydrocarbons, aldehydes and others. Results from Principal Component Analysis (PCA) using the data of major volatile classes as variables showed that the levels (expressed as relative percentage of total peak area) of furans and pyrroles were higher in *espresso* samples, whereas those of pyrazines and ketones were higher in ground samples. Slow roasting speed favoured pyridines formation, while medium and fast roasting speed favoured ketones formation both for ground and *espresso* coffee. The most representative furanic compound in ground samples was furfuryl alcohol, followed by furfuryl acetate, furfural, 2-methylfuran and 5-methylfurfural. In *espresso* samples, the levels of 2-methylfuran, furfural, furfuryl formate, 5-methylfurfural and furfuryl acetate were largely increased, while the proportion of furfuryl alcohol was strongly reduced in comparison to ground coffee. High roast speed increased formation of furfural and 5-methylfurfural in *espresso* coffee.

**Keywords:** Ground coffee; *Espresso* coffee; Furanic compounds; SPME; GC-MS; Volatiles.

## 2.1. Introduction

The volatile fraction of roasted and brewed coffee has been studied for years, and several hundreds of compounds have been reported as constituents of coffee aroma (Liardon & Ott, 1984; Semmelroch & Grosch, 1995, 1996; Mayer *et al.*, 1999; Sanz *et al.*, 2001; Toci & Farah, 2008). Throughout these years, it has also been observed that many parameters can influence coffee volatiles profile, namely, species and origin, roasting degree and method, brewing procedure and beans quality (Mayer *et al.*, 1999; Loapez-Galilea *et al.*, 2006; Gonzalez-Rios *et al.*, 2007; Akiyama *et al.*, 2008; Toci & Farah, 2008). Among these parameters, roasting and brewing are important steps by which a pleasant aroma is formed and released from coffee. Despite this, to our knowledge, only a few studies have been performed investigating the influence of roasting method and brewing on volatile composition, especially regarding to *espresso* coffee, whose consumption has been increasing over the years (Andueza *et al.*, 2003; Andueza *et al.*, 2007; Maeztu *et al.*, 2001; López-Galilea *et al.*, 2006) and which presents peculiar flavour characteristics mainly produced by the ketones (Maeztu *et al.*, 2001; López-Galilea *et al.*, 2006). Sanz *et al.*, (2001) studied the volatile compounds in the presence of foam, which traps the volatilised aromas and doses their emission into the air (Illy & Viani, 1995).

Gonzalez-Rios *et al.*, (2007) showed that furans were the main chemical class found in ground *Arabica* coffee, followed by ketones, pyrazines, pyridines and pyrroles. Regarding *espresso* coffee, Rocha *et al.*, (2004) found that the major family present was furans, followed by pyrazines, aldehydes and pyridines.

Furan and furanic compounds are nowadays receiving special attention. Usually, they are related to the flavour of foods and beverages, providing pleasant characteristics. However, they are also associated to potential harmful effects on human health (Wenzl *et al.*, 2007). The furan concentration in coffee drink depends on its content in coffee powder, as well as on the brewing procedure (Zoller *et al.*, 2007; JECFA, 2011; EFSA, 2011a). Since 2005, a large number of studies have applied HS–SPME to identify and quantify furan in different matrices as well as to evaluate the effect of coffee roasting degree and brewing in its content (Ho *et al.*, 2005; Altaki *et al.*, 2007, 2009, 2011; La Pera *et al.*, 2009; Bicchi *et al.*, 2011).

As regards to coffee roasting methods, it is possible to achieve the same roasting degree using different types of roasters and variations on time, temperature and roast speed (in the case of those using hot air flow to raise the beans temperature). It has been shown that changes in time and temperature of roasting may affect the nonvolatile composition of

coffee (Toci & Farah, 2008). However, little knowledge is available on the influence of changes in the roast speed to achieve similar roasting degrees on the volatile profile of coffee, especially regarding the formation of furanic compounds and their release during *espresso* processing.

The aim of this work was to investigate the profile of furans and other volatile compounds in ground *Arabica* coffee, roasted at different speeds, and in their respective *espresso* brews.

## 2.2. Materials and methods

### *2.2.1. Sampling*

Three blends of Brazilian commercial *Arabica* coffee were used for this study identified as, blends 1, 2 and 3. Samples were roasted separately to give medium roasting degree, according to the Roast Colour Classification System – Agtron, SCAA, USA in an industrial semifluidized bed roaster (OPUS 500 Third Generation, Cia. LILLA, Brazil). Each blend was roasted at three different speeds: fast (4 min) (F), medium (8 min) (M) and slow (15 min) (S) using different temperature and air flow speeds (information was keep secret by the roast industry). Ground roast samples were codified as G1S, G1M, G1F, G2S, G2M, G2F, G3S, G3M, and G3F. Two batches of each sample were analysed, making a total of 18 samples, analysed in triplicate. Samples were ground to pass a 500 µm sieve. *Espresso* coffee was prepared for each of the 18 samples, from 8 g of coffee through which purified water of 88-95 °C was forced at 9-10 atm of pressure and extraction time of 5 ± 1 s and holder filter diameter of 38 mm (Krups, Model: EA 8025 E1). The two batches of each ground sample were analysed as *espresso* samples and codified as E1S, E1M, E1F, E2S, E2M, E2F, E3S, E3M, and E3F. Analyses were performed immediately after brew preparation.

### *2.2.2. HS-SPME conditions*

Three different SPME fibres were tested for extraction of the volatiles present in ground coffee: 100 µm Polydimethylsiloxane (PDMS), 85 µm Polyacrylate (PA), and 75 µm Carboxen/Polydimethylsiloxane (CAR/PDMS) (Supelco). Furthermore, different

headspace exposure fibre times (20, 25, 30, 35 min) were evaluated. Analyses were performed in triplicate.

HS-SPME analyses of ground samples: 1.5 g of ground coffee at 500 µm was placed in a 15 mL vial subsequently sealed with PTFE-silicone septa (Supelco, Bellefonte, PA, USA). Sample vial equilibration was performed at room temperature for 30 min; the CAR/PDMS fibre was then exposed to the headspace above the sample for 30 min followed by 10 min thermal desorption of the adsorbed substances in the injector port.

HS-SPME analyses of *espresso* samples: 5 mL of *espresso* coffee were prepared directly to 15 mL vials containing 1.5 g of NaCl subsequently sealed with PTFE-silicone septa (Duran, Schott, Germany). Extracts were prepared from 8 g of coffee through which purified water of 88-95 °C has been forced at 9-10 atm of pressure for a brew time of 5 ± 1s. Then coffee samples were cooled to 4 ºC and equilibration was performed during 30 min in ultrasonic bath at thermostatically controlled analysis room (20 ºC); the CAR/PDMS fibre was then exposed to the headspace above the sample for 30 min followed by 10 min thermal desorption of the adsorbed substances in the injector port.

### *2.2.3. GC-MS analysis*

Analyses were performed using a Hewlett-Packard (HP), model 6890 (Hewlett-Packard Company, Palo Alto, U.S.A.), GC fitted with a splitless injector suitable for SPME analysis and Agilent 5973 MS detector (Agilent Technologies, Santa Clara, U.S.A.). Helium was used as carrier gas with a flow rate of 1mL $min^{-1}$. The components were separated on a SPB-5 capillary column 60 m x 0.32 mm x 1.0-µm-film thickness (Supelco, Bellefonte, PA). The oven temperature program was 5 min at 40º C and then raised 3 ºC $min^{-1}$ until 200 ºC and kept for 5 min. The injector temperature was 290 ºC (Pinho *et al.*, 2006). Detection was accomplished by MS on the total ion current (TIC) obtained by electron impact at 70 eV. The constituents were identified by comparing the experimental spectra with spectra from Nist 98 data bank (NIST/EPA/NISH Mass Spectral Library, version 1.6, U.S.A.), using a match factor higher than 97%, also by comparison of their GC Kovats index, and in some cases by comparison of their retention times with those of standard compounds.

### *2.2.4. Quantitative Measurements*

The total content of volatile compounds of each headspace analysis was defined by integrating the peak areas of all the identified compounds. The relative percentages of individual compounds were calculated from the total contents of volatiles on the chromatograms. Reproducibility was assessed as the relative standard deviation (RSD %) of a 6-fold analysis.

### *2.2.5. Statistical design*

The influence of *espresso* processing on major volatile chemical classes with special attention to furanic compounds was studied in three blends roasted at three different fluid flow speeds. One-way analysis of Variance (ANOVA) was performed. To ensure data were normally distributed, standardized skewness and standardized kurtosis values were checked. A significance level of $p < 0.05$ was used for all means. *T*-Tukey was applied as the test *a posteriori* with a level of significance of 95 %. Pearson's correlation and PCA were applied to major volatile chemical classes and furanic compounds in order to search for similarities and find relationships among ground samples roasted at three different fluid flow speeds and respective *espresso* samples composition. Analyses were performed by using the SPSS package (v. 20.0), with a significance level of 0.05.

## 2.3. Results and discussion

### *2.3.1. HS-SPME conditions*

The first step of the work involved SPME sampling parameters optimisation. Conditions adopted were those giving the most abundant and reproducible chromatographic peaks for volatiles and furanic compounds. Fibre coating was shown to influence qualitative and quantitative volatile profiles of ground samples obtained by SPME technique. Comparison of the performances of the three fibres tested clearly showed that CAR/PDMS fibre enabled detection of a wider range of compounds and produced higher signal intensities than PDMS and PA fibres. CAR/PDMS not only detected more volatile compounds, since it presented higher Sum of GC peak area but also was more efficient in the detection of furanic compounds, comparing with the other two analysed fibres (Figure 2.1). In fact

most authors used this fibre for the analyses of furan (Altaki *et al.*, 2007; Bicchi *et al.*, 2011).

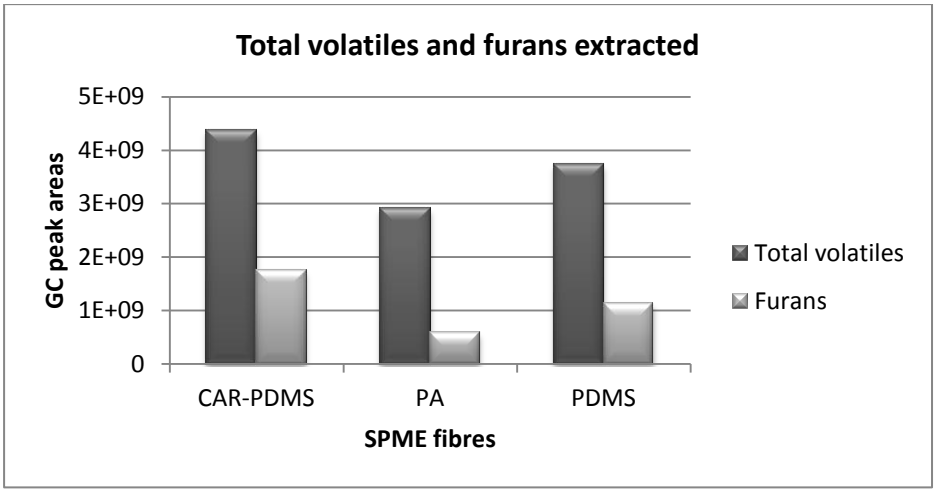



**Figure 2.1:** Comparison of three types of fibre for extraction of volatile compounds from ground coffee samples.

Exposure time for CAR-PDMS fibre was investigated to determine the most suitable conditions for adsorption of volatile compounds in ground coffee. Other factors such as vial volume (15 mL), equilibrium and sampling temperature (20 °C) were kept constant. As observed in previous published works (Pinho *et al.*, 2003; Pinho *et al.*, 2004), keeping sampling temperature constant during equilibrium and exposure time is critical to achieve reproducible results, being the best choice, when possible, the use of 20 °C in thermostatically controlled analysis room. Other important parameter is to keep the same headspace volume for all samples in order to obtain standardization of the procedure. Adsorption time showed a significant effect on the chromatographic peak areas of the extracted compounds, as represented in Figure 2.2. This Figure shows the efficiency of the extraction displayed as the sum of peak areas of total volatile compounds after different exposure times (20, 25, 30, 35 min) of the fibre to the headspace of ground coffee. An exposure time of 30 min was chosen as a compromise between higher sensitivity and shorter time of analysis.

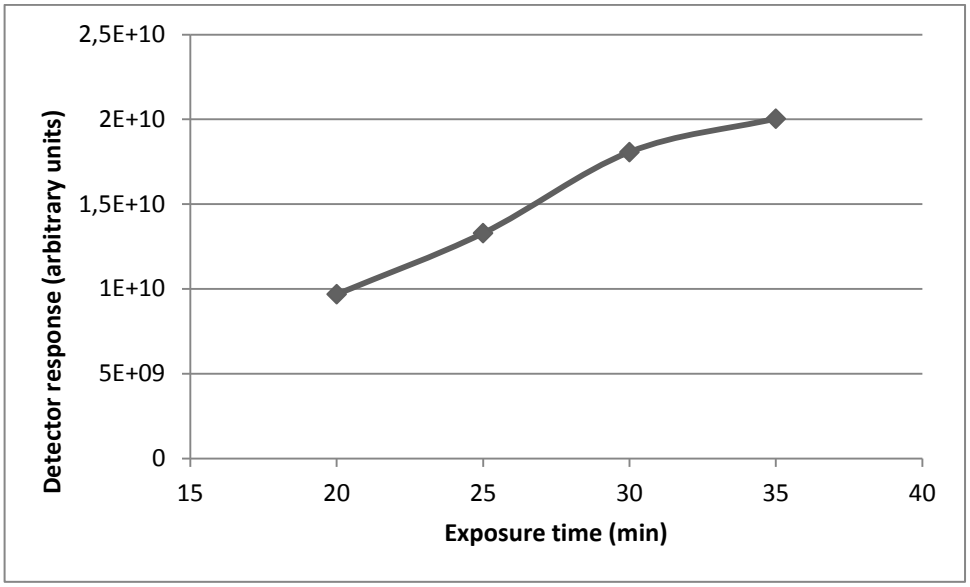



**Figure 2.2:** Effect of adsorption time at room temperature on the volatile compounds extraction efficiency of CAR/PDMS fibre in a ground coffee sample (y-axis - total volatile compounds expressed as peak area arbitrary units).

The reproducibility of the method depended on the compounds, and ranged between 0.92 % and 8.84 % for furans, between 0.09 % and 10.4 % for pyrroles, between 2.2 and 5.3 % for pyridines, between 0.03 % and 7.39 % for pyrazines, between 0.86 % and 8.38 % for ketones, and between 3.8% and 13.6% for other compounds. Similar values were obtained for solid matrices in previous works (Pinho *et al.*, 2004). HS-SPME analysis of *espresso* samples was performed using 5 mL of *espresso* coffee prepared directly to 15 mL vials containing 1.5 g of NaCl. Using this sample preparation procedure the sum of volatiles GC peak area was in the same order of magnitude of that obtained for ground samples.

### *2.3.2. Volatile compounds profile in ground and espresso coffee at different flow roast speed*

A total of 113 and 105 volatile compounds were identified in ground and *espresso* coffees, respectively. They were clustered in the following chemical families: furans, pyrroles, pyridines, pyrazines, ketones, hydrocarbons, aldehydes and others. Table 2.1 shows the relative percentage of the different chemical families of volatile compounds in G1S, G1M, G1F, G2S, G2M, G2F, G3S, G3M, G3F, E1S, E1M, E1F, E2S, E2M, E2F, E3S, E3M, and E3F.

**Table 2.1:** Relative percentage of volatile compounds chemical classes extracted from three ground coffee samples (G1, G2, G3) roasted at three different speeds, slow (S – 15 min), medium (M – 8 min) and fast (F – 4 min) and respective *espresso* coffee*

| **Class** | **G1** | | | | **G2** | | | | **G3** | | | | **GS** | **GM** | **GF** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | G1S | G1M | G1F | *p* | G2S | G2M | G2F | *p* | G3S | G3M | G3F | *p* | *p* | *p* | *p* |
| **Furans** | 29.43a | 29.25a | 35.59a | 0.047 | 29.14a | 34.43a | 37.48a | 0.063 | 31.4a | 31.78a | 41.63b | 0.000 | 0.397 | 0.202 | 0.003 |
| **Pyrroles** | 4.59a | 3.67b | 3.40b | 0.005 | 2.93a | 2.23a | 1.91a | 0.076 | 4.04a | 3.09b | 1.81c | 0.000 | 0.023 | 0.002 | 0.00 |
| **Pyridines** | 23.92a | 16.98b | 12.49c | 0.004 | 18.21a | 12.58b | 12.51b | 0.029 | 22.34a | 15.25b | 10.78c | 0.001 | 0.023 | 0.101 | 0.043 |
| **Pyrazines** | 22.88a | 26.32a | 23.33a | 0.491 | 18.20a | 22.37a | 17.35 | 0.047 | 19.94a | 19.12a | 24.71b | 0.007 | 0.359 | 0.006 | 0.016 |
| **Ketones** | 12.02a | 16.27b | 19.28b | 0.001 | 19.07a | 18.93a | 21.5a | 0.258 | 11.74a | 18.2b | 11.9c | 0.000 | 0.007 | 0.082 | 0.003 |
| **Hydrocarbons** | 0.90a | 1.46b | 1.23a,b | 0.034 | 4.02a | 3.64a | 4.07a | 0.954 | 0.19a | 0.23a | 0.16a | 0.510 | 0.000 | 0.081 | 0.004 |
| **Aldehydes** | 0.86a | 0.92a | 0.28b | 0.013 | 3.89a | 0.46b | 0.77b | 0.001 | 0.50a | 0.41a | 0.01b | 0.001 | 0.000 | 0.127 | 0.001 |
| **Others** | 5.40a | 5.13a | 4.38b | 0.015 | 4.54a | 5.36a | 4.41a | 0.068 | 9.83a | 11.91b | 9.01a | 0.012 | 0.004 | 0.001 | 0.000 |
| **Total GC Area** | 2.18E+10a | 2.39E+10a | 2.22E+10a | 0.565 | 2.33E+10a | 2.83E+10a | 2.43E+10a | 0.201 | 2.10E+10a | 2.06E+10a | 6.01+9b | 0.000 | 0.197 | 0.056 | 0.001 |
| **Class** | **E1** | | | | **E2** | | | | **E3** | | | | **ES** | **EM** | **EF** |
| | E1S | E1M | E1F | *p* | E2S | E2M | E2F | *p* | E3S | E3M | E3F | *p* | *p* | *p* | *p* |
| **Furans** | 38.10a | 39.34a | 40.87a | 0.454 | 43.74a | 50.65a | 49.70 a | 0.177 | 38.09 a | 44.18 b | 46.88 b | 0.076 | 0.085 | 0.029 | 0.146 |
| **Pyrroles** | 7.78a | 11.29a | 11.11a | 0.058 | 9.55a | 8.63a | 10.57a | 0.520 | 9.52 a | 9.35 b | 11.13 b | 0.020 | 0.323 | 0.002 | 0.210 |
| **Pyridines** | 26.13a | 23.60a | 16.22b | 0.019 | 20.89a | 9.96b | 8.39b | 0.002 | 30.69 a | 21.64 b | 16.09 c | 0.005 | 0.005 | 0.008 | 0.020 |
| **Pyrazines** | 12.05a | 15.62b | 15.71b | 0.004 | 6.59a | 7.65a,b | 10.24b | 0.028 | 13.16a | 10.88a | 10.54a | 0.513 | 0.005 | 0.046 | 0.044 |
| **Ketones** | 6.42a | 3.83b | 6.73a | 0.004 | 10.64a | 10.79a | 11.01a | 0.951 | 3.54a | 6.11b | 7.46b | 0.013 | 0.001 | 0.006 | 0.038 |
| **Hydrocarbons** | 1.02a | 1.09a | 0.94a | 0.057 | 0.40a | 4.74b | 0.98a | 0.000 | 0.02a | 0.19b | 0.45c | 0.004 | 0.013 | 0.000 | 0.181 |
| **Aldehydes** | 2.12a | 0.74b | 2.38a | 0.028 | 2.99a | 4.43a | 4.91a | 0.589 | 0.68a | 1.83a,b | 2.76b | 0.015 | 0.005 | 0.006 | 0.240 |
| **Others** | 6.38a | 4.49a | 6.04a | 0.805 | 5.18a | 3.14a | 4.19a | 0.168 | 4.31a | 5.81b | 4.69a | 0.008 | 0.022 | 0.009 | 0.140 |
| **Total GC Area** | 1.81E+10a | 4.9E+09b | 1.41E+10c | 0.001 | 9.41E+09a | 1.33E+10a,b | 5.97E+09b | 0.029 | 4.95E+09a | 1.18E+10b | 1.13E+10b | 0.004 | 0.000 | 0.001 | 0.035 |

* Mean values of two batches analysed in triplicate. One-way ANOVA (analysis of variation) at the significant levels $p$ <0.05. a-c Means with different letters in the same row differ significantly among the three distinct flow roast speed within each bend.

Regarding total volatiles of ground samples, no significant differences were observed between total peak areas, except for G3F that presented significant lower levels. This means that three different blends, roasted at three different roasting speeds, did not influence total GC peak area. However, when analysing volatile classes, with respect to percentage of total chromatographic area, significant differences were observed among samples of the same blend roasted at different speeds, i.e., higher content of pyrazines was found in G2S in comparison to G2M and G2F batches, and among different blends roasted at the same speed, i.e., G1F samples showed higher pyrroles levels than G2F and G3F ones (Table 2.1). Concerning *espresso* samples, significant differences were observed between total peak areas and the volatile classes from the same blend roasted at three different speeds and from different blends roasted at the same flow speed (Table 2.1).

Furans were the major chemical class in all ground and *espresso* samples. The main differences occurred in the three other major classes, namely pyridines, pyrazines, ketones and pyrroles. The nitrogen-containing heterocyclic compounds, including pyridines, pyrazines, and pyrroles, are well-known as *Maillard* reaction products and give characteristic roasted or toasted flavours to roasted coffee (Moon & Shibamoto, 2009). Ground samples from slow roasting speed presented higher percentage of pyridines when compared with medium and fast batches, which presented higher percentages of pyrazines and ketones.

Also in the *espresso* samples, furans were the major chemical class, followed in general by pyridines. In ground batches, furans was the major chemical family of volatile compounds (14.63 - 19.14 %), followed by pyrazines (17.35 - 26.32 %), pyridines (12.58 - 23.92 %), ketones (11.74 - 19.28 %), pyrroles (1.81 - 4.59 %), hydrocarbons (0.23 - 4.07 %) and aldehydes (0.01 - 0.32 %). In relation to *espresso* samples, the highest percentages were also shown by furans (38.09 - 50.65 %), followed by pyridines (9.39 - 30.69 %), pyrazines (6.65 - 15.71 %), pyrroles (7.78 - 11.29 %), ketones (3.83 - 11.01 %), aldehydes (0.58 - 4.91 %) and hydrocarbons (0.02 - 4.74 %). Thus, from a general point of view, it can be observed differences between ground and *espresso* samples mainly for pyridines, pyrazines, ketones and pyrroles amounts. Rocha *et al.*, 2004 analysed commercial *espresso* coffee samples using a PDMS fibre and found that furans represented the major chemical family (around 47 %), followed by pyrazines (around 16 %, pyridines and aldehydes (around 10 % each group), being ketones and phenolic compounds the minor groups of volatile compounds (around 1 % each one). The profile found by these authors is not totally in concordance with results obtained in the present

study, except for furans profile in *espresso* samples. However, it should be noted that a different fibre was used and according to our previous studies this fibre quantified lower amounts of volatiles.

A PCA was performed using percentage of total chromatographic area of furans, pyrroles, pyridines, pyrazines, ketones, hydrocarbons, and aldehydes as variables (Figure 2.3). The Component 1 justified 49.63 % of total variance of results and clearly separated ground and *espresso* samples. Furans and pyrroles were positively correlated with this Component and increased from ground to *espresso* coffee samples, whereas pyrazines and ketones were negatively correlated with this Component and their levels were higher in ground samples than in *espresso* samples.

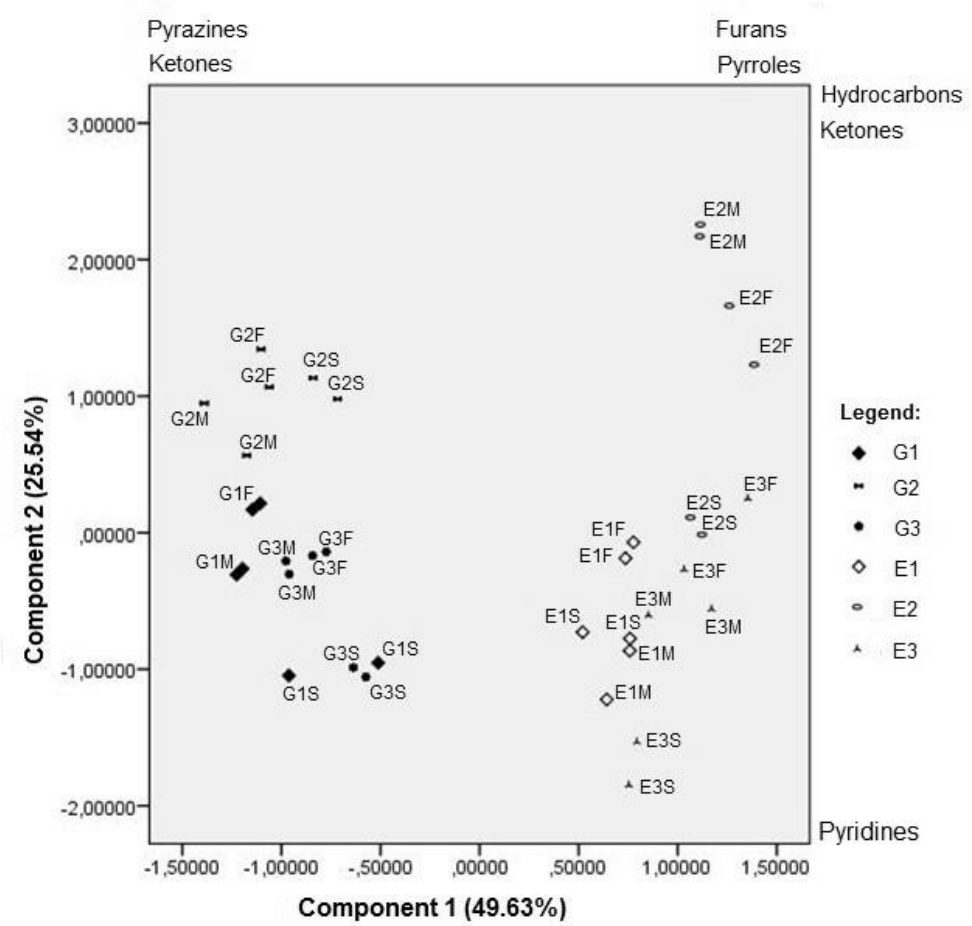



**Figure 2.3:** PCA of volatile compounds chemical classes of three blends (1, 2, 3) of ground (G) and *espresso* (E) coffee samples roasted at three different speeds, slow (S – 15 min), medium (M – 8 min) and fast (F– 4 min).

Pearson's correlations indicated significant correlation between the proportion of furans ($p$ = 0.567) and ketones ($p$ = 0.586) in ground and *espresso* samples. Component 2 justified 25.54 % of total variance of results and is related with blend composition and roasting speed. Findings showed that slow roasting speed favoured pyridine formation, whereas medium and fast roasting speed increased ketones proportion. Pearson's correlations indicated highly significant correlation between the proportion of pyridines ($p$ = 0.813) in ground and *espresso*.

Due to the fact that furans represented the major chemical group in both ground and *espresso* coffees, and because of the health concerning about their potential harmful effect, this study focus on the profile evaluation of these compounds in the different analysed samples.

### *2.3.3. Furanic compounds profile in ground and espresso coffee roasted at different flow speed*

Table 2.2 show the percentage of furanic compounds present in ground and *espresso* samples, respectively. 2-Furfuryl alcohol was the highest of all furanic compounds in ground coffee, representing more than 50 %. These results are in agreement with data presented by Moon & Shibamoto (2009) that investigated the formation of volatile chemicals in coffee beans under various roasting conditions. Furfuryl acetate, furfural, 2-methylfuran and 5-methylfurfural were also appreciable in ground samples.

**Table 2.2:** Relative percentage of furanic compounds extracted from three ground coffee samples (G1, G2, G3) roasted at three different flow speeds, slow (S – 15 min), medium (M – 8 min) and fast (F – 4 min) and respective *espresso* coffee (E1, E2, E3) *

| Furanic compounds | G1 | | | | G2 | | | | G3 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | G1S | G1M | G1F | *p* | G2S | G2M | G2F | *p* | G3S | G3M | G3F | p | p(S) | p(M) | p(F) |
| **Furan** | 0.151a | 0.162a | 0.086[a] | 0.054 | 0.412a | 0.000b | 0.086b | 0.007 | 0.147b | 0.094a,b | 0.000a | 0.005 | 0.021 | 0.003 | 0.010 |
| **2-methyl-furan** | 3.235a | 2.175a | 1.383[a] | 0.076 | 3.521a | 1.507b | 1.009b | 0.009 | 3.954d | 2.455b,c | 0.452a | 0.001 | 0.530 | 0.082 | 0.003 |
| **2-vinylfuran** | 0.406a | 0.144b | 0.056b | 0.009 | 0.134a | 0.093a,b | 0.067b | 0.031 | 0.546c | 0.141b | 0.000a | <0.001 | 0.007 | 0.032 | <0.001 |
| **2-(methoxymethyl)-furan** | 0.386a | 0.271b | 0.000c | <0.001 | 0.354a | 0.224b | 0.000c | 0.001 | 0.398d | 0.214b | 0.000a | <0.001 | 0.323 | 0.015 | <0.001 |
| **Furfural** | 2.347a | 3.324b | 4.498c | 0.001 | 3.127a | 5.037b | 5.460b | 0.003 | 2.622a,b | 3.124b,c | 7.664e | <0.001 | 0.031 | 0.001 | 0.001 |
| **2-furfuryl alcohol** | 15.050a | 16.091a | 21.938b | 0.004 | 14.360a | 19.130a | 18.621a | 0.109 | 15.941a,b | 18.712b,c | 23.832d | 0.001 | 0.197 | 0.256 | 0.010 |
| **Furfuryl formate** | 0.496a | 0.608a | 0.623[a] | 0.110 | 0.483a | 0.670a | 0.478a | 0.048 | 0.386a | 0.492a,b | 0.471a,b | 0.116 | 0.209 | 0.095 | 0.001 |
| **5-methylfurfural** | 1.218a | 2.083b | 2.875c | 0.001 | 1.773a | 3.335a | 2.653a | 0.112 | 1.284a | 1.958a,b | 4.750d | <0.001 | 0.014 | 0.109 | 0.005 |
| **Furfuryl acetate** | 4.459a | 3.723a,b | 3.487b | 0.038 | 3.377a | 3.518a | 2.579a | 0.226 | 4.772c | 3.938b,c | 3.867b,c | 0.014 | 0.022 | 0.662 | 0.006 |
| **Furfuryl propanoate** | 0.171a,b | 0.147a | 0.191b | 0.039 | 0.133a | 0.000b | 0.127a | 0.001 | 0.191d,e | 0.200d,e | 0.218e | 0.200 | 0.020 | 0.001 | 0.004 |

| Furanic compounds | E1 | | | | E2 | | | | E3 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | E1S | E1M | E1F | *p* | E2S | E2M | E2F | *p* | E3S | E3M | E3F | p | p(S) | p(M) | p(F) |
| **Furan** | 0.401a | 0.179b | 0.398a | 0.017 | 1.081a | 1.835b | 0.206c | 0.006 | 0.131a | 0.438a,b | 0.572b | 0.028 | <0.001 | 0.004 | 0.073 |
| **2-methyl-furan** | 7.398a | 3.866b | 5.565a,b | 0.039 | 7.715a | 11.886a | 5.031a | 0.089 | 4.126a | 7.183a | 6.865a | 0.052 | 0.292 | 0.003 | 0.174 |
| **2-vinylfuran** | 0.414a | 0.485a | 0.448a | 0.574 | 0.562a | 0.577a | 0.572a | 0.975 | 0.418a | 0.483a | 0.473a | 0.218 | 0.251 | 0.067 | 0.160 |
| **2-(methoxymethyl)-furan** | 0.735a | 0.653a | 0.330a | 0.102 | 0.696a | 0.472a | 0.626a | 0.069 | 0.862a | 0.457b | 0.283c | 0.001 | 0.053 | 0.364 | 0.018 |
| **Furfural** | 3.015a | 5.254b | 7.145b | 0.007 | 4.723a | 8.119a,b | 13.334b | 0.018 | 3.527a | 4.850b | 8.931c | <0.001 | 0.268 | 0.017 | 0.019 |
| **2-furfuryl alcohol** | 7.087a | 6.755a | 9.647b | 0.008 | 6.946a | 8.374a | 6.778a | 0.745 | 5.690a | 11.173b | 10.740b | 0.004 | 0.796 | 0.001 | 0.012 |
| **furfuryl formate** | 1.078a | 1.476a | 1.724a | 0.135 | 1.172a | 2.023b | 3.089c | 0.005 | 0.624a | 1.278a,b | 1.933b | 0.015 | 0.024 | 0.085 | 0.025 |
| **5-methylfurfural** | 1.775a | 4.552b | 4.885b | 0.003 | 4.380a | 5.317a,b | 7.580b | 0.041 | 2.580a | 3.666b | 5.850c | <0.001 | 0.001 | 0.086 | 0.042 |
| **Furfuryl acetate** | 10.546a,b | 11.583a | 8.075b | 0.026 | 11.320a | 8.162b | 8.212b | 0.018 | 13.583a | 11.122a,b | 8.323b | 0.027 | 0.108 | 0.013 | 0.931 |
| **Furfuryl propanoate** | 0.439a | 1.192a | 0.231a | 0.363 | 0.547a | 0.299a | 0.347a | 0.056 | 0.611a | 0.509a,b | 0.374b | 0.049 | 0.193 | 0.404 | 0.204 |

* Mean values of two batches analysed in triplicate. One-way ANOVA (analysis of variation) at the significant levels $p$ <0.05. a-c Means with different letters in the same row differ significantly among the three different flow roast speed within each bend

Minor furanic compounds were furan, 2-vinylfuran, 2-(methoxymethyl)-furan, furfuryl formate and furfuryl propanoate. In comparison to ground samples, *espresso* ones presented a large increase in 2-methylfuran, furfural, furfuryl formate, 5-methylfurfural and furfuryl acetate, whereas 2-furfuryl alcohol suffered a drastic reduction. Furan derivatives, such as methyl, aldehyde, alcohol or ester analogues, are of great concern because of the formation of possible reactive metabolites. 2-methylfuran is metabolically activated in a similar fashion as the parent furan yielding highly reactive α,β-unsaturated dialdehydes (Becalski *et al.*, 2010). Furfural, together with the corresponding alcohol (furfuryl alcohol), occur in many fruits, tea, coffee and cocoa. They are formed during the acid hydrolysis or heating of polysaccharides containing hexoses or pentoses and the highest amounts have been found in cocoa and coffee (EFSA, 2004b).

Little is known about the mechanism of action in humans, and only little information is available from animal studies (Parent, 2005). In organisms, furfuryl esters (furfuryl acetate) are hydrolysed to furfuryl alcohol and it is subsequently oxidized to furfural by enteric bacteria, and A-esterases, produced by the hepatocytes (Monien *et al.*, 2011). Regarding 5-methylfurfural, additional data are needed to confirm the existence of genotoxic metabolites (EFSA, 2009b). Studies show the conversion of these compounds to 2-furoic acid, and possible decarboxylation to carbon dioxide. However, this reaction may be preceded by epoxidation or hydroxylation of the furan ring producing reactive intermediates (EFSA, 2004b; Monien *et al.*, 2011).

Figure 2.4 shows the PCA carried out with relative proportion of furanic compounds from ground and *espresso* samples, summarizing 76.96 % of the total variance, and it is defined by two Principal Components 1 and 2. The Component 1 accounted for the 55.94 % of total variance data, and it was positively related to furan, 2-methylfuran, 2-vinylfuran, 2-(methoxymethyl)-furan, furfuryl formate, furfuryl acetate and furfuryl propanoate and negatively related with furfuryl alcohol. The Component 2 explained the 21.02 % of total variance data, showing a positive correlation of furfural and 5-methylfurfural. As it can be seen, ground and *espresso* samples are well separated on Component 1 and slow, medium and fast flow speed roast are separated in Component 2. These results highlight that *espresso* process modifies furanic compounds profile and apparently fast roasting speed increases the formation of furfural and 5-methylfurfural.

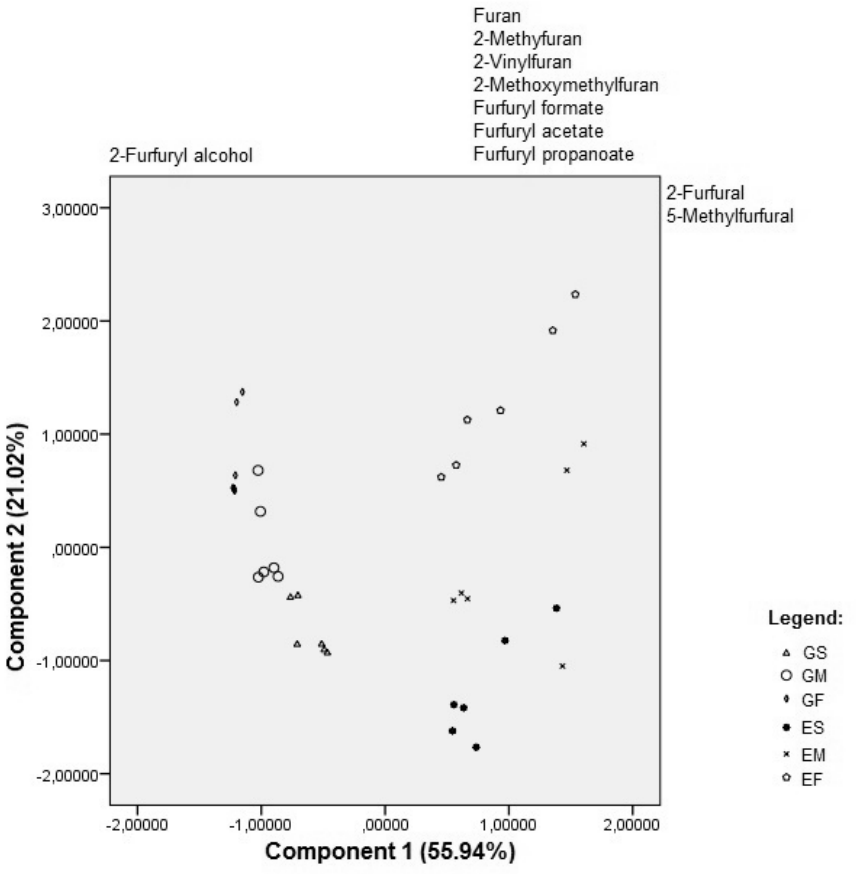



**Figure 2.4:** PCA of furanic compounds of three blends (1, 2, 3) of ground (G) and *espresso* (E) coffee samples roasted at three different speeds, slow (S – 15 min), medium (M– 8 min) and fast (F– 4 min).

## 2.4. Conclusions

The SPME sampling method using CAR/PDMS fibre enabled us to investigate the volatile profile and furanic compounds of ground and *espresso Arabica* coffee, being able to identify more than one hundred volatiles in ground and *espresso* coffees belonging to different chemical classes. Although the general profile of volatile compounds was similar in ground and *espresso* samples, with furans being the major class, statistical analysis indicated that furans and pyrroles were higher in *espresso* samples, whereas pyrazines and ketones were higher in the ground ones. The roasting speed did not influence total furans level in ground and *espresso* samples. On the other hand, the content of total ketones, furfural and 5-methylfurfural increased with the roasting speed, while pyridine percentage decreased with the roasting speed, indicating the importance of the roasting parameters for manipulating the volatile and furanic compounds composition of coffee brews, and hence its aroma and taste.

# CHAPTER 3

## Validation and application of a HS-SPME-GC-MS methodology for quantification of furanic compounds in *espresso* coffee

## Abstract

A HS-SPME-GC-MS method previously optimised for analyses of volatiles in coffee was validated for simultaneous quantification of major furanic compounds (2-furfural, furfuryl alcohol, 5-methylfurfural and furfuryl acetate) and furan, in *espresso* coffee prepared from hermetically closed capsules. High sensitivity was achieved with low LOD and LOQ. Good linearity was observed with correlation coefficients higher than 0.999. Recovery percentages were 101.9 % for furan, 99.70 % for 2-furfural, 97.98 % for furfuryl alcohol, 99.82 % for 5-methylfurfural and 99.93 % for furfuryl acetate. The method was applied to the analyses of *espresso* coffee from hermetically closed capsules. A total of 69 volatiles for Blend Roast and Blend Dark Roast samples, 64 volatiles for *Arabica* Dark Roast samples, 91 volatiles for *Arabica* Light Roast samples, 96 volatiles for Caramel coffee, 90 volatiles for Vanilla coffee and 92 volatiles for Almond coffee. In general, furanic compounds were the major chemical family, ketones, aldehydes, acids, pyrazines, pyrroles, alcohols, pyridines, aromatic compounds, hydrocarbons, and ethers were also detected. Total content of these furanic compounds varied from 105 to 199 μg $mL^{-1}$. The validated method proved to be a reliable methodology for quantification of major furanic compounds and furan present in different types of *espresso* coffee. Although relative percentage of peak area is a good method for discriminate volatiles in different coffee brews with closer composition, the quantification of furanic compounds is more accurate for understand the real intake amount.


**Keywords:** furanic compounds; *espresso* coffee capsules; HS-SPME-GC-MS methodology; *Arabica*; *Robusta*; aromatized coffee.

## 3.1. Introduction

Coffee is the second-most popular beverage in the world after tea (Parliament & Stahl, 1995). One of the most contributing factors for the high acceptability of coffee is its aroma, which involves a high number of volatile compounds (Flament, 2001). Coffee volatile compounds have been analysed by HS-GC-MS, quantified by relative percentage of total area and grouped by chemical families: pyrazines, pyrroles, pyridines, aldehydes and ketones, among others, being furanic compounds, namely, furan, furfural, furfuryl alcohol, 5-methylfurfural and furfuryl acetate, usually abundant in coffee drinks (Petisca *et al.*, 2012; López-Galilea *et al.*, 2006; Altaki *et al.*, 2007).

Furan and its derivatives are receiving increased attention nowadays. These compounds contribute to the flavour of foods and beverages, but on the other hand, they are associated with potential harmful effects on human health, being mitigation strategies a real challenge (Monien *et al.*, 2011; Rendic & Guengerich, 2012).

The Joint Expert Committee on Food Additives (JECFA) have allocated an ADI of 0.5 mg $kg^{-1}$ bw for furfural, furfuryl alcohol and furfuryl acetate among others (JECFA, 2001). Moreover, the World Health Organisation (WHO) published a report in 2011 with the ADI for furan, which correspond to 1 µg $kg^{-1}$ bw (WHO, 2011).

According to the literature data, coffee is the beverage that presents the highest amounts of furan levels. As reported by Kuballa *et al.*, 2005 and reviewed by the Codex Alimentarius Commission (JECFA, 2011; WHO, 2011), the furan concentration in coffee drinks depends on its content in ground coffee, as well as on the brewing procedure. Coffee brews from *espresso*-type machines present considerably higher amounts of furan (74 to 109 ng $g^{-1}$) than other coffee brews, i.e., coffee produced by standard home coffee-making machines contains 46 to 105 ng $g^{-1}$ and by manual brewing contains 19.9 to 51.3 ng $g^{-1}$ (Zoller *et al.*, 2007).

Nowadays, there is a large variety of *espresso* coffee such as 100 % Coffee *Arabica*, blends of *Coffea Arabica* and *Coffea Robusta*, subjected to different degrees of roast, and artificially aromatised coffee. According to the Associação Industrial e Comercial do Café (Dutra, 2010) about 80 % of the Portuguese population consumes *espresso* coffee. Regarding these, 78 % have *espresso* coffee outside home and 22 % drinks *espresso* coffee at home. The number of home consumptions is increasing due to the spread of *espresso* coffee machines from easy access, and the acquisition of *espresso* coffee packed in capsules in local stores, which enables *espresso* coffee consumption at lower price. As consequence, the consumption of coffee from hermetically packed capsules is

also increasing. This system guarantees fresh *espresso* coffee brews for long time and protect coffee from the damaging effects of light, air and humidity until consumption, because it is hermetically closed (Altaki *et al.*, 2011).

There are numerous studies focused on the detection and quantification of furan in coffee (Altaki *et al.*, 2007, 2009, 2011; FDA, 2004; Ho *et al.*, 2005; Korhonová *et al.*, 2009; La Pera *et al.*, 2009; López-Darias *et al.*, 2011; Bicchi *et al.*, 2011; Arisseto *et al.*, 2011), whereas the quantification of furanic compounds in this beverage remains almost unstudied. It is still a challenge to develop an analytical method that enables routine quantification of furan and its derivatives in food and beverages (Parent, 2005; Gaspar & Lopes, 2009; Becalski *et al.*, 2010).

The analysis of furanic compounds in food samples is difficult due to its high volatility (López-Galilea *et al.*, 2006). In 2004, FDA introduced a static (S)-HS-GC-MS to quantify furan by the standard addition approach. This method is time-consuming; it has relatively low sensitivity and requires a sampling temperature of 80 ºC. From 2005, several authors applied HS-SPME to identify furan in different matrices (Altaki *et al.*, 2007, 2011; Ho *et al.*, 2005; Korhonová *et al.*, 2009; La Pera *et al.*, 2009; López-Darias *et al.*, 2011; Bicchi *et al.*, 2011; Bianchi *et al.*, 2006; Pérez-Palacios *et al.*, 2012a). These authors used HS-SPME with a CAR/PDMS fibre coupled to GC-MS, using $d_4$-furan as an IS and an external calibration curve as quantification method. This approach enabled higher sensitivities than S-HS using lower sampling temperature, which is remarkable taking into account that above 40 ºC furan starts to form spontaneously (Bicchi *et al.*, 2011; Pérez-Palacios *et al.*, 2012a). Moreover, Altaki *et al.* (2011) found the best furan response at 25ºC.

The major goal of this study was the validation of a HS-SPME-GC-MS method previously optimised for evaluation of volatile compounds profile in *espresso* coffee for simultaneous quantification of major furanic compounds and furan (Petisca *et al.*, 2012). Another goal of this work was the application of the validated method to the analysis of *espresso* coffee samples prepared from hermetically closed capsules of 100 % Coffee *Arabica*, blends of C. *Arabica* and C. *Robusta* and artificially aromatised coffee.

## 3.2. Materials and Methods

### *3.2.1. Chemicals and reagents*

$D_4$-furan (98 %) was purchased by ISOTEC (Ohio, USA). Furfuryl alcohol (99 %), furfuryl acetate (99 %) and 5-methylfurfural (99 %) were supplied by Sigma Aldrich (Steinheim, Germany). Furfural was provided by Merck (99 %) (Darmstadt, Germany). Sodium Chloride PA-ACS was obtained from José Manuel Gomes dos Santos, LDA (Odivelas, Portugal). Methanol was supplied by Merck (Darmstadt, Germany) and ultrapure water (0.055 μS $cm^{-1}$) was obtained by using a Seral Milli-Q system for Millipore (Supor DCF, Gelman Sciences, Chentelham, Australia). SPME device and CAR/PDMS fused silica fibre were supplied by Supelco (Bellafonte, PA, USA). Before use, fibre was conditioned as recommended by the manufacturer.

### *3.2.2. Experimental design*

Seven different types of *espresso* coffee commercialized as hermetically closed capsules were selected for this study. Two blends of *Arabica* and *Robusta* with different roasting degree (coded as blend roasted (BR) and blend dark roasted (BDR)), 100 % *Arabica* dark roasted (ADR), 100 % *Arabica* light roast (ALR), and three different varieties of aromatized *espresso* coffee capsules, namely caramel (CAR), vanilla (VAN) and almond (ALM), were analysed. All analysed samples came from the same manufacturer and their origins are as follows: BDR (*Arabica* coffee beans from Eastern Africa, Columbia, Central America and Brazil, and *Robusta* coffee beans from Africa); BR (*Arabica* coffee beans from Central America and Brazil and *Robusta* coffee beans from Africa); ADR and ALR (*Arabica* coffee beans from Central America and Brazil). Two batches of each coffee type were analysed in quintuplicate. Samples were kept in a dry place, protected from light, at room temperature, in order to maintain all properties of *espresso* coffee capsules. For preparation of *espresso* coffee, all capsules containing ca. 5.5 g of coffee, were brewed with an appropriate coffee machine (19 x$10^5$ Pa), to obtain the first 5 mL of *espresso* coffee, directly to a 15 mL vial and hermetically sealed with a silicone-PTFE septum. The equipment was cleaned after each use with 1 L of hot water (95 ºC) passage through the system, in order to ensure the absence of volatiles contamination between samples.

### *3.2.3 Standard solutions*

Standard stock solutions of furan (83.61 µg $mL^{-1}$), furfural (772.5 µg $mL^{-1}$), furfuryl alcohol (766.4 µg $mL^{-1}$), 5-methylfurfural (794.3 µg $mL^{-1}$) and furfuryl acetate (1178 µg $mL^{-1}$) in methanol were prepared. A working solution (1:2) of each analyte was made by adding 0.5 mL of stock solution to 0.5 mL of HPLC grade water. The working solution was stored at 0 ºC and renewed daily.

The stock solution of $d_4$-furan (2 mg $mL^{-1}$) was stable for at least two weeks if kept at 4 ºC. Working solutions (0.1 mg $mL^{-1}$) were prepared daily by adding 0.05 mL of stock solution to a 2 mL HS vial containing 0.95 mL of water.

Calibration curves were constructed using a set of mixtures of furan, furfural, furfuryl alcohol, 5-methylfurfural and furfuryl acetate in water of analytical grade, under the same conditions used for sample analyses. The mixtures were prepared by diluting different volumes of the working solution in HPLC grade water completed to 1 mL final volume. In addition, 0.1 mL of $d_4$-furan working solution was added. Analytes concentration was calculated using the ratio of furanic compound / $d_4$-furan peak areas *versus* furanic compound concentration.

### *3.2.4. GC – MS conditions*

Chromatographic analysis was performed using the methodology described in the previous Chapter (Chapter 2; Section 2.2.3.: GC-MS analysis). Additionally, the furanic compounds were quantified in the Single Ion Monitoring (SIM) mode, using *m/z* 68, *m/z* 72, *m/z* 96, *m/z* 98, *m/z* 110 and *m/z* 140 characteristic ions for furan, $d_4$-furan, furfural, furfuryl alcohol, 5-methylfurfural and furfuryl acetate, respectively. The injection was done in splitless mode at 280 ºC. Fibre blanks were run daily to ensure the absence of contaminants or carry-over.

### *3.2.5 HS – SPME method*

The HS-SPME procedure used for furanic compounds in *espresso* coffee prepared from hermetically closed capsules was as follows: the sample (5 mL) was placed into a 15 mL HS vial, containing 2 g of sodium chloride and a magnet, and the vial was immediately closed. 0.1 mL of $d_4$-furan work solution (0.1 mg $mL^{-1}$) was added, and the vial was kept at -4 ºC during 10 min, in order to avoid losses due to their high volatility. Sample vial was placed into an ultrasonic bath (FUNGILAB, Barcelona, Spain) for 30 min, to equilibrate the analytes between the matrix and the HS. A SPME fibre coated with CAR/PDMS (75 µm thickness, Supelco Co., Bellefonte, PA, USA) was used to extract the volatile compounds. Prior to analysis, the SPME fibre was preconditioned at 300 ºC for 60 min in the GC injection port. The fibre was inserted into the sample vial through the septum and exposed to the HS for 30 min at room temperature (25 ºC) under constant agitation (750 rpm). Thereafter, the SPME fibre was inserted into the injector and analytes were desorbed for 10 min at 280 ºC.

Procedural blanks of clean water were routinely analysed to ensure the absence of contaminants in the HS-SPME equipment.

### *3.2.6 Analytical method validation*

The identification criteria for furanic compounds were based on their retention times and by the comparison of the analytes mass spectra with spectra from NIST library with a similarity ≥ 90 %. A deviation of the ion intensity ratios within 20% of the mean values of the calibration standards was considered acceptable. Intraday and interday precision were calculated using the analysed coffee samples. For intraday precision, samples were analysed five times consecutively and for interday precision, samples were analysed ten times in three nonconsecutive days. Precision was determined as RSD (%).

The LOD were calculated as the concentration corresponding to three times the standard deviation of the background noise, whereas the LOQ values were calculated as the concentration corresponding to ten times the standard deviation of the background noise.

The accuracy of the method was evaluated through recovery studies that were performed by spiking BR samples with different concentrations of furanic compounds, 0.208 µg $mL^{-1}$ for furan, 19.22 µg $mL^{-1}$ for furfural, 19.07 µg $mL^{-1}$ for furfuryl alcohol, 19.76 µg $mL^{-1}$ for 5-methylfurfural and 29.31 µg $mL^{-1}$ for furfuryl acetate. In addition, 0.1 mL of $d_4$-furan working solution (0.1 mg $mL^{-1}$) was added.

### *3.2.7 Statistical design*

All results are presented as mean and standard deviation of quintuplicate analyses performed in two batches for each type of *espresso* coffees. Statistical test One-Way ANOVA was used, in order to verify whether the differences between the content of furanic compounds in different types of *espresso* coffees were significant. *T*-Duncan was applied as the test *a posteriori* with a level of significance of 95 %. Normal distribution was checked by Shapiro–Wilk Test and homocedasticity of variances was checked by Levene Test. Pearson's correlation was applied to investigate correlation between the content of each furanic compound and its relative percentage considering the total of area of *espresso* volatile compounds. Statistical analyses were carried out using SPSS (v. 20.0).

## 3.3. Results e Discussion

### *3.3.1. Validation of a HS-SPME-GC-MS method for quantification of major furanic compounds*

The analytical performance of the HS-SPME-GC-MS method for reliable quantification of major furanic compounds in *espresso* coffees was evaluated. Calibration curve parameters, LOD, LOQ, repeatability or run-to-run precision and reproducibility or day-to-day precision, were determined. Quantitative analyses of HS require calibration curves performed by spiking the analyte in blank matrices. However, roasted coffee free of furanic compounds is not available. The alternative was to analyse headspace quantitative composition by adding standards to water (the most abundant compound of *espresso* coffee) and applying the same conditions used to analyse *espresso* coffee samples (Bicchi *et al.*, 2011).

Quality parameters of the method are summarized in Table 3.1.

**Table 3.1:** Quality parameters of the purposed HS-SPME-GC-MS method (LOD, LOQ, working range and linearity) for quantification of major furanic compounds and furan in *espresso* coffee (n=5 for intraday assays and n=10 for interday assays).

| | **Furan** | **2-furfural** | **Furfuryl alcohol** | **5-methylfurfural** | **Furfuryl acetate** |
|---|---|---|---|---|---|
| LOD (μg $mL^{-1}$) | $1.0 \times 10^{-5}$ | $9.0 \times 10^{-5}$ | $9.0 \times 10^{-5}$ | $1.0 \times 10^{-4}$ | $1.4 \times 10^{-4}$ |
| LOQ (μg $mL^{-1}$) | $1.0 \times 10^{-4}$ | $9.0 \times 10^{-4}$ | $9.0 \times 10^{-4}$ | $1.0 \times 10^{-3}$ | $1.4 \times 10^{-3}$ |
| Working range (μg $mL^{-1}$) | 0.0001 – 13.935 | 0.0009 – 128.75 | 0.0009 – 99.967 | 0.0010 – 132.39 | 0.0014 – 56.099 |
| $r^2$ | 0.9990 | 0.9992 | 0.9992 | 0.9994 | 0.9993 |
| $S_{y/x}$ | 0.056 | 0.0136 | 0.017 | 0.020 | 0.023 |
| $a \pm S_a$ | -0.008±0.023 | 0.042±0.005 | 0.014±0.007 | 0.016±0.008 | 0.031±0.009 |
| $b \pm S_b$ | 0.134±0.004 | 0.030±0 | 0.039±0 | 0.045±0 | 0.051±0 |

LOD, limits of detection; LOQ, limits of quantification. $S_{y/x}$ - standard deviation of y-residuals of regression. a- intercept, b– slope; $S_b$ and $S_a$ - standard deviations of the slope and intercept, respectively.

Calibration curves with working ranges varying from 0.0001 and 13.935 μg $mL^{-1}$ for furan, 0.0009 and 128.75 μg $mL^{-1}$ for furfural, 0.0009 and 99.967 μg $mL^{-1}$ for furfuryl alcohol, 0.0010 and 132.39 μg $mL^{-1}$ for 5-methylfurfural and 0.0014 and 56.099 μg $mL^{-1}$ for furfuryl acetate were constructed, good linearity was obtained with a correlation coefficient ($r^2$) higher than 0.999. However, since a significant proportion of errors at the lower end of the calibration line can coexist with acceptable correlation coefficients, and these errors are underestimated in analyzing the dispersion of the regression parameters (Miller & Miller, 2005; Mansilha et al., 2010). The calculation of random errors in the y-direction ($S_{y/x}$) as well as the standard deviations for the slope ($S_b$) and intercept ($S_a$) associated with the line was also evaluated. The parameters of the calibration curves (slope and intercept) and their respective errors, as well as the regression error, are presented in Table 3.1. The validation conditions of the calibration curves were satisfied (Miller & Miller, 2005).

Low LOD (0.00001 μg $mL^{-1}$ for furan, 0.00009 μg $mL^{-1}$ for 2-furfural, 0.00009 μg $mL^{-1}$ for furfuryl alcohol, 0.00010 μg $mL^{-1}$ for 5-methylfurfural and 0.00014 μg $mL^{-1}$ for furfuryl acetate) and LOQ (0.0001 μg $mL^{-1}$ for furan, 0.0009 μg $mL^{-1}$ for furfural, 0.0009 μg $mL^{-1}$ for furfuryl alcohol, 0.0010 μg $mL^{-1}$ for 5-methylfurfural and 0.0014 μg $mL^{-1}$ for furfuryl acetate) were achieved. LOD and LOQ for furan were lower than that described by other authors for coffee brew (Arisseto *et al.*, 2011; EFSA, 2011a). Comparison with literature can only be made with furan since quantification of other furanic compounds was not found.

### *3.3.2. Sample collection for recovery studies and methodology application*

In order to optimise the sampling procedure, two proves were carried out. The first one consisted on collecting an aliquot of 5 mL from a 40 mL *espresso* coffee (since in Portugal the volume of an *espresso* coffee usually ranges between 30 and 50 mL) sample to a 15 mL vial containing 2 g of NaCl and a magnet, following the methodology previously explained. The second one consisted on collecting the first 5 mL of coffee sample directly to the 15 mL vial containing 2 g of NaCl and a magnet. Analyses were made in triplicate for each sample and results are presented in Table 3.2.

The amount of furanic compounds was similar in both experiments. Consequently both trials could be used to quantify furanic compounds in *espresso* coffees. However, it was chosen to collect the first 5 mL of *espresso* sample, since lower standard deviation values were found when carrying out this procedure.

**Table 3.2:** Comparison of furanic compounds levels present in the two sample collection procedures. Results are summarized as mean and standard deviation (n=3). Significant levels $p<0.05$.

| Compound | Conc. (µg $mL^{-1}$) 5mL aliquot from 40 mL *espresso* sample | Conc. (µg $mL^{-1}$) First 5 mL *espresso* sample | *p* |
|---|---|---|---|
| **Furan** | 0.23 ± 0.01 | 0.23 ± 0.02 | 0.274 |
| **Furfural** | 70.8 ± 1.5 | 70.6 ± 1.2 | 0.122 |
| **Furfuryl alcohol** | 57.7 ± 1.1 | 57.7 ± 0.5 | 0.127 |
| **5-methylfurfural** | 44.5 ± 1.0 | 44.5 ± 0.4 | 0.960 |
| **Furfuryl acetate** | 25.7 ± 0.9 | 25.6 ± 0.7 | 0.114 |

Table 3.3 show the results for recovery studies. In order to evaluate the accuracy of the method they were performed at one concentration level for each analyte (0.208 µg $mL^{-1}$ for furan, 19.22 µg $mL^{-1}$ for furfural, 19.07 µg $mL^{-1}$ for furfuryl alcohol, 19.76 µg $mL^{-1}$ for 5-methylfurfural and 29.31 µg $mL^{-1}$ for furfuryl acetate). Mean recoveries achieved were 101.9 % for furan, 99.70 % for furfural, 97.98 % for furfuryl alcohol, 99.82 % for 5-methylfurfural and 99.93 % for furfuryl acetate. With respect to intraday precision, RSD was lower than 0.1 % for retention time and lower than 5 % for peak area. For interday precision, RSD was lower than 1.07 % for retention time and lower than 10.6 % for peak area. These results fulfil the EFSA (2011a) requirements for quantification of furan compound (LOQ less than 5 µg $kg^{-1}$, precision less than 20 % RSD and a minimum recovery of 80 %) and validate the analytical procedure developed in the present study, being well applicable for the reliable quantification of major furanic compounds and furan in *espresso* coffee samples.

**Table 3.3:** HMF and furfural recovery studies (spiked level, recovery percentage and reproducibility) using blend roast samples (n=3).

| | | Furan | Furfural | Furfuryl alcohol | 5-methylfurfural | Furfuryl acetate |
|---|---|---|---|---|---|---|
| **Spiked level (µg mL$^{-1}$)** | | 0.208 | 19.22 | 19.07 | 19.76 | 29.31 |
| **Recovery %** | | 101.9 | 99.70 | 97.98 | 99.82 | 99.93 |
| **RSD % Intraday** | **RT** | 0.05 | 0.06 | 0.01 | 0.01 | 0.01 |
| | **Area** | 1.71 | 1.28 | 3.24 | 5.00 | 1.24 |
| **RSD % Interday** | **RT** | 1.07 | 0.04 | 0.01 | 0.01 | 0.01 |
| | **Area** | 1.79 | 1.78 | 7.47 | 10.6 | 7.31 |

RSD: Relative standard deviation.

### *3.3.3. Application of the HS-SPME-GC-MS method for quantification of major furanic compounds in espresso coffee from hermetically closed capsules*

The HS-SPME-GC-MS methodology allowed analyses of furanic compounds and other volatiles by TIC (Figure 3.1), where major peaks were from furanic compounds. SIM of furan, $d_4$-furan, furfural, furfuryl alcohol, 5-methylfurfural and furfuryl acetate was used for quantification purposes.

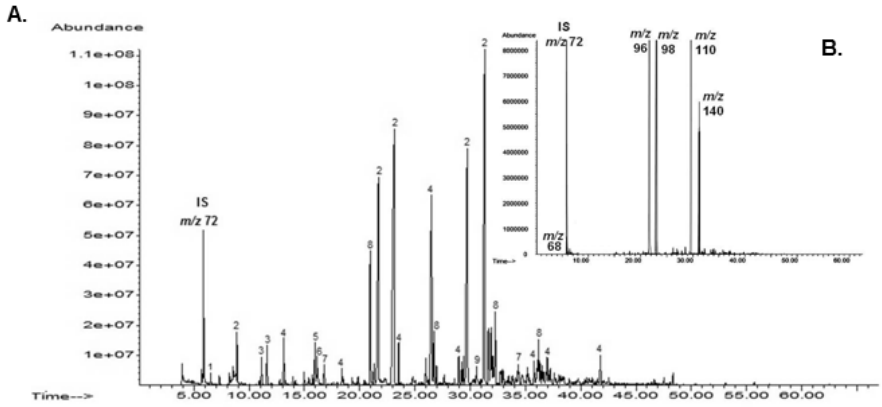



**Figure 3.1:** A. HS-SPME-GC-MS chromatogram of an *espresso* coffee sample with major peaks numbered by chemical families: 1 - acids, 2 - furans, 3 - aldehydes, 4 - ketones, 5 - pyridines, 6 - pyrroles, 7 - aromatic rings, 8 - pyrazines, 9 – alcohols. B. major furanic compounds and furan were selected by their *m/z* and quantified: *m/z* 68 – furan; *m/z* 72 – $d_4$-furan; *m/z* 96 – furfural; *m/z* 98 furfuryl alcohol; *m/z* 110 -5-methylfurfural; *m/z* 140 – furfuryl acetate.

HS-SPME-GC-MS analysis in *espresso* coffee and artificially aromatized *espresso* coffee indicated a total of 69 volatiles for BR and BDR samples, 64 volatiles for ADR samples, 91 volatiles for ALR samples, 96 volatiles for CAR samples, 90 volatiles for VAN samples and 92 volatiles for ALM samples. Volatiles were clustered according to their chemical families: ethers, alcohols, hydrocarbons, aromatic hydrocarbons, acids, pyridines, pyrroles, aldehydes, ketones, pyrazines, and furanic compounds (Table 3.4).

ADR and ALR samples presented lower sum of volatile compounds peak area than BR and BDR samples, whereas CAR, VAN and ALM showed higher sum of volatile compounds' peak area than all non-aromatized samples. In general, furanic compounds were the major chemical family (except in ALM espresso, where the major group were aldehydes), followed by pyrazines, ketones, aldehydes, pyrroles, pyrazines and acids. Ethers, alcohols, hydrocarbons and aromatic hydrocarbons presented minor percentages. Similar results have been found by Petisca *et al.*, (2012), and López-Galilea *et al*,. (2006) for *espresso* coffee.

Table 3.4 also presents detailed relative percentage of major furanic compounds and furan. In general the furanic compounds abundance (relative percentage of area) in decreasing order was furfuryl alcohol, furfuryl acetate, furfural, 5-methylfurfural, and furan, except ADR and ALR *espresso* samples (100% *Arabica*) that presented higher relative percentage of furfuryl acetate. Hovell *et al.*, (2010) showed that some furanic compounds can be used to discriminate *Arabica* and *Robusta* coffees, namely furfural, furfuryl alcohol, 5-methylfurfural and furfuryl acetate. In our previous study, performed in *espresso* coffees from 100% *Arabica* variety, the decreasing order for furanic compounds abundance was furfuryl acetate followed by furfuryl alcohol, furfural, 5-methylfurfural and furan, close to that obtained for ADR and ALR samples (Petisca *et al.*, 2012). Although furan is not a major furanic compound its quantification was also performed due to their possible health effects.

**Table 3.4:** Relative percentage of each chemical family of *espresso* coffee volatile compounds with detailed information about relative percentage of furan and major furanic compounds. Results are presented as mean and standard deviation of five replicates for two lots of each sample. *Espresso* coffee was prepared from hermetically closed capsules of blend roast (BR), blend dark roast (BDR), 100% *Arabica* dark roast (ADR), 100% *Arabica* light roast (ALR), caramel (CAR), vanilla (VAN) and almond (ALM).

| Chemical families | BR | BDR | ADR | ALR | CAR | VAN | ALM |
|---|---|---|---|---|---|---|---|
| Ethers | n.d. | n.d. | n.d. | n.d. | 0.08±0.08 | 0.05±0.05 | n.d. |
| Alcohols | n.d. | 0.21±0.27 | n.d. | n.d. | 0.42±0.29 | 0.06±0.04 | 0.07±0.07 |
| Hydrocarbons | 0.67±0.24 | 0.22±0.14 | 0.26±0.21 | 1.11±0.29 | 0.91±0.59 | 0.63±0.40 | 0.83±0.58 |
| Arom. Hydrocar. | 0.47±0.48 | 0.21±0.08 | 0.15±0.06 | 0.58±0.60 | 0.60±0.36 | n.d. | 10.12±8.78 |
| Acids | 4.13±0.37 | 3.56±0.92 | 1.47±0.11 | 4.44±0.75 | 1.71±0.30 | 13.58±0.95 | 1.63±0.31 |
| Pyridines | 2.49±0.28 | 3.65±1.05 | 3.21±0.91 | 2.23±0.29 | 8.91±3.52 | 6.80±1.01 | 1.03±0.21 |
| Pyrroles | 3.41±0.28 | 5.24±0.78 | 3.14±0.59 | 6.53±1.01 | 1.37±0.16 | 3.63±0.49 | 1.88±0.45 |
| Aldehydes | 3.09±1.02 | 3.09±1.14 | 3.71±0.86 | 3.47±0.36 | 7.35±2.59 | 6.18±2.91 | 41.04±8.36 |
| Ketones | 7.35±0.59 | 8.18±1.17 | 8.04±0.59 | 23.78±4.64 | 14.51±1.38 | 13.52±2.72 | 4.73±0.26 |
| Pyrazines | 16.23±1.37 | 14.98±2.40 | 20.28±3.77 | 20.90±2.03 | 7.20±0.71 | 9.07±1.33 | 10.73±0.44 |
| Furanic comp. | 62.17±1.28 | 60.72±0.97 | 59.74±1.34 | 36.96±4.15 | 56.94±2.54 | 46.49±3.86 | 27.95±1.57 |
| **Sum of volatile comp. peak área (arbitrary units)** | **5.42 x10$^{10}$** | **5.58 x10$^{10}$** | **4.45 x10$^{10}$** | **4.31 x10$^{10}$** | **7.82 x10$^{10}$** | **8.52 x10$^{10}$** | **1.04 x10$^{11}$** |
| Furan | 0.03±0.00 | 0.03±0.00 | 0.04±0.01 | 0.02±0.00 | 0.02±0.00 | 0.02±0.01 | 0.01±0.00 |
| Furfural | 10.55±0.15 | 10.55±0.83 | 9.60±1.11 | 2.06±0.27 | 7.91±0.40 | 7.06±1.36 | 4.53±0.18 |
| Furfuryl alcohol | 19.76±2.98 | 21.23±1.58 | 17.53±3.35 | 2.48±0.34 | 21.53±2.24 | 20.38±1.97 | 10.74±0.53 |
| 5-methylfurfural | 9.60±1.49 | 8.16±2.06 | 7.94±1.33 | 2.09±0.27 | 5.71±0.29 | 4.77±0.98 | 0.37±0.07 |
| Furfuryl acetate | 17.89±1.75 | 17.12±1.93 | 18.80±0.86 | 20.90±2.24 | 12.12±1.73 | 10.27±2.09 | 8.55±1.35 |

The concentration of furanic compounds in *espresso* samples is presented in Table 3.5. It is possible to observe that for BR, BDR, ADR and ALR samples the major furanic compound was furfural (35.12 – 70.21 µg $mL^{-1}$), followed by furfuryl alcohol (31.2 – 57.7 µg $mL^{-1}$), 5-methylfurfural (23.8 – 44.8 µg $mL^{-1}$), furfuryl acetate (13.3 – 25.8 µg $mL^{-1}$) and furan (0.22 – 0.24 µg $mL^{-1}$). In samples with addition of aroma, the major furanic compound was furfuryl alcohol (48.8 – 69.9 µg $mL^{-1}$), followed by furfural (38.8 – 44.6 µg $mL^{-1}$), 5-methylfurfural (21.4 – 24.8 µg $mL^{-1}$), furfuryl acetate (17.1 – 17.9 µg $mL^{-1}$) and furan (0.230 µg $mL^{-1}$). The furan levels found in this study are close to the levels found by other authors for *espresso* coffees (JECFA, 2001; WHO, 2011; Kuballa *et al.*, 2005; Zoller *et al.*, 2007, Altaki *et al.*, 2011) although furan levels can vary from sample to sample, due to several factors, namely brewing temperature, the type of extraction method, the type of fibre used and roasted coffee beans.

Statistical test One-Way-ANOVA was used, in order to verify whether the differences between the content of furanic compounds in different types of *espresso* coffees were significant. This method was applied because data for each type of coffee presented normal distribution (Shapiro–Wilk Test) and homocedasticity of variances (Levene Test). These results show that each type of espresso coffee present a different quantitative profile of furanic compounds. Additionally, Pearson's correlation coefficients indicate that no correlation was found between the content of each furanic compound expressed as µg $mL^{-1}$ of *espresso* coffee and its relative percentage considering the total area of *espresso* volatile compounds.

**Table 3.5:** Major furanic compounds content and furan (μg $mL^{-1}$) in *espresso* coffee prepared from hermetically closed capsules of blend roast (BR), blend dark roast (BDR), 100% *Arabica* dark roast (ADR), 100% *Arabica* light roast (ALR), caramel (CAR), vanilla (VAN) and almond (ALM).

| Samples / Compounds | BR | BDR | ADR | ALR | CAR | VAN | ALM | *p* |
|---|---|---|---|---|---|---|---|---|
| **Furan** | 0.23±0.01a | 0.24±0.02b | 0.22±0c | 0.23±0d | 0.23±0.01e | 0.23±0.01d | 0.23±0.01d | <0.001 |
| **2-Furfural** | 37.0±1.5a | 52.0±0.7b | 35.1±0.7c | 70.2±1.0d | 44.6±1.3e | 44.3±1.9e | 38.8±1.3f | <0.001 |
| **Furfuryl alcohol** | 34.8±1.4a | 41.9±1.2b | 31.2±0.9c | 57.7±1.4d | 59.5±1.4e | 69.9±0.5f | 48.8±0.5g | <0.001 |
| **5-Methylfurfural** | 23.9±0.5a,b | 32.8±0.7c | 23.8±1.1a,b | 44.8±0.5d | 23.1±1.3a | 24.8±0.8b | 21.4±1.1e | <0.001 |
| **Furfuryl acetate** | 13.3±0.7a | 19.7±0.8b | 14.4±0.7c | 25.8±0.6d | 17.9±0.5e | 17.1±0.3e | 17.7±0.7e | <0.001 |
| **Sum of major Furanic compounds** | 109 | 147 | 105 | 199 | 145 | 156 | 127 | |

[a-g] Means with different letters in the same line are significantly different for each type of coffee. $p < 0.05$ means significant differences.

## 3.4. Conclusions

In conclusion, between the different types of espresso coffee samples the profile of volatile compounds is highly variable. Consequently, the daily intake of these compounds by consumers varies greatly. The validated method proved to be a reliable methodology for quantification of major furanic compounds and furan present in *espresso* coffee. Although relative percentage of peak area is a good method for discriminate volatiles in different coffee brews (López-Galilea *et al.*, 2006) and compare samples with similar composition (Petisca *et al.*, 2012), the quantification of furanic compounds in different types of coffee revealed to be more accurate to understand the real intake amount and in the future can be used to evaluate the effect of roast degree, coffee composition (blend *Arabica*+*Robusta vs* 100% *Arabica*) and artificial aromatisation on the quantity of major furanic compounds and furan.

# PART III

## Furanic compounds in coated deep-fried products simulating normal preparation and consumption

# CHAPTER 4

## Quantification of volatile and less volatile furanic compounds in coated deep-fried products: optimisation of extraction conditions by response surface methodology

## Abstract

Two methods were validated for quantification of volatile and less volatile furanic compounds in coated deep fried samples processed and handled as usually consumed. The deep-fried food was grinded using a device that simulates the mastication, and immediately analysed.

Volatile furanic compounds were quantified by HS-SPME-GC/MS. Parameters affecting the efficiency of HS-SPME procedure were selected by RSM, using a $2^3$ full-factorial central composite design (CCD). Optimal conditions were achieved using 2 g of sample, 3 g of NaCl and 40 min of absorption time at 37 ºC.

The development and validation of an extraction procedure for the quantification of HMF in coated deep-fried products by HPLC-DAD is also described. The method entailed the extraction of HMF with ethyl acetate/hexane (4:1) followed by a concentration step with 40 mM sodium formate (pH=3)/methanol (1:1). The optimum combination of the extraction variables was also achieved by RSM. Sample amount and concentration solvent volume showed a notable influence on HMF yield, while the effect of extraction solvent volume seemed to be less marked. From experimental results, 5 g of sample, 10 mL of the extraction solvent, and 550 µL of the concentration solvent were selected as optimal combination.

Consistency between predicted and experimented values was observed for the two methods and quality parameters of each method were established. Furan, furfural, furfuryl alcohol and 2-pentylfuran levels in coated deep-fried fish were 5.59, 0.27, 10.48 and 1.77 µg $g^{-1}$, respectively. HMF content was 1.25±0.21 µg $g^{-1}$.


**Keywords:** Coated deep-fried products; Furanic compounds; Extraction procedure optimisation; Method optimisation; Process and consuming conditions; Response Surface Methodology.

## 4.1. Introduction

Cereal flour and vegetable oil mixtures constitute one of the main ingredients of many foods, such as frozen coated products. This type of foodstuff is composed by a food matrix covered by a layer made with flour, oil, water, starch, salt, and spices among others. The coated deep-fried products are highly appreciated and consumed by the young population. In addition, these products are easily and quickly prepared. However, there are no literature data showing the levels of furanic compounds in coated deep-fried products.

Thermal processes used in the preparation of coated deep-fried products have a strong impact on its final quality, being related with sensory properties, such as palatability, colour, taste, aroma, and texture. However, some detrimental consequences of thermal processes come from the formation of potential mutagenic, carcinogenic, and cytotoxic compounds that are absent in fresh and untreated food. Well-known examples of these compounds are heterocyclic amines, acrylamide, and furans (Capuano & Fogliano, 2011; Melo *et al.*, 2008; Viegas *et al.*, 2012a).

The formation of furan and its derivatives has been associated with thermal treatment of *Maillard* reaction precursors or lipids (Maga, 1979; Mottram, 1991). Alkylsubstitute furans are formed through the thermal oxidation of lipids (Tang *et al.* 1983), while furfuryl alcohol, furfural, and HMF derive from fructose, pentose, and hexose sugars, respectively (Swasti & Murkovic, 2011; Gaspar & Lucena, 2009).

Furan is formed during commercial or domestic thermal treatment of food and it is considered a possible human carcinogen (Group 2B) by the IARC (IARC,1995). Consequently, the presence of this compound in foodstuffs is of concern.

Furan content in 4186 food samples (coffee, infant formula, baby food, cereal, meat, fish, vegetal, dairy and fruit products) has been reported by EFSA (EFSA, 2010) from 2004 to 2009 and later updated (EFSA, 2011a). These EFSA reports (2010, 2011a) note that 92 % of the results concerning furan content in foods were obtained for samples analysed as purchased and it claims that future evaluation of furan content should be performed in the samples as they are consumed. Thus, the exact cooking conditions such as time, temperature and handling information should be mentioned.

Not only furan is found in thermally treated products, but furan derivatives are also present. Namely, furfural, furfuryl alcohol, and 2-pentylfuran were found in smoked-cured bacon by Yu *et al.*, (2008). Giri *et al.*, (2010) found 2-ethylfuran, 2-butylfuran, 2-acetylfuran, 2-pentylfuran, furfural, and furfuryl alcohol in paste fish. Furan and its

derivatives contribute to the flavour of foods and beverages. These compounds have low thresholds and provide characteristic pleasant odour, such as cocoa, butter or fruity (Belitz & Grosch, 1997). Furanic compounds contribute to the characteristic odour of French fries (Wagner & Grosch, 1998) and roasted beef (Cerny & Grosch, 1992). However, there are also studies revealing the toxicity of these furanic compounds in animals and humans (Sujatha, 2008; Arts *et al.*, 2004; Goldsworthy *et al.*, 2001; Wilson *et al.*, 1992).

Due to its high volatility, furan levels in foods are usually determined, with high accuracy, by HS methods, followed by GC–MS monitoring *m/z* 39 and *m/z* 68. Moreover, international organisations recommend the use of isotopically labelled furan (monitoring *m/z* 72 for $d_4$-furan) as IS for quantification purposes (EFSA, 2004a; FDA, 2004).

The effect of time and temperature during the HS procedures has been highly investigated and remains a controversial subject. The first reports on furan analysis indicated HS incubation and extraction temperatures of 80 ºC for at least 30 min (EFSA, 2004a; FDA, 2004), however, in 2006 FDA decreased the incubation temperature from 80 to 60 ºC, due to the formation of additional furan amounts, cover-up the real furan levels present in samples (Senyuva & Gokmen, 2005).

Most recent EFSA publications (EFSA, 2009c, 2010) indicated the use of HS or HS-SPME coupled to GC–MS for furan analysis. HS-SPME methodology has proved to be an excellent alternative to HS for the analysis of volatile compounds in food samples (Ferreira *et al.*, 2009; Ramos *et al.*, 2009). Advantages of HS-SPME are the possibility of using lower extraction temperature (Wardencki *et al.*, 2004) and obtaining higher sensitivity values. Senyuva & Gokmen (2005) found that furan response rises exponentially as the equilibration temperature increases from 40 to 80 ºC. On the other hand, Altaki *et al.*, (2007) reported a decrease in the relative furan response by increasing the extraction temperature from 25 to 40 ºC. Nevertheless, Kaseleht & Leitner (2008) advised to use extraction temperatures close to the human body temperature since they reproduce volatile compounds people feel when consuming.

The CAR/PDMS fibre presents advantages in terms of sensitivity (Altaki *et al.*, 2007; Bianchi *et al.*, 2006; Goldmann *et al.*, 2005) and it is the most commonly used for the analysis of this type of compounds. Goldmann *et al.*, (2005) reported a rapid and robust SPME-GC–MS method that entailed the following steps: addition of $d_4$-furan to the sample, NaCl-assisted extraction into the HS, cryofocussing, and finally fibre desorption and GC–MS analysis. They also indicated that furan should be analysed in refrigerated samples. Additionally, for an accurate quantification of furan, calibration curve should be prepared and analysed applying the same HS sampling conditions (Vranová *et al.*, 2007).

Altaki *et al.,* (2009) have proposed an automated HS-SPME-GC–ion trap-MS method for furan determination in food.

HMF is less volatile than other furanic compounds and has been analysed in cereal products, honey, fruit products, and coffee (Capuano & Fogliano, 2011; Abraham *et al.*, 2011), generally by HPLC-DAD (Gaspar & Lucena, 2009; Akpinar *et al.*, 2011; Durmaz & Gökmen, 2010), with the simultaneous determination of furfural in some cases (Durmaz & Gökmen, 2010; Rufián-Henares *et al.*, 2006b; Chambel *et al.*, 1997).

HMF is known to be a good indicator to control the heat load of industrial-processed foods (Capuano & Fogliano, 2011; Abraham *et al.*, 2011). The growing attention of the scientific community with regard to the potentially toxic effects of this compound requires new efforts to be made to establish rapid, reliable, and sensitive methods for determining HMF in different food matrices (Durmaz & Gökmen, 2010).

Different extraction methods for HMF are described in literature, namely, suspension in water followed by protein precipitation in TCA, which was selected as the extraction procedure in cookies (Ait-Ameur *et al.*, 2006), and aqueous extraction with simultaneous clarification using *Carrez* I and II reagents, which have been used for the extraction of HMF from breakfast cereal (Rufián-Henares *et al.*, 2006b) and baby foods (Gökmen & Senyuva, 2006). The use of *Carrez* I and II as clarifying agents instead of chemical acids TCA is recommended for cereal products because of the possible production of HMF from glucose present in the food matrices at low pH (Abraham *et al.,* 2011).

Teixidó *et al.*, (2006) obtained the best extraction yield in different food samples by using dichloromethane. Gaspar & Lucena, (2009) extracted HMF from samples using the HPLC mobile phase consisting of water, acetonitrile, and perchloric acid. In roasted hazelnut, ethyl acetate was the most efficient solvent for HMF extraction (Durmaz & Gökmen, 2010), whereas in roasted flour/oil mixtures, carbon tetrachloride is described as the most appropriate solvent followed by extraction of this organic phase with water and addition of *Carrez* I and II to the aqueous phase (Akpinar *et al.*, 2011).

The optimisation of the different extraction parameters is usually performed by univariate strategies which could generate some limitations since the interactions between factors are not considered. The RSM explores the relationship between several explanatory variables and one or more response variables by means of a mathematical model able to properly predict the values of the response. This is a very useful tool for selecting the optimum conditions when there are interactions between variables (Bianchi *et al.*, 2006, Ghafoor *et al.*, 2009; Leardi, 2009).

The main goals of this study were the validation of a method for the simultaneous quantification of furan and its volatile derivatives in coated deep-fried samples by using HS-SPME coupled to GC–MS and the development and validation of an extraction procedure for HPLC-DAD quantification of HMF, a less volatile furanic compound in coated deep-fried products. It was also aimed (i) to reproduce usual preparation and consumption throughout the analysis and (ii) to select the optimum combination of analytical variables using RSM.

## 4.2. Material and methods

### *4.2.1. Chemicals and standards*

$D_4$-furan (98 %) was provided by ISOTEC (Ohio, USA). Furan (99 %), furfuryl alcohol (99 %) were supplied by Sigma-Aldrich (Steinheim, Germany). Furfural was purchased by Merck (99 %) (Darmstadt, Germany). 2-pentylfuran (98 %) was provided by ALFAAESAR (Karlsrula, Germany). Methanol was supplied by MERCK (Darmstadt, Germany) and ultrapure water (0.055 µS $cm^{-1}$) was obtained by using a Serial Milli-Q system for Millipore (Supor DCF, Gelman Sciences, Chentelham, Australia). HMF (98 %) was supplied by Sigma-Aldrich (Steinheim, Germany). Ethyl acetate, hexane, sodium formate, formic acid, and methanol were supplied by Merck (Darmstadt, Germany). Frozen coated fish and sunflower oil were obtained from a local store.

### *4.2.2. Standard solutions*

Stock solution of $d_4$-furan 2 mg $mL^{-1}$ was prepared by injecting 2 mg $mL^{-1}$ of refrigerated $d_4$-furan with a syringe through the septum of a 10 mL HS vial filled with 10 mL of methanol and sealed. The exact weight of methanol and $d_4$-furan was recorded, expressing the concentration in mg $mL^{-1}$ and taking into account the density of methanol. This solution is stable for at least two weeks if kept at 4 ºC. Working solutions 1 mg $mL^{-1}$ were prepared daily by adding 500 µL of stock solution to a 1 mL HS containing 500 µL of water using the same procedure.

A standard calibration solution containing 8.69, 0.52, 10.84 and 0.07 mg $mL^{-1}$ for furan, furfural, furfuryl alcohol and 2-pentylfuran, respectively, was prepared by the addition of 100, 5, 100 and 5 µL of furan, furfural, furfuryl alcohol and 2-pentylfuran, respectively, into a 15 mL HS vial containing 15 mL of methanol. The exact weight of methanol and each added furanic compound was recorded expressing the concentration in µg $µL^{-1}$.

Concerning HMF a stock solution was prepared in methanol at *ca*. 1.2 mg $mL^{-1}$. A working solution at *ca*. 0.056 mg $mL^{-1}$ was made by injecting 50 µL of the stock solution into a vial containing 1 mL of the 40 mM sodium formate (pH=3)/methanol (1:1) mixture. Consecutive dilutions of the standard calibration solution in methanol were made. Thus, standard calibration curves (0.0008–0.056 µg $\mu L^{-1}$) were constructed for HMF.

### *4.2.3. Sample preparation*

Before preparation, coated fish were kept frozen at -10 ºC. Frozen coated fish pieces were fried in sunflower oil using a domestic deep fryer (KENWOOD DF-150, 1 l) at 180 ºC during 4 min, according to the manufacturer. Coated deep-fried fish was drained, placed on a plate covered with a paper towel for removing external oil, and grinding by using a device named ‘‘masticator shears straight’’ (BUENO HERMANOS, S.A., La Rioja, Spain, ISO 9001-2000 Quality Certified Company) which simulates the chewing process.

### *4.2.4. HS-SPME-GC/MS procedure*

#### 4.2.4.1. Furanic compounds extraction

The optimised HS-SPME procedure used for quantification of furanic compounds in coated deep-fried fish samples was as follows: 2 g were transferred to a 50 mL HS vial, containing 5 mL of water and 3 g of NaCl. 100 µL of $d_4$-furan work solution was added, and the vial immediately sealed at once and kept at -4 ºC during 10 min, in order to avoid losses due to the high volatility of the compounds under study. Afterwards, the vial was placed into an ultrasonic cleaner (FUNGILAB, Portugal) during 15 min, favouring the equilibrium between the matrix and the HS (Zhang & Pawliszyn, 1993). To extract furanic compounds a CAR/PDMS SPME fibre (75 µm thickness, Supelco Co., Bellefonte, PA, USA) was used.

Prior to analysis, the SPME fibre was preconditioned at 300 ºC for 60 min in the GC injection port. The fibre was inserted into the sample vial through the septum and exposed to the HS for 40 min at 37 ± 1 ºC under constant agitation (600 rpm). Thereafter, the SPME fibre was inserted and desorbed for 10 min at 280 ºC, in splitless mode, with 1 mL $min^{-1}$ flow.

#### 4.2.4.2. GC–MS conditions

Chromatographic analysis was performed using an Agilent 6890 GC (Agilent, Avondale, PA, USA) coupled to a MS detector (Agilent 5973). Volatiles were separated on a 5% phenyl-methyl silicone (HP-5) bonded phase fused-silica capillary column (Hewlett–Packard, Palo Alto, CA, USA; 60 m x 320 µm i.d., film thickness 1 µm), operating at 80 kPa column head pressure, resulting in a flow of 1 mL $min^{-1}$ at 40 ºC. The oven temperature programme was isothermal for 5 min at 40 ºC, raised to 135 ºC at a rate of 3ºC $min^{-1}$ and then raised to 220 ºC at 20ºC $min^{-1}$. The transfer line to the MS was maintained at 250 ºC. Mass spectra were obtained by electronic impact at 70 eV, with a multiplier voltage of 2056 V, collecting data at a rate of 1 scan $s^{-1}$ over the *m/z* range 30–500. Furanic compounds were identified by comparison with the mass spectrum from Nist 98 data bank (NIST/ EPA/NISH Mass Spectral Library, version 1.6, USA) and retention indices of commercial reference compounds. The furanic compounds were also detected by *m/z* characteristic ion, using *m/z* 68, *m/z* 72, *m/z* 96, *m/z* 98 and *m/z* 138 for furan, $d_4$-furan, furfural, furfuryl alcohol and 2-pentylfuran, respectively. Fibre blanks were run daily to ensure the absence of contaminants or carry-over.

#### 4.2.4.3. Quality control and criteria

A daily sensitivity test was carried out to check for possible changes in the absorption capacity of the SPME fibre and the GC–MS response.

Detection and quantification limits were based on a signal-to-noise ratio of 3:1 and 10:1, respectively. Since it is not possible to achieve blank samples (coated deep-fried fish products without furanic compounds) these parameters were determined using diluted standard solutions (n = 5) placed in a 50 mL HS vial containing 5 mL of water and 3 g of NaCl, following the same preparation and analysis conditions of the samples. The RSD of run-to-run and day-to-day analyses was performed in one day and in two more non-consecutive days using five replicate analyses of the same sample.

#### 4.2.4.4. Calibration curves

Furan and its derivatives were quantified by external calibration curve method and by standard addition method. Five consecutive dilutions of the standard calibration solution in methanol (1:10 v/v) were prepared. 100 µL of the corresponding standard solution and a fixed volume (100 µL) of $d_4$-furan working solution were placed in a 50 mL HS vial containing 5 mL of water and 3 g of NaCl, following the same preparation and analysis conditions of samples. For each individual furanic compound a calibration curve (furanic compound /$d_4$-furan peak areas *vs.* furanic compound amount) was constructed, obtaining $r^2$ values of 0.9999. The final results, expressed in µg $g^{-1}$, take into account the exact weight of the sample portion in the HS vial.

The standard addition procedure was also used for quantifying furanic compounds. Samples were spiked with appropriate amounts of furan, furfural, furfuryl alcohol and 2-pentylfuran over the range 0.05–40, 0.002–2.5, 0.005–50, and 0.0003– 0.3 µg $g^{-1}$, respectively. For this purpose, 100 µL of the corresponding standard solution and 100 µL of $d_4$-furan working solution were spiked into the HS vial containing 2 g of sample, 5 mL of water and 3 g of NaCl, following the same preparation and analysis conditions of samples. The quantity of each furanic compound was calculated by using the calibration curves constructed (furanic compound /$d_4$-furan peak areas *vs*. µg furanic compound added/ g sample).

#### 4.2.4.5. Experimental design

Trials were conducted to optimise HS-SPME conditions for the quantitative analyses of furanic compounds in coated deep-fried fish using RSM. It was applied a full-factorial CCD, consisting of a complete $2^3$-factorial design with six centre points and two axial points on the axis of each design variable at a distance of $\alpha = 1.682$ from the design centre. Hence the complete design had 20 combinations, including six replicates of the centre point. The response was the sum of furanic compounds/$d_4$-furan peak areas. Experiments were carried out with sample amount, NaCl amount and absorption time, varying from 2 to 5 g, from 1 to 3 g and from 10 to 40 min, respectively. Table 4.1 shows the complete experimental design, which was performed by Design Expert trial-Version 7 (Stat-Ease Inc., Minneapolis, MN).

**Table 4.1:** Coded and uncoded values of the independent variables of the central composite design for the HS-SPME method optimisation.

| | Independent variables | | | | | |
|---|---|---|---|---|---|---|
| | CODED | | | UNCODED | | |
| Standard order | $X_1$ Sample amount (g) | $X_2$ NaCl amount (g) | $X_3$ Absorption temperature (º C) | $X_1$ Sample amount (g) | $X_2$ NaCl amount (g) | $X_3$ Absorption temperature (º C) |
| 1 | 1 | 1 | 1 | 5 | 3 | 40 |
| 2 | 1.682 | 0 | 0 | 6 | 2 | 28 |
| 3 | 1 | 1 | -1 | 5 | 3 | 15 |
| 4 | 0 | 0 | 0 | 4 | 2 | 28 |
| 5 | 0 | 1.682 | 0 | 4 | 4 | 28 |
| 6 | 0 | 0 | 0 | 4 | 2 | 28 |
| 7 | 1 | -1 | 1 | 5 | 1 | 40 |
| 8 | 1 | -1 | -1 | 5 | 1 | 15 |
| 9 | -1 | 1 | 1 | 2 | 3 | 40 |
| 10 | 0 | 0 | 0 | 4 | 2 | 28 |
| 11 | -1.682 | 0 | 0 | 1 | 2 | 28 |
| 12 | 0 | 0 | 0 | 4 | 2 | 28 |
| 13 | -1 | 1 | -1 | 2 | 3 | 15 |
| 14 | 0 | 0 | -1.682 | 4 | 2 | 6 |
| 15 | 0 | 0 | 0 | 4 | 2 | 28 |
| 16 | 0 | -1.682 | 0 | 4 | 0 | 28 |
| 17 | 0 | 0 | 0 | 4 | 2 | 28 |
| 18 | 0 | 0 | 1.682 | 4 | 2 | 49 |
| 19 | -1 | -1 | -1 | 2 | 1 | 15 |
| 20 | -1 | -1 | 1 | 2 | 1 | 40 |

### *4.2.5. HPLC/DAD analysis of HMF*

#### 4.2.5.1. HMF Extraction

After carrying out previous trials and the subsequent statistical design for optimising, the extraction procedure used for HMF determination in the selected food samples was as follows: 5 g of the grinded coated deep-fried fish was mixed with 10 mL of the extraction solvent, ethyl acetate/hexane (4:1, v/v). The mixture was shaken for 1 min, centrifuged (5810R Centrifuge, Eppendorf AG, Hamburg, Germany) (10 min, 3 000 rpm), and filtered. This filtrate was mixed with 550 µL of the concentration solvent, which was one part of 40 mM sodium formate adjusted to pH=3 with formic acid (98–100 %) to one part of methanol 100 %, and shaken vigorously. The final biphasic system was allowed to separate by centrifugation (5 min, 10 000 rpm). The upper organic phase was eliminated, and the lower aqueous phase (300 µL) was used for analysis.

#### 4.2.5.2. HMF Analysis

A 20 µL portion of the final extract was injected onto an Ultracarb ODS column (5 µm, 250 mm length, 4.6 mm i.d.) for HPLC-DAD analysis. It was used an analytical HPLC unit (Jasco, Tokyo, Japan) equipped with Jasco PU-2080 HPLC pumps, an MD-2010 Plus multiwavelength detector, and a type 7725i Rheodyne injector with a 20 µL loop. Borwin PDA Controller Software (JMBS Developments, Le Fontanil, France) was also used.

Chromatographic separation was performed using a gradient elution of sodium formate 40 mM adjusted to pH=3 with formic acid (A) and methanol 100 % (B). The linear gradient program used was t= 0–5 min, 95 % A; t= 20 min, 60 % A; t= 27 min, 95 % A; t= 27–30 min, column rinse and reequilibration. The flow rate was 0.7 mL $min^{-1}$, and separations were carried out at room temperature. DAD was set at 280 nm, and peak identification in coated deep-fried fish samples was carried out by comparing retention times and spectra of unknown peaks with reference standards.

#### 4.2.5.3. Statistical Design

Trials were conducted to optimise conditions for the extraction of HMF in coated deep-fried fish by using RSM. Table 4.2 shows the complete experimental design developed by CCD and how it was carried out with three independent variables, sample amount (2–5 g), extraction solvent volume (5–10 mL), and concentration solvent volume (550–1 000 µL). It consisted of a complete $2^3$ factorial design with six center points and two axial points on the axis of each design variable at a distance of α= 1.682 from the design center, giving 20 combinations, including six replicates of the center point. The response was the HMF peak area. Data analysis, response surfaces and contour diagrams, was performed by Design Expert trial-Version 7 (Stat-Ease, Inc., Minneapolis, MN).

**Table 4.2:** Independent variables and their coded values used for optimisation of variables combination for HMF quantification in coated deep fried products.

| | | | **Coded levels** | | | | |
|---|---|---|---|---|---|---|---|
| **Independent variable** | **Unit** | **Symbol** | **-1.682** | **- 1** | **0** | **1** | **+1.682** |
| **Sample amount** | **g** | **$X_1$** | **0.98** | **2** | **3.5** | **5** | **6.02** |
| **Extraction solvent volume** | **mL** | **$X_2$** | **3.29** | **5** | **7.50** | **10** | **11.70** |
| **Concentration solvent volume** | **µL** | **$X_3$** | **396.70** | **550** | **775** | **1000** | **1153.40** |

#### 4.2.5.4. HMF Quantification

Increasing levels of HMF standard (5.6–44.2 µg) were added to the coated deep-fried fish samples, which were analysed following the established conditions. Thus, curves (in µg HMF added / g sample *vs*. peak area) were constructed and used for the HMF quantification.

#### 4.2.5.5. Quality Control and Criteria

Quality control of the HPLC-DAD method was performed through the routine analysis of procedural blanks and quality control standards and samples to ensure the absence of contaminants and the possible carry over between samples and to assess the quality of the results. In addition, a daily sensitivity test was carried out to check the possible changes in the response. The identification criteria for HMF were based on the retention

times and wavelength. A deviation of the area peak within 20 % of the mean values of the calibration standards was considered acceptable.

LOD and LOQ based on a signal-to-noise ratio of 3:1 and 10:1, respectively, were determined using standard solutions (n= 5). For calculating the RSD run-to-run and day-to-day, five replicate analyses of samples were analysed in one day and in two more different days, respectively. In order to study the linearity, coated deep-fried fish samples were spiked with appropriate amounts of HMF (5.6–44.2 µg) and were extracted using the established conditions. Percentage recovery of HMF from the spiked samples was also calculated.

## 4.3. Results and discussion

### *4.3.1. HS-SPME-GC/MS analysis of volatile furanic compounds*

#### 4.3.1.1. Simulating eating conditions

In order to determine the quantity of furanic compounds in coated deep-fried fish, the usual preparation and eating process was simulated and coupled with usual HS-SPME-GC–MS procedure, such as sample refrigeration and IS addition (Goldmann *et al.*, 2005). The frozen coated fish product was cooked following manufacturer recommendations, consisting on deep-frying at 180 ºC during 4 min. After removing oil, the chewing process was simulated grinding the coated deep-fried fish using the “masticator shears straight”, and eight bites were padronised. Then, the sample was transferred to the 50 mL vial containing water (5 mL) and NaCl (3 g). Immediately 100 µL of $d_4$-furan work solution was added and the vial was sealed. It took 2 ± 0.2 min since the coated deep-fried fish is placed on a plate until the vial is sealed.

After then, the vial was kept at -4 ºC during 10 min, followed by sonication during 15 min. Finally, the fibre was exposed to the HS.

The absorption temperature was set up at 37 ± 1 ºC as suggested by Kaseleht & Leitner, (2008). Preliminary studies performed in the laboratory indicated an increase of furanic compounds formation when temperature increased from 37 to 50 ºC (Pérez-Palacios *et al.*, 2012a), i.e., the sum of furan, furfural, furfuryl alcohol and 2-pentylfuran was significantly higher ($p$ = 0.005) at 50 ºC (30.20 AU x $10^7$) than at 37 ºC HS temperature (14.96 AU x $10^7$). Thus, 37 ºC was chosen as HS absorption temperature, to prevent the possible influence of temperature on furan formation.

*4.3.1.2. Model prediction adequacy*

The variables, sample and NaCl amount and absorption time were optimised for furanic compounds quantification in coated deep-fried fish. Four preliminary assays were conducted using 5 g of sample, 2 g of NaCl and 30 min of absorption time. These conditions were selected taking into account sample and NaCl amount used by Goldmann *et al.* (2005) for dry foods and the optimum absorption time found by Altaki *et al.* (2007).

Table 4.3 shows results from CCD experiments using ANOVA by means of Fisher´s *F* test. *F* value of 6.51 means the significance of the model. There is only a 0.24 % chance that a model *F* value so large could occur due to noise. Values of Prob > *F* for $X_2$, $X_3$, $X_2X_3$ and $X_1X_3$ are lower than 0.05, which indicates they are significant model terms. The lack of fit *F* value of 0.72 is good and shows that the model is valid for the present study (Montgomery, 2000; Pinho *et al.*, 2011). Moreover, the obtained $R_{pred}^2$ and $R_{adj}^2$ (0.6350 and 0.5688, respectively) were in concordance (Chauhan & Gupta, 2004; Le Man *et al.*, 2010).

**Table 4.3:** ANOVA for RSM for the sum of furanic compounds peak/$d_4$-furan peak areas in coated deep fried products.

| Source | Sum of Squares | Degrees of freedom | Mean Square | *F* value | Prob > *F* | Remarks |
|---|---|---|---|---|---|---|
| **Model** | 0.00315466 | 6 | 0.00052578 | 6.50871832 | 0.0024 | significant |
| $X_1$ | 9.3282E-05 | 1 | 9.3282E-05 | 1.15475791 | 0.3021 | significant |
| $X_2$ | 0.00031615 | 1 | 0.00031615 | 3.91364882 | 0.0695 | significant |
| $X_3$ | 0.00154066 | 1 | 0.00154066 | 19.0721578 | 0.0008 | significant |
| $X_1 X_2$ | 0.0001281 | 1 | 0.0001281 | 1.58577346 | 0.2301 | |
| $X_1 X_2$ | 0.00049376 | 1 | 0.00049376 | 6.11233395 | 0.0280 | |
| $X_2 X_3$ | 0.00058272 | 1 | 0.00058272 | 7.21363798 | 0.0187 | |
| **Residual** | 0.00105015 | 13 | 8.078E-05 | | | |
| **Lack of Fit** | 0.00056381 | 8 | 7.0476E-05 | 0.72454965 | 0.6742 | not significant |
| **Pure Error** | 0.00048634 | 5 | 9.7268E-05 | | | |
| **Total** | 0.00420481 | 19 | | | | |

4.3.1.3. Selecting the optimum conditions

Figure 4.1 shows the surface and contour plots on the response function (sum of furanic compounds/$d_4$-furan peak areas) as affected by the three variables (sample and NaCl amount and absorption time), two variables are varying at time while the third stays constant.

Figure 4.1 A shows the contour map of the combined effect of sample and NaCl amount in the response, whereas the combined effect of sample amount and absorption time is shown in Figure 4.1 B. As can be seen, the sum of furanic compound /$d_4$-furan peak areas is slight affected by the amount of sample. Figure 4.1 C shows the contour map of the effect of NaCl amount added and absorption time on the response. The sum of furanic compounds/$d_4$ furan peak areas increased as NaCl amount and absorption time rise. Therefore, the furanic compounds yield mainly depends on NaCl amount and absorption time.

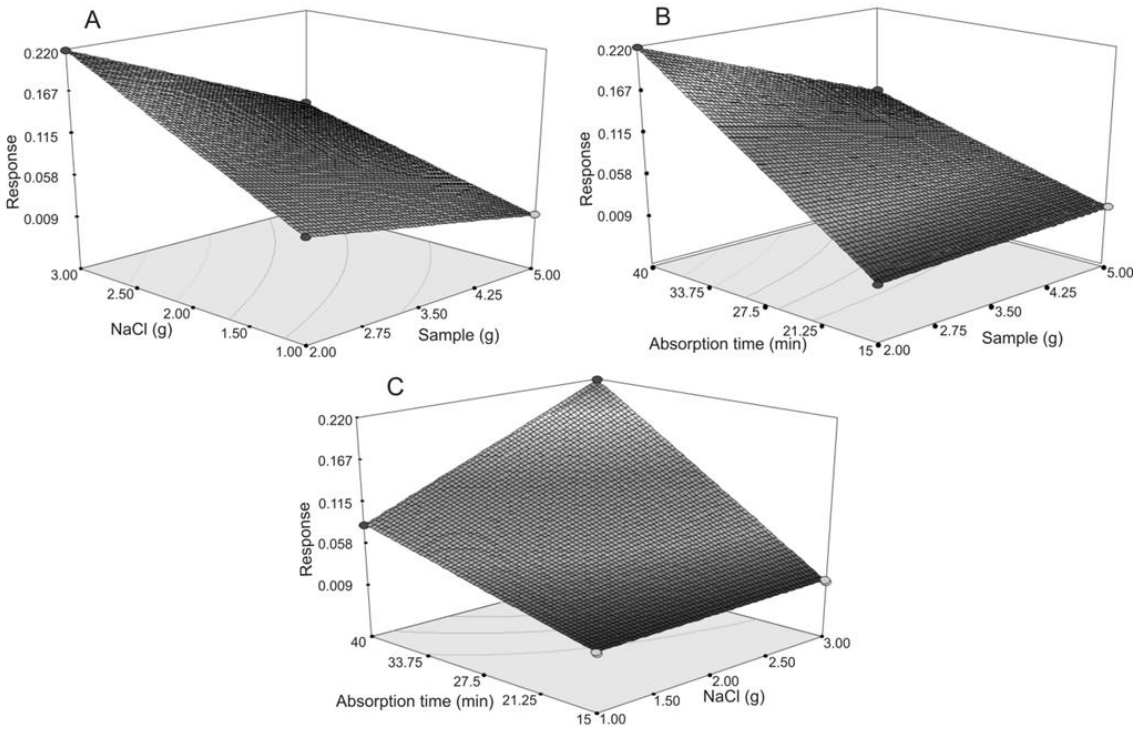



**Figure 4.1:** Response surface plots on the sum of furanic compounds /$d_4$-furan peak areas in coated deep-fried products as affected by sample amount, NaCl amount and absorption time: (A) Sample and NaCl amount at constant absorption time (27.5 min); (B) Sample amount and absorption time at constant NaCl amount (2 g); (C) NaCl amount and absorption time at constant sample amount (3.5 g).

The optimum combination of the three studied variables, which provide the maximum sum of furanic compound peak area/$d_4$-furan area consists on 2 g of sample, 3 g of NaCl and 40 min of absorption time (Table 4.4). Other authors that studied one variable at time for furan quantification in liquid and semi solid samples, chose 20 % NaCl (w/w) and 30 min as the optimal conditions (Altaki *et al.*, 2007). Kim *et al.,* (2010) selected a sample amount of 5 g for liquid, semi solid and paste state. Using CCD experiments for the development and validation of a method for determination of furan in baby food, Bianchi *et al.,* (2006) chose 30 ºC and 30 min for extraction in order to obtain shorter analyses time.

**Table 4.4:** Optimum combination of HS-SPME parameters and their experimental and predicted response.

| HS-SPME variables | | Optimum values |
|---|---|---|
| **Sample amount (g)** | | **2** |
| **NaCl amount (g)** | | **3** |
| **Absorption time (min)** | | **40** |
| **Response[A]** | **Experimental** | **0.025** |
| | **Predicted** | **0.023** |

[A]sum of furanic compouns/$d_4$-furan peak areas

4.3.1.4. Model validation and confirmation

Observed and predicted values of the sum of furanic compounds peak area/$d_4$-furan peak area under the optimum combination of sample and NaCl amount and absorption time are shown in Table 4.5 It can be observed the consistency between the experimental and predicted values (0.023 *vs*. 0.025, respectively). Moreover, in order to confirm the validity of the experimental model, a duplicate set of coated deep-fried fish samples were analysed using the selected optimum conditions. As expected, furan, furfural, furfuryl alcohol and 2-pentylfuran were detected (Figure 4.2).

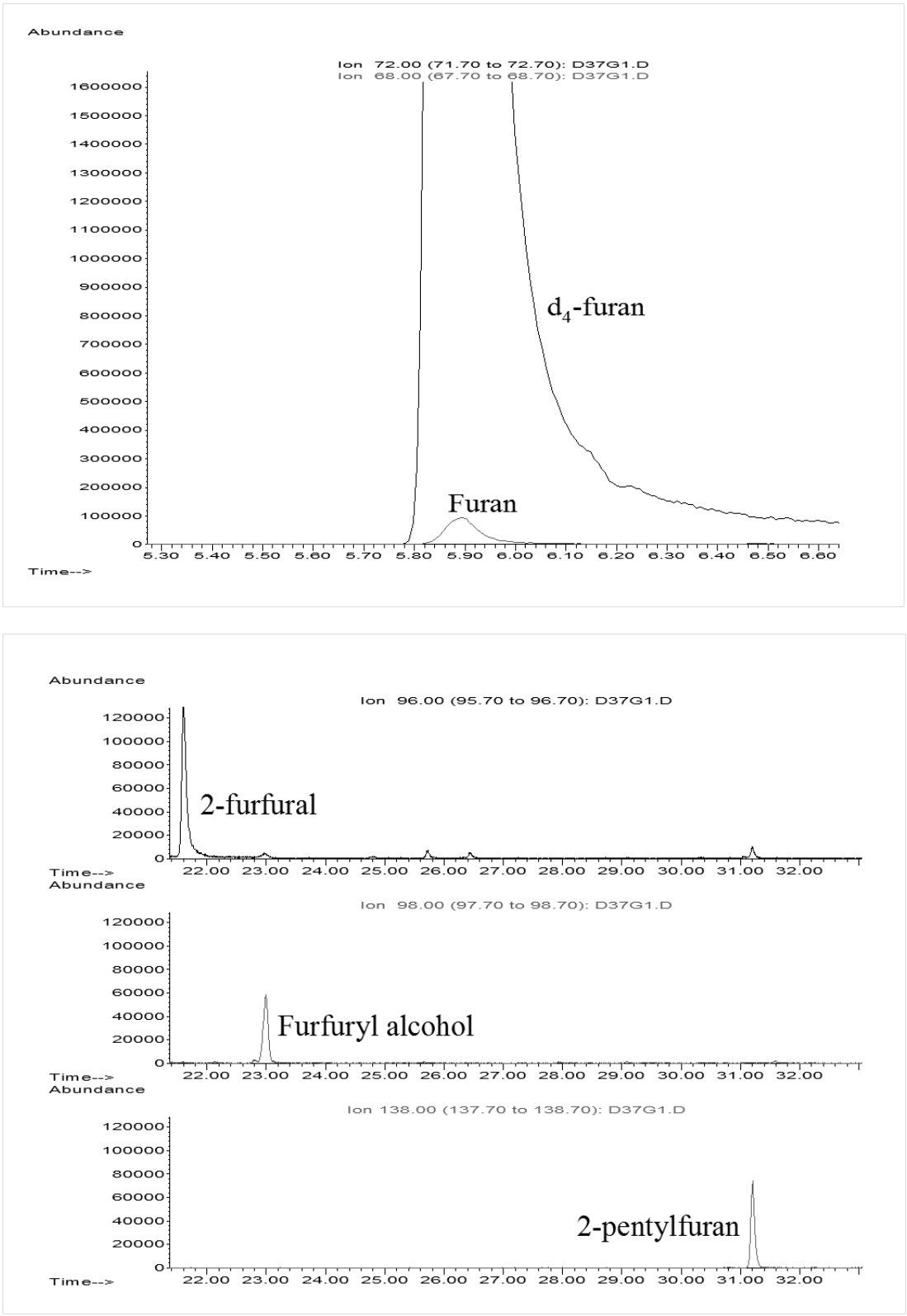



**Figure 4.2:** Typical GC–MS SIM chromatograms of furanic compounds determined in coated deep-fried fish products under the optimal combination of the HS-SPME validated method.

To examine the performance of the proposed methodology, quality parameters for each individual furanic compound were determined using standard solutions placed in a 50 mL HS vial containing 5 mL of water and 3 g of NaCl, following the same preparation and analysis conditions of the samples (Table 4.5). LOD and LOQ, expressed as µg furanic compound / g sample were calculated, obtaining 0.66 and 2.22 for furan, 0.01 and 0.04 for furfural, 1.50 and 5.01 for furfuryl alcohol, and 0.06 and 0.21 for 2-pentylfuran. Good precision was achieved for run-to-run assays with a RSD of 1.17, 1.76, 11.30 and 3.21 % for furan, furfural, furfuryl alcohol and 2-pentylfuran, respectively. Good precision was also achieved for day-to-day assays with a RSD of 4.86, 15.28 %, 13.54 and 8.70 % for furan, furfural, furfuryl alcohol and 2-pentylfuran, respectively. Good linearities with correlation coefficients ($r^2$) of 0.9999 were obtained.

**Table 4.5:** Quality parameters of each furanic compound detected under the optimised HS-SPME method.

| | **LOD[a]** | **LOQ[b]** | **Run-to-run** | **Day-to-day** | **Linearity** |
|---|---|---|---|---|---|
| | (µg furan/g sample) | (µg furan/g sample) | (RSD[c], %) | (RSD[c], %) | ($r^2$) |
| **Furan** | **0.66** | **2.22** | **1.17** | **4.86** | **> 0.9999** |
| **Furfural** | **0.01** | **0.04** | **1.76** | **15.28** | **> 0.9999** |
| **Furfuryl alcohol** | **1.50** | **5.01** | **11.30** | **13.54** | **> 0.9999** |
| **2-pentylfuran** | **0.06** | **0.21** | **13.21** | **8.70** | **> 0.9999** |

[a] Limit of detection based on a signal-to-noise ratio of 3:1.
[b] Limit of quantification based on a signal-to-noise ratio of 10:1.
[c] Relative standard deviation.

Finally, the quantification of each furanic compound was carried out by external calibration curve method and by standard addition method. The content of furan, furfural, furfuryl alcohol and 2- pentylfuran in coated deep-fried fish products was 5.59 ± 1.43, 0.27 ± 0.07, 10.48 ± 1.18, 1.77 ± 0.12 µg $g^{-1}$ sample, respectively, when measured by external calibration, and 6.22 ± 3.92, 0.60 ± 0.69, 9.39 ± 3.21, 0.01 ± 0.00 µg $g^{-1}$ sample, respectively, by standard addition. No significant differences were obtained between results obtained by the two methods except for 2-pentylfuran ($p$ = 0.995, n = 4 paired Student's *t*-test). In addition, higher standard deviation was found when the standard addition method was applied, which points out the higher suitability of external calibration

for quantifying furan and its derivatives in coated deep-fried products. The furan levels in coated deep-fried fish were similar to those reported in coffee samples, which is the food showing the highest furan content (EFSA, 2010). The high content of furan in coated deep-fried fish products can be related with the ingredients composition (wheat flour, cereals presenting high content of carbohydrates, and polyunsaturated fatty acids) and the deep-fried processing using oil at high temperatures. Van Lancker *et al.*, (2009) demonstrated that oils caused high furan retention.

The furfural content in coated deep-fried fish was similar to that reported by Mesías-García *et al.,* (2010) in baby foods. The quantities of 2-pentylfuran were also comparable to that found in crispbread and mock-turtle (EFSA, 2009c). Lower levels of furfuryl alcohol were obtained in the coated deep-fried fish analysed when compared with coffee samples amounts (Swasti & Murkovic, 2011).

### *4.3.2. HPLC DAD analysis of HMF*

#### 4.3.2.1. Selection of the Solvents for the HMF Extraction

Firstly, it was assured that the HPLC-DAD conditions developed in this study allowed the detection of the HMF standard. Secondly, extraction was tested suspending 500 mg of sample in 5 mL of water and the subsequent clarification with 0.250 mL of each of the *Carrez* I and II solutions (Rufián-Henares *et al.,* 2006b). Using this procedure, which has been developed for breakfast cereals, HMF was not detected in coated deep-fried fish samples, which might be due to the different matrix characteristics.

The use of organic solvents is recommended for the extraction of HMF from fatty foods. Thus, different extraction solvents described in the scientific literature were tested (namely diethyl ether, ethyl acetate, hexane, chloroform, and also the HPLC mobile phases, 40 mM sodium formate (pH=3) and methanol), with and without clarifying with *Carrez* I and II and with and without filtration before injection.

Finally, HMF was allowed to be detected by using the mixture ethyl acetate/hexane (4:1, v/v) as an extraction solvent, following by a concentration step with the HPLC mobile phases (40 mM sodium formate (pH=3)/methanol, 1:1, v/v). HMF is highly soluble in polar solvents like water, methanol, and ethanol. On the other hand, it has been previously shown that HMF strongly adsorbed to hydrophobic functional surfaces where they could only be desorbed by using nonpolar solvents (Gökmen & Senyuva, 2006).

Thus, in roasted hazelnut, Durmaz & Gökmen (2010) found the more accurate results by using ethyl acetate for extracting HMF into oil, followed by a liquid–liquid extraction using 70 % methanol, which is in agreement with the method developed in this study.

Based on literature data, the clarification with *Carrez* I and II and the filtration before injection were also tested in this study. The obtained results showed no improvement with the addition of the *Carrez* solutions (436 194 *vs*. 631 000 µAU, with and without using *Carrez* I and II solutions, respectively), and the filtration step seemed to be detrimental (232 597 *vs*. 445 737 µAU, with and without filtrating, respectively). These findings agree with those found by Chambel *et al.* (1997), who observed that the use of *Carrez* I and II or a passage through a Sep-pack C-18 cartridge was not necessary and caused losses of HMF. As a consequence, in this study, the HMF extraction was carried out with ethyl acetate/hexane (4:1, v/v) and the subsequent addition of 40 mM sodium formate (pH=3)/methanol (1:1, v/v), without using both *Carrez* solutions and filtration.

#### 4.3.2.2. Model Prediction Adequacy

The variables, sample amount, extraction solvent volume, and concentration solvent volume, were aimed to be optimised for HMF quantification in coated deep-fried fish. The full-factorial CCD with 20 experiments is shown in Table 4.6. It also involves six replicates of the center point in order to measure the precision property and verify any change in the estimation procedure. The observed and predicted response functions (HMF peak area) are also shown in Table 4.6.

**Table 4.6:** CCD and experimented and predicted results for the HMF peak area in coated deep-fried products.

| Standar order | Independent variables | | | Response | |
|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | Experimenal | Predicted |
| 1 | -1 | -1 | -1 | 459911 | 449272 |
| 2 | 1 | -1 | -1 | 761142 | 719560 |
| 3 | -1 | 1 | -1 | 421979 | 404765 |
| 4 | 1 | 1 | -1 | 816835 | 819824 |
| 5 | -1 | -1 | 1 | 470983 | 433647 |
| 6 | 1 | -1 | 1 | 549866 | 532732 |
| 7 | -1 | 1 | 1 | 331642 | 338877 |
| 8 | 1 | 1 | 1 | 606441 | 582732 |
| 9 | -1.682 | 0 | 0 | 247876 | 265778 |
| 10 | 1.682 | 0 | 0 | 667445 | 698119 |
| 11 | 0 | -1.682 | 0 | 349054 | 395934 |
| 12 | 0 | 1.682 | 0 | 398858 | 400553 |
| 13 | 0 | 0 | -1.682 | 816650 | 839600 |
| 14 | 0 | 0 | 1.682 | 601467 | 627092 |
| 15 | 0 | 0 | 0 | 616436 | 581861 |
| 16 | 0 | 0 | 0 | 509224 | 581861 |
| 17 | 0 | 0 | 0 | 590736 | 581861 |
| 18 | 0 | 0 | 0 | 635632 | 581861 |
| 19 | 0 | 0 | 0 | 629347 | 581861 |
| 20 | 0 | 0 | 0 | 518123 | 581861 |

Table 4.7 shows the analysis of the variance by means of Fisher's *F* test. The model *F* value was 19.53, indicating the significance of the model and that there is only a 0.01 % possibility that a so large model *F* value could occur due to noise. $X_1$, $X_3$, $X_1X_3$, $X_1^2$, $X_2^2$, and $X_3^2$ presented values of Prob > *F* less than 0.05 which denotes that they are significant terms. The lack of fit *F* value of 0.58 is nonsignificant (> 0.05) and means that the model is valid for the present study (Montgomery, 2000; Pinho *et al.,* 2011). There is a 71.88 % chance that a so large lack of fit *F* value could take place due to noise. In addition, the obtained $R_{pred}^2$ and $R_{adj}^2$ (0.7973 and 0.8977, respectively) were in concordance, and a ratio of 16.278 was attained, which indicates an adequate signal. Thus, the response surface quadratic model is adequate and significant.

**Table 4.7:** ANOVA for RSM for the HMF peak area in coated deep-fried products.

| Source | Sum of Squares | Degrees of freedom | Mean Square | *F* value | Prob > *F* | Remarks |
|---|---|---|---|---|---|---|
| **Model** | **2.61428E+12** | **9** | **2.90475E+11** | **19.535103** | **< 0.0001** | **significant** |
| $X_1$ | **1.3499E+12** | **1** | **1.3499E+12** | **90.783399** | **< 0.0001** | **significant** |
| $X_2$ | **154092888.2** | **1** | **154092888.2** | **0.0103631** | **0.9209** | |
| $X_3$ | **3.26136E+11** | **1** | **3.26136E+11** | **21.933386** | **0.0009** | **significant** |
| $X_1 X_2$ | **62694467305** | **1** | **62694467305** | **4.2163394** | **0.0671** | |
| $X_1 X_3$ | **87678712525** | **1** | **87678712525** | **5.8965843** | **0.0356** | **significant** |
| $X_2 X_3$ | **7557367682** | **1** | **7557367682** | **0.5082494** | **0.4922** | |
| $X_1^2$ | **1.07585E+11** | **1** | **1.07585E+11** | **7.2352992** | **0.0227** | **significant** |
| $X_2^2$ | **3.63361E+11** | **1** | **3.63361E+11** | **24.436801** | **0.0006** | **significant** |
| $X_3^2$ | **2.47318E+11** | **1** | **2.47318E+11** | **16.632674** | **0.0022** | **significant** |
| **Residual** | **1.48694E+11** | **10** | **14869407143** | | | |
| **Lack of Fit** | **54481349477** | **5** | **10896269895** | **0.5782802** | **0.7188** | **not significant** |
| **Pure Error** | **94212721957** | **5** | **18842544391** | | | |
| **Total** | **2.76297E+12** | **19** | | | | |

4.3.2.2. Selecting the Optimum Conditions

Figure 4.3 shows the surface and contour plots on the response function (HMF peak area) as affected by the three studied variables (sample amount, extraction solvent volume, and concentration solvent volume); two variables are varying with time, while the third is keeping constant. Figure 4.3 A shows the contour map of the combined effect of sample amount and extraction solvent volume in the HMF peak area whereas the combined effect of sample amount and concentration solvent volume is shown in Figure 4.3 B. The contour map of the effect of the volumes of the extraction and concentration solvents on the HMF peak area is shown in Figure 4.3 C.

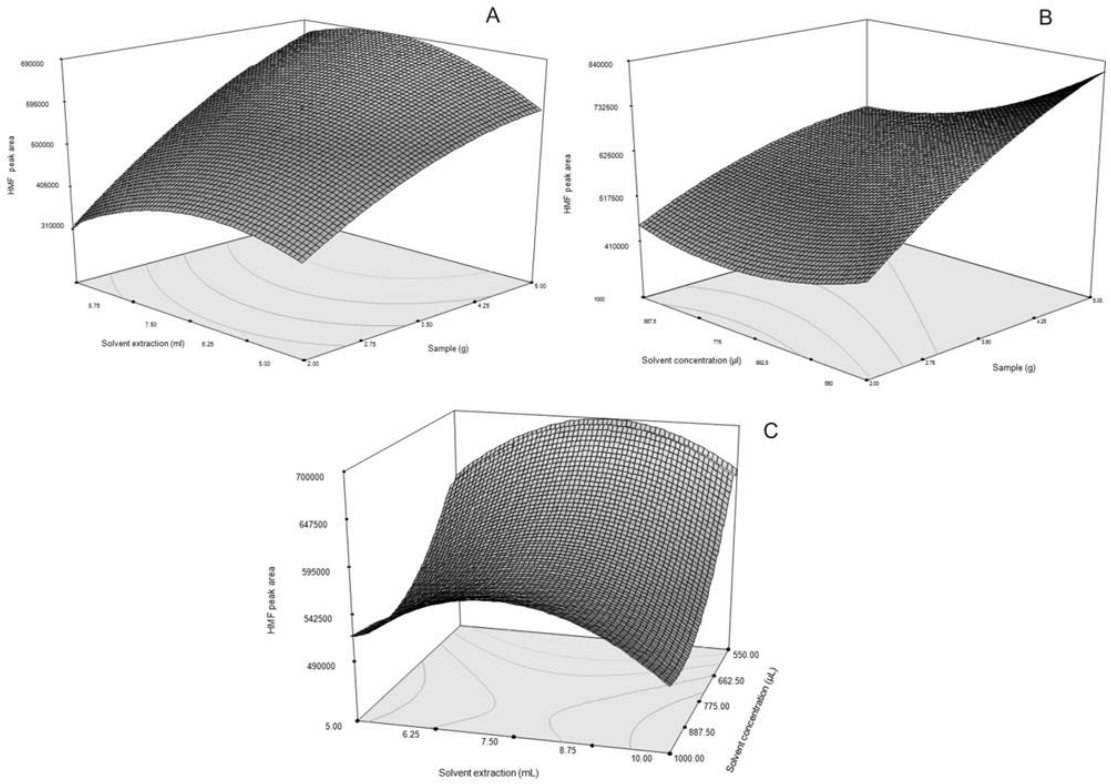



**Figure 4.3:** Response surface plots on the HMF peak area in coated deep-fried products as affected by sample amount, extraction solvent volume, and concentration solvent volume. A - Sample amount and extraction solvent volume at constant concentration solvent volume (775 µL), B - sample amount and concentration solvent volume at constant extraction solvent volume (7.5 mL), and C - extraction solvent volume and concentration solvent volume at constant sample amount (3.5 g).

As can be seen, within the ranges tested, the response was highly affected by the amount of sample and the concentration solvent volume, whereas the extraction solvent volume was not so notable. The HMF peak area increased as sample amount increased, concentration solvent volume decreased, and when the volume of extraction solvent was higher than 7.5 mL. As a result, the optimum combination of the three studied variables, which should provide the maximum HMF quantity in coated deep-fried fish, consists of 5 g of sample, 10 mL of ethyl acetate/hexane (4:1), and 550 µL of 40 mM sodium formate (pH=3)/methanol (1:1) (Table 4.8.).

**Table 4.8:** Model Validation

| Sample amount (g) $X_1$ | Extraction solvent volumen (ml) $X_2$ | Concentration solvent volumen (µl)) $X_3$ | Desirability | Response[A] | |
|---|---|---|---|---|---|
| | | | | Experimental | Predicted |
| **5** | **10** | **550** | **0.99** | **877202** | **819823** |

[A] HMF peak area

As can be seen in Figure 4.4, the HMF peak area obtained after carrying out the optimum combination of variables was higher in comparison to that found when using one of the conditions tested (2 g of sample, 5 mL of ethyl acetate/hexane (4:1), and 1 000 µL of 40 mM sodium formate (pH=3)/methanol (1:1)). Observed and predicted quantities of HMF peak area under the optimum combination of sample amount, extraction solvent volume, and concentration solvent volume are shown in Table 4.8. The consistency between the experimental and predicted values (877 202 *vs*. 819 823, respectively) can be observed. To our knowledge, this is the first work studying the effect of the extraction parameters and selecting the optiminum combination of variables in order to develop and validate a method for the HMF quantification.

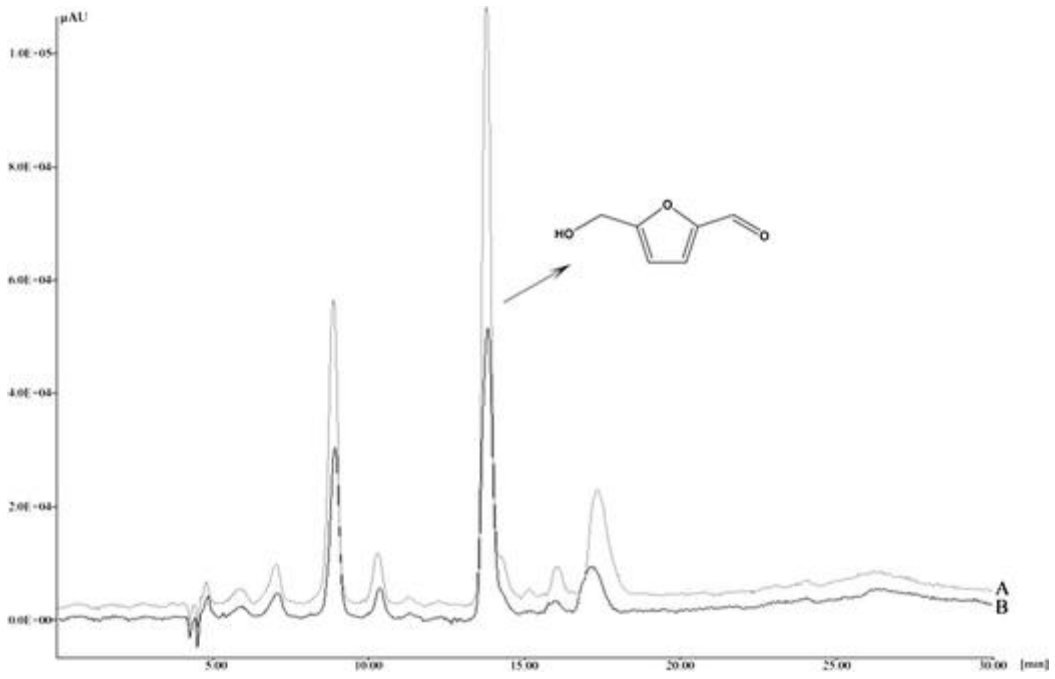



**Figure 4.4:** Overlapped UV spectra of the chromatographic peak of HMF extracted from coated deep-fried fish under the optimum combination of analytical (A: 5 g of sample, 10 ml of extraction solvent and 550 µl of concentration solvent) and other conditions (B: 2 g of sample, 5 ml of extraction solvent and 1 000 µl of concentration solvent).

#### 4.3.2.3. Model validation and confirmation

To examine the performance of the proposed methodology, quality parameters were calculated. Good linearity with correlation coefficients ($r^2$) higher than 0.9999 were obtained. The slopes of the regression lines obtained for the pure standard calibration curve and for the standard addition to sample curve were significantly different ($p$=0.995, n=4, paired Student's *t*-test). This evidences the matrix effect and allows concluding the use of calibration curves through the addition of increased HMF standard levels to sample for quantification purposes. The matrix effect phenomenon has also been reported in other food, such as honey, sugar, vinegar, and apple juice (EFSA, 2010). LOD and LOQ were calculated, obtaining 0.0076 and 0.025 μg $g^{-1}$ sample, respectively, lower than those previously described by others (Durmaz & Gökmen 2010; Chambel *et al.,* 1997). Adequate precision was achieved with a RSD of 4.15 % for run-to-run and 15.95 % for day-to-day. Percentage recovery of HMF was 94 %.

Moreover, in order to confirm the validity of the experimental model, the HMF quantity was analysed in coated deep-fried fish products with a duplicate set at the selected optimum conditions, obtaining 1.25 ± 0.21 μg $g^{-1}$, which are quite in agreement with some of the HMF concentration reported by other authors, who found a high variability, for example, between 0.49 and 74.60 μg $g^{-1}$ in cookies (Ait-Ameur *et al.,* 2006), between 0.08 and 7 μg $g^{-1}$ in pasta (Sesidoni *et al.,* 1999), and between 2.20 and 87.70 μg $g^{-1}$ in bread (Ramírez-Jiménez *et al.,* 2001).

## 4.4. Conclusions

This study presents the validation of two analytical methods for quantification of volatile and less volatile furanic compounds in coated deep-fried products simulating the eating process, performing the preparation as usually cooked and consumed. Simultaneous determination of furan, furfural, furfuryl alcohol and 2-pentylfuran in coated deep-fried products by HS-SPME-GC–MS and HPLC/DAD for HMF quantification. The use of CCD allows reaching the optimum combination of analytical variables in each method. Consistency between predicted and experimented values was observed.

Results obtained for furanic compounds in coated deep-fried fish indicate that this type of product should be included in the group of food products with high furan content, such as coffee samples.

# CHAPTER 5

## Impact of cooking and handling conditions on volatile and less volatile furanic compounds in coated deep-fried fish products

## Abstract

This study evaluates the influence of cooking and handling conditions on the quantity of furanic compounds (furan, furfural, furfuryl alcohol, 2-pentylfuran, HMF) in breaded fish products. Oven-baking and reheating in the microwave lead to low furanic compounds formation in comparison with deep-frying. The use of olive oil for deep-frying promoted higher levels of furanic compounds than sunflower oil. The amounts of these compounds diminished as the temperature and time of deep-frying decreased as well as after a delay after deep-frying. Thus, the generation of furanic compounds can be minimized by adjusting the cooking method and conditions, such as using an electric oven, deep-frying in sunflower oil at 160 ºC during 4 min, or waiting 10 min after cooking. However, these conditions that reduce furanic compounds levels also reduce the content of volatile compounds related to the aroma and flavour of fried foods. In this sense, new efforts should be done to reduce the formation of furanic compound without being detrimental to the volatile profile.

**Keywords:** Furanic compounds; Volatile compounds; Coated products; Cooking conditions; Handling conditions.

## 5.1. Introduction

The development of aroma and flavour in cooked products is a complex process in which different compounds react to produce intermediary or volatile compounds. Frying induces oxidation and *Maillard* reactions, which are essential for the final aroma and flavour attributes of the food (Bastida & Sánchez-Muniz, 2001; Romero *et al.*, 2000) and simultaneously responsible for the formation of undesirable and compounds (Mottram, 1998; Nawar, 1998).

Furanic compounds are recognized as important contributors to the characteristic odour of fried products (Wagner & Grosch, 1998; Cerny & Grosch, 1992), particularly in coated products, due to the intense heat effect on carbohydrates and polyunsaturated fatty acids. These compounds have low thresholds and provide pleasant odour characteristic, such as cocoa, butter or fruity (Belitz & Grosch, 1997). However, furan is considered a possible human carcinogen (Group 2B) by IARC (IARC,1995). Furan can be formed from various precursors naturally present in foods, namely, ascorbic acid, carbohydrates, amino acids, fatty acids, and carotenoids (Perez Locas & Yaylayan, 2004; Becalski & Seaman, 2005; Märk *et al.*, 2006; Limacher *et al.*, 2007, 2008; Fan *et al.*, 2008). Most of the results concerning furan content in foods (coffee, infant formula, baby food, cereal, meat, fish, vegetal, dairy and fruit products) have been obtained from samples analysed as purchased, however, it is claimed that further evaluation of furan content in foods should be performed in the samples as they are consumed (EFSA, 2010). Thus, the effect of usual cooking conditions, namely time, temperature and handling information should be studied. Additionally, furan derivatives should also be analysed since they are also present in thermally treated products, for example furfural, furfuryl alcohol and 2-pentylfuran were found in smoked-cured bacon by Yu *et al.* (2008). Giri *et al.* (2010) found 2-ethylfuran, 2-butylfuran, 2-acetylfuran, 2-pentylfuran, furfural and furfuryl alcohol in paste fish. There are also studies revealing the toxicity of these furanic compounds in animals and humans (Sujatha, 2008; Arts *et al.*, 2004; Goldsworthy *et al.*, 2001; Wilson *et al.*, 1992).

Concerning HMF, it is less volatile than other furanic compounds and has been mainly analysed in cereal products, honey, fruit products and coffee (Capuano & Fogliano, 2011; Abraham *et al.*, 2011). This furanic compound is an intermediate product in the *Maillard* reaction (Berg & Van Boekel, 1994; Morales *et al.*, 1997) and is also formed from the degradation of sugars at high temperatures (Kroh, 1994). HMF is usually used to evaluate the quality of processing and it also raises toxicological concerns (Capuano & Fogliano, 2011; Abraham *et al.*, 2011).

The influence of the various factors involved in a culinary process on the nutritive value of processed foods is of major concern (Guidurus *et al.*, 2010). In house holding conditions the controllable variables are the cooking process, the oil variety and time / temperature of processing. Several studies about the modifications of fat and oil composition during heating and frying under very different conditions have been carried out (Dobarganes *et al.*, 2000). However, papers on the changes in the foods are less abundant, and most of them focused on fat uptake and water loss mechanisms during frying (Debnath *et al.*, 2003). Moreira *et al.*, (1997) studied the effect of oil temperature (130, 160 and 190 ºC) on the final oil content of *tortilla* chips. Miranda *et al.* (2010) evaluated how the cooking method (baking and deep frying in olive or sunflower oil) affects fatty acids profiles of frozen breaded foods.

Recent results obtained for furanic compounds in coated deep-fried fish have indicated that this type of product should be included in the group of food products with high furan content, such as coffee samples (Pérez-Palacios *et al.*, 2012b). Thus, this work aims to study the effect of cooking and handling conditions (deep-frying at different combinations of time and temperature; deep-frying in different oils and dry oven-baking; reheated in the microwave oven; time after cooking) on the content of furanic compound in a breaded fish product, in order to provide data on the reduction of these compounds. Moreover, the influence of the cooking conditions on the profile of volatile compounds was also investigated. Coated fish products are very consumed by kids and teenagers due to its high sensory acceptance, its assumed good health characteristics (i.e., high polyunsaturated fatty acid content) and its quick and easy preparation.

## 5.2. Material and Methods

### *5.2.1. Chemical and standards*

$D_4$-furan (98 %) was provided by ISOTEC (Ohio, USA). Furan (≥ 99%), furfuryl alcohol (99%) were supplied by Sigma-Aldrich (Steinheim, Germany). Furfural was purchased by Merck (99 %) (Darmstadt, Germany). 2-pentylfuran (98 %) was provided by ALFAAESAR (Karlsrula, Germany). HMF (98 %) was supplied by Sigma-Aldrich (Steinheim, Germany). Ethyl acetate, hexane, sodium formate, formic acid, and methanol were supplied by Merck (Darmstadt, Germany), and ultrapure water (0.055 μS $cm^{-1}$) was obtained by using a Serial Milli-Q system for Millipore (Supor DCF, Gelman Sciences, Chentelham, Australia). Frozen breaded fish products, sunflower and olive oils (extra virgin with 0.8 % acidity) were obtained from a local stored.

*5.2.2. Experimental design*

Frozen breaded fish products were prepared by deep-frying in sunflower (SF) (n= 6) and olive oil (OL) (n= 6) using a domestic deep-fryer (KENWOOD DF-150; 1l) at 180 ºC during 4 min, and by baking using an electric oven (ELECTRIC Co MF22VD, 22l), at 200 ºC during 17 min (turning over after first nine min) (OV) (n = 6). These conditions are recommended by the manufacturer. Deep-frying and oven temperature was monitored using a cooking thermometer (Model 26003 DeltaTRAK, USA). In addition, other batch of breaded fish products (MO) (n= 6) were deep-fried in sunflower oil (at 180 ºC during 4 min), placed into the fridge (4 ºC) during 16h and reheated in a domestic microwave (HAIER M01700, 17l) at 750 W during 40 sec.

Moreover, the breaded fish products were deep-fried in sunflower oil at nine different combinations (n= 6) of temperature (160, 180 and 200 ºC) and time (2, 4 and 6 min).

In order to study the stability of the volatile compounds, three batches of breaded fish products were deep-fried in sunflower oil and analysed straightaway (n= 6) (ST0) and with a delay of 10 min (n= 6) (ST1) and 20 min (n= 6) (ST2) kept at ambient temperature (18 - 20 ºC).

All samples were slightly drained after frying, placed on paper towel for removing external excess oil, and grinding by using a device named “masticator shears straight” (BUENO HERMANOS, S.A., La Rioja, Spain, ISO 9001-2000 Quality Certified Company) which simulates the chewing process. The oil was replaced every 6 frying sessions. All samples were processed individually.

*5.2.3. Standard solutions*

Standard solutions were made using the same procedure described previously (Chapter 4, Section 4.2.2. Standard solutions). Briefly, stock solution of $d_4$-furan 2 mg $mL^{-1}$ was prepared by injecting 20 µL of refrigerated $d_4$-furan with a syringe through the septum of a 10 mL HS vial filled with 10 mL of methanol and sealed. Working solutions 1 mg $mL^{-1}$ were prepared daily by adding 500 µL of stock solution to a 2 mL vial containing 500 µL of water using the same procedure.

A standard calibration solution containing 8.69, 0.52, 10.84 and 0.07 mg $mL^{-1}$ for furan, furfural, furfuryl alcohol and 2-pentylfuran, respectively, was prepared into a 15 mL HS vial containing 15 mL of methanol. The exact weight of methanol and each added furanic compound was recorded. Additionaly, stock solution of HMF in methanol 1.2 mg $mL^{-1}$ was

prepared. A standard working solution 0.056 mg $mL^{-1}$ was made by injecting 50 µL of the stock solution into a vial containing 1 mL of the 40 mM sodium formate (pH=3)/methanol (1:1) mixture. The exact weight of methanol and HMF was recorded, expressing the concentration in µg $µL^{-1}$. Consecutive dilutions of the standard calibration solution in methanol were made.

### *5.2.4. Volatile analysis*

The procedure used for the quantification of furanic compounds was described by Pérez-Palacios *et al.,* (2012b). Straightaway after grinding, 2 g of sample were transferred to a 50 mL HS vial, containing 5 mL of water and 3 g of NaCl. 100 µL of $d_4$-furan work solution was added, and the vial immediately sealed at once and kept at – 4 ºC during 10 min. Afterwards, the vial was placed into an ultrasonic cleaner (FUNGILAB, Portugal) during 15 min. To extract furanic compounds a CAR–PDMS SPME fibre (75 µm thickness, Supelco Co., Bellefonte, PA, USA) was used. Prior to analysis, the SPME fibre was preconditioned at 300 ºC for 60 min in the chromatograph injection port. The fibre was inserted into the sample vial through the septum and exposed to the HS for 40 min at 37 ± 1 ºC under constant agitation (600 rpm). Thereafter, the SPME fibre was inserted and desorbed for 10 min at 280 ºC, in the splitless mode, with 1 mL $min^{-1}$ flow.

Chromatographic analysis was performed using the method described in Chapter 4; Section 4.2.4.2. GC-MS conditions.

Furan and its derivatives were also detected by *m/z* characteristic ion, using *m/z* 68, *m/z* 72, *m/z* 96, *m/z* 98 and *m/z* 138 for furan, $d_4$-furan, furfural, furfuryl alcohol and 2-pentylfuran, respectively. They were quantified by external calibration curve method. Five consecutive dilutions of the standard calibration solution in methanol (1:10, v/v) were prepared. 100 µL of the corresponding standard solution and a fixed volume (100 µL) of $d_4$-furan working solution were placed in a 50 mL HS vial containing 5 mL of water and 3 g of NaCl, following the same preparation and analysis conditions of samples. For each individual furanic compound a calibration curve (furanic compound /$d_4$-furan peak areas *vs.* furanic compound amount) was constructed, obtaining $r^2$ values of 0.9999. The final results, expressed in µg $g^{-1}$, take into account the exact weight of the sample portion in the HS vial.

### *5.2.5. HMF analysis*

The HMF analysis was done as described by Pérez-Palacios *et al.,* (2012a). Five grams of the grinded coated products was mixed with 10 mL ethyl acetate/hexane (4:1, v/v). The mixture was shaken for 1 min, centrifuged (5810R Centrifuge, Eppendorf AG, Hamburg, Germany) (10 min, 3 000 rpm), and filtered. This filtrate was mixed with 550 µL of 40 mM sodium formate adjusted to pH=3 with formic acid (98 – 100 %):methanol 100 % (1:1, v/v), and shaken vigorously. The final biphasic system was allowed to separate by centrifugation (5 min, 10 000 rpm). The upper organic phase was eliminated, and the lower aqueous phase (300 µL) was used for analysis in the HPLC-DAD system as described in Chapter 4; Section 4.2.5.2 HMF analysis.

Moreover, increasing levels of HMF standard (5.6–44.2 µg) were added to the coated deep-fried fish samples, which were analysed following the established conditions. Thus, curves (in microgram HMF added *per* gram sample *vs*. peak area) were constructed and used for the HMF quantification.

### *5.2.6. Statistical analysis*

Influence of cooking and handling condition (deep-frying in SF/OL; OV; reheating in the microwave (MO); time after deep-frying) were determined by ANOVA. The effect of time and temperature of deep-frying were analysed according to the General Linear Model. When a significant effect ($p$ <0.05) was detected, paired comparisons between means were conducted using the Tukey's test. Analyses were done by using the SPSS package (v. 20.0).

## 5.3. Results and Discussion

### *5.3.1. Furanic compound levels as affected by cooking and handling conditions*

Table 5.1 shows the content of furanic compounds in breaded fish products cooked by different methods (deep-frying in sunflower (SF)/olive oil (OL), baking in the electric oven (OV), reheating in microwave after deep-frying in sunflower oil (MO)).

**Table 5.1:** Content of furanic compunds (µg $g^{-1}$ sample) in breaded fish products deep-fried in sunflower (SF) and olive (OL) oils, oven-baked (OV) and reheated in the microwave after deep-frying in sunflower oil (MO)*.

| | SF | OL | OV | MO | *p* |
|---|---|---|---|---|---|
| Furan | 5.51 ± 0.37$^{a}$ | 4.02 ± 0.61$^{b}$ | 4.36 ± 0.75$^{ab}$ | ND | < 0.001 |
| Furfural | 0.23 ± 0.01$^{b}$ | 0.57 ± 0.01$^{a}$ | ND | 0.06 ± 0.02$^{c}$ | < 0.001 |
| Furfuryl alcohol | 10.54 ± 0.20$^{b}$ | 18.87 ± 1.25$^{a}$ | 4.67 ± 0.19$^{d}$ | 7.13 ± 0.23$^{c}$ | < 0.001 |
| 2-pentylfuran | 1.50 ± 0.32$^{b}$ | 2.23 ± 0.13$^{a}$ | 0.25 ± 0.09$^{c}$ | 1.09 ± 0.05$^{b}$ | < 0.001 |
| HMF | 1.78 ± 0.08$^{b}$ | 4.47 ± 0.98$^{a}$ | 0.64 ± 0.09$^{bc}$ | ND | < 0.001 |
| TOTAL | 19.46 ± 0.88$^{b}$ | 30.36 ± 2.68$^{a}$ | 9.99 ± 1.08$^{c}$ | 8.15 ± 0.29$^{c}$ | < 0.001 |

* The results are expressed as means values ± standard deviation.
On the same row, means with different letters differ significantly ($p$ <0.05).
ND: non detected.

The highest levels of total furanic compounds were found in OL products (30.36 µg $g^{-1}$ sample), followed by SF samples (19.46 µg $g^{-1}$ sample), whereas OV and MO batches showed the minor content (9.99 and 8.15 µg $g^{-1}$ sample, respectively), due to levels of furfural, furfuryl alcohol, 2-pentylfuran and HMF. However, the highest content of furan was found in SF products (5.51 µg $g^{-1}$ sample), followed by OV and OL ones (4.36 and 4.02 µg $g^{-1}$ sample, respectively), while this compound was not presented in MO samples. In addition, HMF was also not detected in MO products and furfural was not found in OV samples. In spite of reaching higher heating temperature when oven-baking (200 ºC) than when deep-frying (180 ºC), higher levels of furanic compounds were found in SF and OV samples than in OV ones, showing the impact of the oil on the quantity of furanic compounds in deep-fried products. A major evaporation of these volatile compounds occurs in the oven than in the deep-fryer filled with the oil, which could explain this finding. In fact, Van Lacker *et al.*, (2009) reported that oils caused high furan retention. Oil composition also seems to be of importance. Thus, olive oil better enhances the formation of furanic compounds than sunflower oil, which can be related to the higher antioxidant capacity in sunflower than in olive oil before and after frying (Quiles *et al.*, 2002). However, Ramírez *et al.,* (2004) found higher area units of 2-pentylfuran in pork loin samples fried in sunflower than in olive oil. Baking fish breaded products in the electric oven minimised the levels of furanic compounds, although this effect was not so marked in the case of furan. However, Roberts *et al.,* (2008) did not detected furan in packaged convenience foods after cooking in both conventional and microwave ovens. The practice of deep-frying followed by keeping in the fridge and reheating in the microwave reduced the content of furanic compounds, especially of furan and HMF, which could be explained by the evaporation of these compounds during the reheating in the microwave. It also

could be due to their losses at low temperatures without being subsequently re-generated in the microwave. This result is in agreement with the studies of furan loss on reheating carried out by Roberts *et al.,* (2008). Nevertheless, HMF concentration was not significantly different between almonds roasted in the microwave, oven, or oil (Agila & Barringer, 2012). Studies carried out in environmental food contaminants, such us polycyclic aromatic hydrocarbons, polychlorinated dibenzo-*p*-dioxins and dibenzofurans, have found that the cooking processes are only of limited value as a means of reducing the concentrations of these compounds (Perelló, 2009; 2010), which mainly depend on the food item (Domingo, 2011).

The influence of time and temperature of deep-frying on furanic compounds levels is exposed in Table 5.2.

**Table 5.2:** Content of furanic compunds (µg $g^{-1}$ sample) in breaded fish products deep-fried in sun flower oil at different combinations of time (t) and temperature ($T^a$)*.

| | 160 ºC | | | 180 ºC | | | 200 ºC | | | *p* (t) | *p* ($T^a$) | *p* (t-$T^a$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **2 min** | **4 min** | **6 min** | **2 min** | **4 min** | **6 min** | **2 min** | **4 min** | **6 min** | | | |
| Furan | 3.64 ± 0.03$^{d}$ | 3.78 ± 0.09$^{cd}$ | 4.17 ± 0.44$^{bcd}$ | 3.76 ± 0.02$^{d}$ | 5.51 ± 0.37$^{a}$ | 5.08 ± 0.26$^{ab}$ | 4.05 ± 0.62$^{bcd}$ | 4.94 ± 0.78$^{abc}$ | 5.15 ± 0.33$^{ab}$ | <0.001 | 0.001 | <0.001 |
| Furfural | ND | ND | 0.14 ± 0.01$^{c}$ | ND | 0.23 ± 0.01$^{c}$ | 0.64 ± 0.07$^{ab}$ | 0.03 ± 0.02$^{c}$ | 0.49 ± 0.11$^{b}$ | 0.82 ± 0.22$^{a}$ | <0.001 | <0.001 | <0.001 |
| Furfuryl alcohol | 4.75 ± 0.40$^{e}$ | 4.58 ± 0.17$^{e}$ | 7.02 ± 0.85$^{de}$ | 5.07 ± 0.46$^{e}$ | 10.54 ± 0.20$^{cd}$ | 17.30 ± 1.82$^{ab}$ | 5.37 ± 0.42$^{e}$ | 14.27 ± 0.38$^{bc}$ | 20.19 ± 4.55$^{a}$ | <0.001 | <0.001 | <0.001 |
| 2-pentylfuran | 1.56 ± 0.08$^{c}$ | 2.24 ± 0.35$^{bc}$ | 3.88 ± 1.03$^{a}$ | 1.81 ± 0.07$^{bc}$ | 1.50 ± 0.32$^{c}$ | 3.21 ± 0.70$^{ab}$ | 2.12 ± 0.41$^{bc}$ | 2.15 ± 0.64$^{bc}$ | 3.33 ± 0.55$^{ab}$ | <0.001 | 0.271 | <0.001 |
| HMF | 0.82 ± 0.14$^{d}$ | 0.96 ± 0.06$^{d}$ | 1.76 ± 0.08$^{d}$ | 1.34 ± 0.28$^{d}$ | 1.78 ± 0.08$^{d}$ | 3.61 ± 0.71$^{bc}$ | 2.06 ± 0.21$^{cd}$ | 3.77 ± 0.17$^{b}$ | 9.69 ± 1.50$^{a}$ | <0.001 | <0.001 | <0.001 |
| TOTAL | 10.65 ± 0.53$^{e}$ | 11.76 ± 0.75$^{e}$ | 1.80 ± 0.09$^{cd}$ | 11.76 ± 0.86$^{e}$ | 19.52 ± 0.92$^{c}$ | 29.29 ± 3.05$^{b}$ | 13.85 ± 1.82$^{d}$ | 25.73 ± 1.76$^{bc}$ | 38.83 ± 6.32$^{a}$ | <0.001 | <0.001 | <0.001 |

* The results are expressed as means values ± standard deviation.
$^{a-d}$Different letters within the same row differed significantly ($p<0.05$).
ND: non detected.

As can be observed, total furanic compounds levels significantly ($p$ <0.001) raised as both time and temperature increased, and this trend is also followed by the individual compounds, except for 2-pentylfuran, which was not influenced by the cooking temperature. Thus, the highest values of furanic compounds were found when deep-frying at 200 ºC during 6 min (38.83 µg $g^{-1}$ sample) and the lowest at 160 ºC during 2 and 4 min and at 180 ºC during 2 min (10.65, 11.76 and 11.76 µg $g^{-1}$ sample, respectively). These minor quantities are near to those obtained when oven-baking and reheating in the microwave (9.99 and 8.15 µg $g^{-1}$ sample, respectively) (Table 5.1), and lower in comparison with products deep-fried following manufacturer recommendations (180 ºC during 4 min) (19.52 µg $g^{-1}$ sample). Thus, it is possible to minimize the generation furanic compounds when deep-frying this kind of products by adjusting the cooking conditions. Agila & Barriger (2012) also found an increase of HMF in almonds when increasing roasting time and temperature. Until now furfural and HMF have been evaluated as indicators of the severity of heat treatment or length of storage in several foods (Gökmen & Açar, 1999; Morales *et al.*, 1997; Teixidó *et al.*, 2006). In addition, results of this study point out that, not only furfural and HMF, also furan and furfuryl alcohol could be used as heat cooking signs in breaded products.

Apart from the influence of the cooking conditions on the generation of furanic compounds, the handling after processing is also of importance. Since these compounds are highly volatile, the decrease in their concentrations by evaporation during domestic handling should be substantial. In this sense, the stability of the furanic compounds after cooking was evaluated in the deep-fried breaded fish products of this study (Figure 5.1).

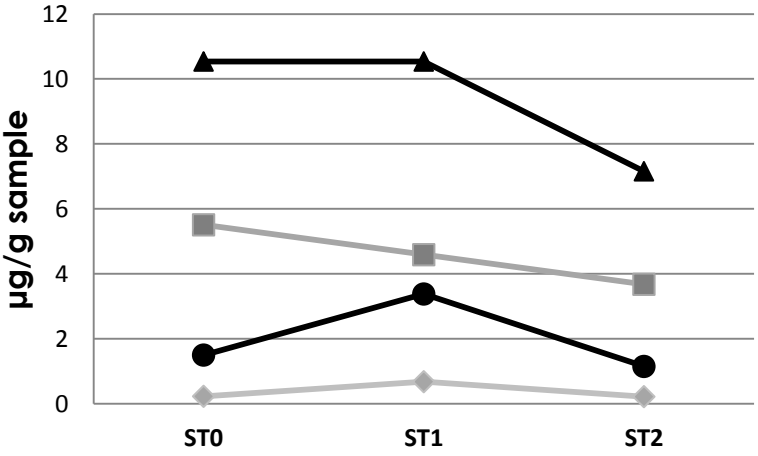



**Figure 5.1:** Content of furan (■), furfural (♦), furfuryl alcohol (▲) and 2-pentylfuran (●) in deep-fried breaded fish samples analysed immediately after cooking (ST0) and with a delay of 10 (ST1) and 20 min (ST2).

The levels of furan significantly decreased with the time (from 5.51 µg $g^{-1}$ in ST0, to 4.59 and 3.68 µg $g^{-1}$ sample in ST1 and ST2, respectively). Other authors have also reported that furan is not stable in foods after preparing or opening the commercial products, and that its loss is clearly related to the product temperature, time of exposure to the atmosphere and the food matrix (Goldmann *et al.* 2005; Kim *et al.*, 2009). The content of furfuryl alcohol kept constant between ST0 and ST1 products (10.54 and 10.56 µg $g^{-1}$ sample, respectively) and decreased in ST2 ones (7.16 µg $g^{-1}$ sample) (Figure 5.1). The levels of furfural and 2-pentylfuran experimented an increase between ST0 (0.23 and 1.50, respectively) and ST1 samples (0.68 and 3.38 µg g-1 sample, respectively), and decreased in ST2 products (0.22 and 1.15 µg g-1 sample, respectively). Thus, the losses of furanic compounds in coated products seem to be more notable 20 min after deep-frying.

### *5.3.2. Profile of volatile compounds as affected by cooking and handling conditions*

A total of 60 volatile compounds were detected, being clustered in the following chemical families: aldehydes (2-methylpropanal; 3-methylbutanal; 2-methylbutanal; hexanal, 2-hexenal; heptanal; benzaldehyde; octanal; benzeneacetaldehyde; 2-octenal; benzaldehyde; nonanal; nonenal; 2-decenal; 2,4-decadienal; 2-dodecenal), alcohols (2-methyl-1-butanol; 1-pentanol; 2-pentanolacetate; 1-hexanol; 1-butanol-3-methyl, acetate; 1-octen-3-ol; 2-ethylhexanol), ketones (2-pentanonone; 2,3-pentanedione; 2-heptanone), aliphatic hydrocarbons (2-methylpentane; 3-methylpentane; hexane; heptane; 2-octene; decane; undecane; dodecene; dodecane), aromatic hydrocarbons (benzene; methylbenzene; chlorobenzene; 1,3-dimethylbenzene; ethenylbenzene; 1-ethyl,3,5-dimethylbenzene; limonene; naphthalene),esters (acetic acid, ethyl ester; propanoic acid, methyl ester; butanoic acid, methyl ester; butanoic acid, 2-methylprotylester; butanoic acid, butyl ester; acetic acid, hexil ester; butanoic acid, 3-methyl, butyl ester; octanoic acid, methyl ester; butanoic acid, hexyl ester; octanoic acid, ethyl ester), furans (furan; furfural; furfuryl alcohol; 2-pentylfuran) and pyrazines (methylpyrazine; dimethylpyrazine; ethylpyrazine). Results on the analysis of volatile compounds in this study are expressed as total area counts of the different chemical families.

The effect of different cooking methods on the profile of volatile compounds was also evaluated in breaded fish products (Table 5.3).

**Table 5.3:** Abundance (total area count × $10^7$)of chemical families of volatile compounds detected in breaded fish products deep-fried in SF and OL oils, OV and MO*.

| | SF | OL | OV | MO | *p* |
|---|---|---|---|---|---|
| **Aldehydes** | 22.86 ± 2.37$^{a}$ | 18.28 ± 0.64$^{b}$ | 4.78 ± 0.56$^{c}$ | 7.12 ± 0.61$^{c}$ | <0.001 |
| **Polycyclichydrocarbons** | 188.97 ± 15.69$^{a}$ | 7.61 ± 0.87$^{b}$ | 9.63 ± 0.02$^{b}$ | 9.29 ± 2.09$^{b}$ | <0.001 |
| **Alcohols** | 2.40 ± 0.19 | ND | ND | ND | <0.001 |
| **Ketones** | 3.01 ± 0.76 | 1.96 ± 0.82 | ND | ND | <0.001 |
| **Aromatic hydrocarbons** | 8.24 ± 1.91$^{b}$ | 14.50 ± 1.25$^{a}$ | 5.43 ± 0.55$^{bc}$ | 3.14 ± 0.07$^{c}$ | <0.001 |
| **Furans** | 9.36 ± 1.05$^{a}$ | 5.26 ± 1.33$^{b}$ | ND | 2.86 ± 0.12$^{c}$ | <0.001 |
| **Pyrazines** | 10.96 ± 1.52$^{a}$ | 7.18 ± 0.79$^{b}$ | ND | ND | <0.001 |
| **Esters** | 2.80 ± 0.43$^{a}$ | ND | 1.54 ± 0.06$^{b}$ | ND | <0.001 |
| **TOTAL** | 249.36 ± 22.63$^{a}$ | 54.21 ± 5.74$^{b}$ | 22.41 ± 1.25$^{c}$ | 21.98 ± 2.54$^{c}$ | <0.001 |

* The results are expressed as means values ± standard deviation.
$^{a-c}$Different letters within the same row differed significantly ($p$ <0.05).
ND: non detected.

The highest levels of total volatile compounds were found in SF products (249 AU x $10^7$), followed by OL samples (54 AU x $10^7$) and OV and MO batches showing the minor values (22 AU x $10^7$), due to most chemical families of volatile compounds showed higher levels in SF than in the other batches. This result shows the notable impact of deep-frying in sunflower oil on the formation of volatile compounds in coated products. In fact, Pokorny (1999) stated that the volatile oxidation products of linoleic acid are the most important flavour compounds found in fried foods, while the oxidation products of oleic acid are less important in contributing to the fried flavour. In addition, these findings could be also explained by the rapid rate that volatile compounds evaporate once they have been formed, being greater in breaded fish products cooked in the oven or reheated in the microwave than in deep-fried products.

The influence of time and temperature of deep-frying on the profile of volatile compounds is exposed in Table 5.4.

**Table 5.4:** Abundance (total area count × $10^7$) of chemical families of volatile compounds detected in breaded fish products deep-fried at different combinations of time (t) and temperature ($T^a$)*.

| | 160 ºC | | | 180 ºC | | | 200 ºC | | | *p* (t) | *p* ($T^a$) | *p* (t-$T^a$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **2 min** | **4 min** | **6 min** | **2 min** | **4 min** | **6 min** | **2 min** | **4 min** | **6 min** | | | |
| **Aldehydes** | 13.13 ± 0.88$^{ef}$ | 11.44 ± 0.45$^{f}$ | 35.26 ± 0.85$^{c}$ | 15.21 ± 0.10$^{e}$ | 22.86 ± 2.37$^{d}$ | 69.78 ± 4.16$^{a}$ | 18.17 ± 1.49$^{de}$ | 23.32 ± 3.37$^{d}$ | 61.53 ±3.15$^{b}$ | <0.001 | <0.001 | <0.001 |
| **Aliphatic hydrocarbons** | 9.09 ± 0.98$^{c}$ | 165.39 ± 70.75$^{b}$ | 4.21 ± 0.36$^{c}$ | 8.86 ± 1.23$^{c}$ | 188.97 ± 15.69$^{b}$ | 11.83 ± 0.73$^{c}$ | 13.94 ± 0.31$^{c}$ | 443.81 ± 118.15$^{a}$ | 9.38 ± 1.51$^{c}$ | <0.001 | 0.029 | <0.001 |
| **Alcohols** | 0.93 ± 0.11$^{b}$ | 0.87 ± 0.15$^{b}$ | 1.27 ± 0.41$^{b}$ | 1.49 ± 0.12$^{b}$ | 2.40 ± 0.19$^{a}$ | 1.43 ± 0.39$^{b}$ | 1.23 ± 0.08$^{b}$ | ND | 1.11 ± 0.13$^{b}$ | 0.736 | 0.001 | <0.001 |
| **Ketones** | ND | 1.71 ± 0.09$^{bc}$ | 1.87 ± 0.66$^{b}$ | 2.28 ± 0.28$^{b}$ | 3.01 ± 0.76$^{b}$ | 5.61 ± 0.54$^{a}$ | 2.71 ± 0.72$^{b}$ | 3.53 ± 1.07$^{bc}$ | 5.25 ± 0.83$^{ab}$ | <0.001 | <0.001 | <0.001 |
| **Aromatic hydrocarbons** | 9.23 ± 0.39$^{b}$ | 3.79 ± 0.19$^{c}$ | 12.47 ± 1.47$^{a}$ | 7.68 ± 0.07$^{b}$ | 8.24 ± 1.91$^{b}$ | 12.25 ± 0.77$^{a}$ | 7.36 ± 0.72$^{b}$ | 9.15 ± 1.02$^{b}$ | 12.16 ± 0.77$^{a}$ | <0.001 | 0.401 | <0.001 |
| **Furans** | 1.72 ± 0.15$^{d}$ | 1.13 ± 0.41$^{d}$ | 7.99 ± 0.06$^{c}$ | 1.88 ± 0.39$^{d}$ | 9.36 ± 1.05$^{c}$ | 18.56 ± 1.53$^{a}$ | 3.07 ± 0.42$^{d}$ | 12.94 ± 1.03$^{b}$ | 17.22 ± 0.05$^{a}$ | <0.001 | <0.001 | <0.001 |
| **Pyrazines** | 0.79 ± 0.26$^{d}$ | ND | 5.53 ± 0.82$^{c}$ | ND | 10.96 ± 1.52$^{b}$ | 20.85 ± 1.66$^{a}$ | 0.87 ± 0.06$^{d}$ | 12.05 ± 0.25$^{b}$ | 20.26 ± 0.13$^{a}$ | <0.001 | <0.001 | <0.001 |
| **Esters** | ND | ND | ND | ND | 2.80 ± 0.43 | ND | ND | 2.91 ± 0.27 | ND | <0.001 | 0.014 | <0.001 |
| **TOTAL** | 34.84 ± 2.73$^{ef}$ | 184.23 ± 71.06$^{bc}$ | 68.98 ± 4.87$^{d}$ | 36.93 ± 2.08$^{ef}$ | 251.79 ± 22.36$^{b}$ | 142.36 ± 9.63$^{c}$ | 47.21 ± 3.62$^{e}$ | 501.14 ± 123.47$^{a}$ | 127.6 ± 6.18$^{c}$ | <0.001 | <0.001 | <0.001 |

* The results are expressed as means values ± standard deviation.
Different letters within the same row differed significantly ($p<0.05$).
ND: non detected

As can be seen, the generation of total volatile compounds increased with temperature, and all chemical families presented the same trend. However, the effect of time of deep-frying was not so plain, finding higher levels of volatile compounds when deep-frying during 4 than 6 min, and obtaining the minor levels in coated products deep-fried during 2 min. This result is due to the values of aliphatic hydrocarbons, and specifically to 2- and 3-methylpentane content, showing significant higher values when deep-frying during 4 min at 200, 180 and 160 ºC (444, 189 and 165 AU x $10^7$) than when applying the other combinations of time and temperature (from 4.21 to 13.94 AU x $10^7$). Nevertheless, the content of the rest of chemical families of volatile compounds significantly rose as the time increased. This finding indicates a great generation of 2- and 3-methylpentane when deep-frying during 4 min, as well as their degradation when deep-frying during more than 4 min. Due to their high threshold values, the presence of these volatile compounds seems to have a limited influence on products aroma (Ansorena *et al.*, 2001), consequently, differences in these aliphatic hydrocarbons might not influence notably on the flavour of deep-fried breaded fish products.

Apart from the influence of the cooking conditions on the generation of volatile compounds, their stability after cooking was also evaluated (Figure 5.2).

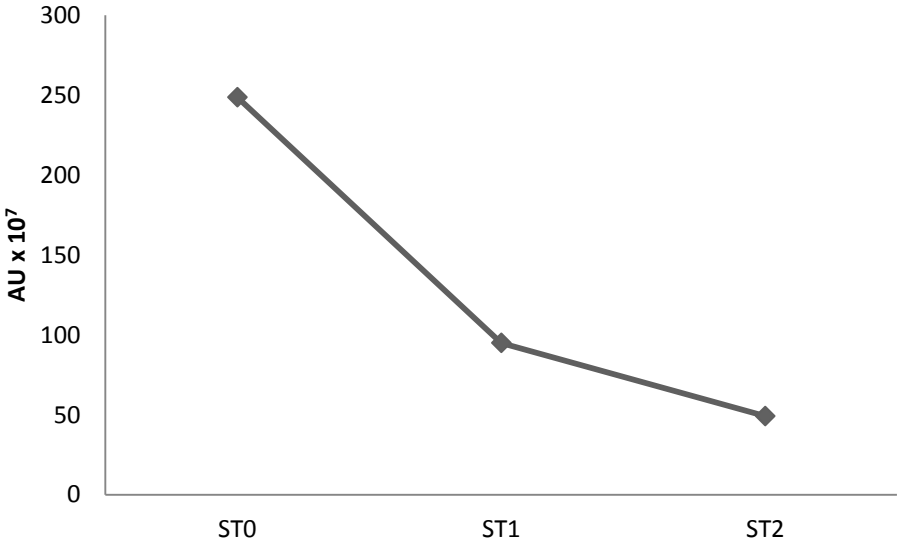



**Figure 5.2:** Abundance of total volatile compounds in deep-fried breaded fish samples analysed immediately after cooking (ST0) and with a delay of 10 (ST1) and 20 min (ST2).

As can be observed, the total content of volatile compounds significantly decreased from ST0 (252 AU x $10^7$) to ST1 (95 AU x $10^7$), due to the diminishing of aliphatic hydrocarbons, while the rest of chemical families kept their values. From ST1 to ST2 the values of total volatile compounds went on decreasing (95 and 49 AU x $10^7$, respectively) because of most chemical families experimented a significant decrease, however, the levels of polycyclic hydrocarbons maintained throughout this time.

Aldehydes, alcohols, pyrazines, pyridines and furans are thought to play an important role in the flavour of the fried samples (Elmore *et al.*, 1999; Timón *et al.*, 2004). Thus, the effect of cooking and handling conditions on the content of these chemical families of volatile compounds might lead to differences in aroma and flavour attributes of breaded fish products.

## 5.4. Conclusions

Cooking and handling conditions exert an important effect on volatile and furanic compounds levels in breaded fish products. Oven-baking and reheating in the microwave are preferred than deep-frying for reducing the generation of furanic compunds. In addition, furanic compound levels might be also minimized by adjusting deep-frying conditions (in sunflower oil at 160 ºC - 4 min or 180 ºC - 2 min) and the time after cooking (10 min). However, other volatile compounds related to the aroma and flavour of fried food, were also reduced.

# PART IV

## Furanic compounds in bakery products: Effect of baking process and occurence in commercial products

# CHAPTER 6

## Effect of baking process in Hydroxymethylfurfural and Furfural content of model cakes: Mitigation strategies and its impact on volatile profile

## Abstract

This study shows the optimisation and validation of an extraction method for HMF and furfural from cakes, achieving the better procedure by using water/methanol (70/30) and clarification with *Carrez* I and II reagents. In addition the effect of both baking method and time on HMF and furfural content and also on the profile of volatile compounds in cakes was studied. Baking procedure and time influence the content of HMF and furfural and the volatile profile of cakes. HMF and furfural content increased in microwave and convection oven cakes with the increase of baking time, while baking time did not influence these compounds when using steam oven. The highest contents of HMF and furfural were achieved in convection oven baked cakes. Similar profiles of volatile compounds were obtained in cakes baked in convection oven and steam oven, while, microwaved cakes presented lower relative percentage of volatile compounds. Thus, it can be pointed out that steam oven is an appropriate mitigation strategy to obtain cakes with low content of HMF and rich in aroma compounds.

**Keywords:** Model cakes, Baking process, HMF, Furfural, HPLC-DAD, HS-SPME-GC-MS

## 6.1. Introduction

Baking is a complex process that involves physical, chemical, and biochemical changes (Sablani *et al.*, 1998), which are essential for the development of the aroma, taste and colour surface in the baked products. It is mainly influenced by cooking time, temperature and moisture of the system (Purlis, 2010). The surface colour developed in bakery products is an important parameter associated with aroma, taste, and appearance characteristics relevant from the consumers' perspective.

The formation of colour during baking is called browning and is the result of non-enzymatic chemical reactions, known as *Maillard* reactions which produce coloured compounds during the baking process. Browning development during baking is also related with other sensorial features such as aroma generation, and with nutritional aspects, such as the formation of undesirable compounds (Purlis, 2010; Isleroglu *et al.*, 2012). In literature there are several works related with the characterisation of physicochemical properties of food after the baking process (Walker *et al.*, 2012; Chevallier *et al.*, 2002; Piazza & Masi, 1997) and the formation of undesirable compounds, such as HMF and acrylamide (Gökmen *et al.*, 2007; Ait-Ameur *et al.*, 2006). HMF and furfural are furanic compounds generated during the advanced stages of *Maillard* reaction, commonly measured as quality parameters to evaluate the severity of the heat treatment. There are several studies reporting the levels of HMF and furfural in different food products such as fortified wines (Pereira *et al.*, 2011), cereals (Rufián-Henares *et al.*, 2009), milks (Chávez-Servín *et al.*, 2006), coat deep-fried products (Pérez-Palacios *et al.*, 2012b, 2013) and bakery products (Ait-Ameur *et al.*, 2006, 2007, 2008; Delgado-Andrade *et al.*, 2009).

Baking can be performed using different types of oven, namely, static convection oven (Walker *et al.*, 2012), microwave oven (Sumnu, 2001) and steam oven (Isleroglu *et al*., 2012). However, the influence of oven type and baking time in the generation of HMF and furfural in cakes containing sugars, flour, citrus juices, eggs, and oil as well as the impact of mitigation strategies on aroma compounds are not understood. The objectives of this work consisted in comparing the formation of HMF and furfural in model cakes using static convection oven, microwave oven and steam oven, searching for baking conditions that produce low levels of HMF and furfural and evaluate the impact of these baking processes in cake volatile profile. To achieve these goals an efficient extraction methodology was validated and applied for HPLC analyses of HMF and furfural in model cakes produced under controlled baking conditions. Moreover, the volatile profile of these cakes were also analysed by HS-SPME-GC-MS.

## 6.2. Material and Methods

### *6.2.1. Chemical and Reagents*

HMF (98 %) was supplied by Sigma-Aldrich (Steinheim, Germany). Furfural was purchased by Merck (99%) (Darmstadt, Germany). Methanol was supplied by Merck (Darmstadt, Germany) and ultrapure water (0.055 µS $cm^{-1}$) was obtained by using a Serial Milli-Q system for Millipore (Supor DCF, Gelman Sciences, Chentelham, Australia). Reagents used for protein precipitation (15% potassium ferrocyanide (w/v) (*Carrez* I); 30 % zinc acetate (w/v) (*Carrez* II); trichloroacetic acid solution (TCA) 40 % (w/v); oxalic acid solution (0.15 M) were from Merck (Darmstadt, Germany).

Separate standard stock solutions of HMF and furfural containing *ca*. 1.0 mg $mL^{-1}$ in methanol were prepared. A working solution (0.05 mg $mL^{-1}$) for each analyte was made by adding 0.25 mL of stock solution to 4.75 mL of HPLC grade water. The working solution was stored at 0 ºC and renewed daily.

### *6.2.2. Experimental design*

Validation of HPLC method for analyses of HMF and furfural was performed on model cakes prepared in two types of oven, microwave (Haier, Model HR-6752T(E), Italy) and static convection oven (Electric Co, Model MF22VD, 22L). The following recipe was chosen for model cakes: 80.0 g of sugar, 90.0 g of wheat flour, 1.5 g of chemical leavening, 10.0 g of grated lemon peel, 1 medium egg (50.0 g), 30.0 g of orange juice and 40.0 g of sunflower oil. The ingredients were thoroughly mixed. Dough was rolled out to 3 mugs, to add up *ca*. 100 g per mug. A total of 18 mugs were prepared, 9 cooked in convection oven and 9 cooked in microwave. Each mug was cooked individually. The following conditions were use: 50 minutes at 180ºC for convection oven samples and 1 minute and 30 seconds at maximum potency for microwave samples. Cake pH was 6.9±0.2 evaluated using a pH meter (MicropH 2001, Crison, Barcelona, Spain).

The effect of baking process in HMF and furfural content and volatile profile was evaluated in model cakes prepared as described above and cooked in microwave, convection and steam ovens applying three different cooking times. Samples were i) microwaved at the maximum potency (1250 W), during 1 min and 30 sec, 2 min and 2 min and 30 sec, and ii) baked in steam and in a convection ovens, at 180 ºC during 20, 40 and 60 min. Three mugs were analysed for each time condition. After preparation, all samples

were crushed with a commercial crusher (Flama, Model 1705 FL, Portugal) and stored in flasks at 4 ºC and analysed in two working days.

### *6.2.3. Quantification of HMF and furfural by HPLC/Diode Array Detection*

#### 6.2.3.1. Extraction method

Three extraction procedures were selected according to literature (Ait-Ameur *et al*., 2006; Ferrer et al., 2002; Rufián-Henares *et al.*, 2006b) and tested to compare the extraction yield of HMF and furfural. For this purpose, extraction of HMF and furfural before quantification by HPLC was performed with oxalic acid and with water (Ferrer *et al.*, 2002) followed by clarification with TCA (Ait-Ameur *et al.*, 2006, 2008), these two procedures were coded as M1 and M2, respectively, whereas, extraction with water and clarification with *Carrez* I and II reagents (Rufián-Henares *et al.*, 2006b) was coded as M3. Modifications of this last procedure were tried, namely, replacement of water extraction by different mixtures of water:methanol – 60:40; 70:30, 80:20 - followed by clarification with *Carrez* I and II reagents. These procedures were coded as M4, M5, and M6, respectively.

The selected procedure was based on the method proposed by Rufián-Henares *et al.* (2006b) (M3) with some modifications. Ten grams of sample suspended in 5 mL water:methanol (70:30) were stirred during 1 min. Following, 0.250 mL *Carrez* I and *Carrez* II solutions were added and centrifuged at 5 000 rpm (4 ºC) during 15 min, recovering the supernatant to a 15 mL flask. Two more extractions were made by adding 2 mL of water:methanol (70:30) until 10 mL of supernatant collection. Two millilitres aliquot of this solution was centrifuged at 8000 rpm during 15 min and injected on the HPLC system.

#### 6.2.3.2. HPLC methodology

The chromatographic method was adapted from the method proposed by Ait-Ameur *et al.* (2006) for cookies: a 20 µL portion of the final extract was injected using an autosampler (Jasco AS – 2057 Plus, Japan) onto an Ultracarb ODS column (5 µm, 250 mm length, 4.6 mm i.d.) for HPLC-DAD analysis. It was used an analytical HPLC unit (Jasco, Tokyo, Japan) equipped with Jasco PU-2080 HPLC pumps, and a type 7725i Rheodyne injector with a 20 µL loop. The identity and purity of HMF and furfural were confirmed by a photodiode array detector (Waters – 2996). Peak identification in the chromatograms was carried out by comparing retention times and spectra of unknown peaks with reference

standards. Borwin PDA Controller Software (JMBS Developments, Le Fontanil, France) was also used.

The mobile phase was composed of sodium acetate (0.04 M) (Merck, Darmstadt, Germany) and methanol (Merck, Darmstadt, Germany) (70:30), adjusted to pH= 4.0 with acetic acid (99.8 %) (Fisher Scientific, Leicestershire, UK). The flow rate was 0.8 mL $min^{-1}$. All analyses were performed in triplicate, including the extraction procedure. The quantification was made using sample curve calibration in order to minimise matrix effects. Results are expressed as mg $kg^{-1}$ cake.

6.2.3.3. Quality control and criteria

LOD and LOQ based on a signal-to noise ratio of 3:1 and 10:1, respectively, were determined using standard solutions (n= 5). To check the linearity of the method calibration curves with six points were prepared using standard solutions in the range of 0.018 to 10 mg $kg^{-1}$ for HMF and 0.004 to 5 mg $kg^{-1}$ for furfural. The linearity of the method was also evaluated by spiking model cakes (prepared in microwave 1 min and 30 seconds and without detected levels of furanic compounds) in the same range of concentration of HMF and furfural and performing the selected extraction procedure.

Model cakes without detected levels of furanic compounds (baked 1 min and 30 seconds in microwave) were also used for evaluation of RSD of run-to-run and day-to-day assays, three replicate analyses of samples were analysed in one day and in two more different days, respectively (n= 9). A deviation of the area peak within 20 % of the mean values of the calibration standards was considered acceptable.

The accuracy of the method was evaluated through recovery studies that were performed by spiking matrix samples without detected levels of HMF and furfural with different concentrations of HMF (0.395, 0.789 and 5 mg $kg^{-1}$) and furfural (0.402, 0.804 and 5 mg $kg^{-1}$). Percentage recovery of HMF and furfural at the three different spiked levels was calculated.

*6.2.4. Analysis of volatile compounds by HS-SPME-GC-MS methodology*

The extraction of volatile compounds was performed by HS-SPME immediately after cake baking following the method described in (Pérez-Palacios *et al.*, 2012b). Briefly, 2 g of sample were transferred to a 50 mL HS vial, containing 5 mL of water and 3 g of NaCl and the vial was immediately sealed and kept at -4 ºC during 10 min. After that, the vial was

placed into an ultrasonic bath cleaner during 15 min. To extract volatile compounds a CAR–PDMS SPME fibre from Supelco (Bellafonte, PA, USA) was used. The fibre was inserted into the sample vial through the septum and exposed to the HS for 40 min at 37 ± 1 ºC under constant agitation (600 rpm). Thereafter, the SPME fibre was inserted and desorbed for 10 min at 280 ºC, in the splitless mode, with 1 mL $min^{-1}$ flow.

Chromatographic analysis was performed using an Agilent 6890 GC (Agilent, Avondale, PA, USA) coupled to a MS detector (Agilent 5973). Volatiles were separated on a 5 % phenyl-methyl silicone (HP-5) bonded phase fused-silica capillary column (Hewlett – Packard, Palo Alto, CA, USA; 60 m x 320 µm i.d., film thickness 1 µm). Mass spectra were obtained by electronic impact at 70 eV, with a multiplier voltage of 2056 V, collecting data at a rate of 1 scan $s^{-1}$ over the *m/z* range 30–500. The constituents were identified by comparing the experimental spectra with spectra from NIST 98 data bank (NIST/EPA/NISH Mass Spectral Library, version 1.6, USA), and also by comparison of their GC Kovats index and in some cases by comparison of their retention times with those of standard compounds. The total content of volatile compounds of each headspace analysis was defined by integrating the peak areas of all the identified compounds. The relative percentages of individual compounds were calculated from the total contents of volatiles on the chromatograms.

### *6.2.5. Statistical design*

Cake samples were analysed in triplicate. All results are presented as mean and standard deviation. One-way ANOVA was applied to data from the extraction methods, the different baking conditions and the volatile profile. Two-way ANOVA was performed to establish the effect of baking time on model cakes from static convection and steam ovens. *T*-Tukey was applied as the test *a posteriori* with a level of significance of 95 %. To ensure data were of normal distribution, standardized skewness and standardized kurtosis values were checked. Statistical analyses were carried out using SPSS (v. 20.0).

## 6.3. Results and discussion

### *6.3.1. Validation of the HPLC method for analysis of HMF and furfural in model cakes*

Table 6.1 presents the results obtained performing the extraction of HMF and furfural in model cakes baked 50 minutes at 180 ºC in a convection oven applying M1, M2 and M3 procedures.

Statistical treatment of results indicated that M1 extracted less amounts of HMF than M2 and M3. Concerning furfural, lower extraction was obtained with M1 and M2 when compared with M3 procedure, which involves extraction with water and clarification with *Carrez* I and II reagents. This method was modified using M4, M5 and M6 procedures to increase extraction.

**Table 6.1:** Extraction methods tested for HMF and Furfural analysis in model cakes baked in a convection oven at 180º during 50 min. Results are presented as mean (mg $kg^{-1}$) ± standard deviation.

| | M1 | M2 | M3 | M4 | M5 | M6 | *p* |
|---|---|---|---|---|---|---|---|
| HMF (mg $kg^{-1}$) | 16.44±0.06$^{a}$ | 18.27±0.07$^{b}$ | 18.31±0.10$^{b}$ | 24.59±0.05$^{c}$ | 25.21±0.03$^{d}$ | 22.66±0.05$^{e}$ | <0.001 |
| Furfural (mg $kg^{-1}$) | 0.366±0.00$^{a}$ | 0.358±0.00$^{a}$ | 0.493±0.00$^{b}$ | 1.963±0.00$^{c}$ | 2.076±0.00$^{d}$ | 1.896±0.00$^{e}$ | <0.001 |

M1 - extraction with oxalic acid and clarification with TCA; M2 - extraction with water and clarification with TCA; M3 - extraction with water and clarification with *Carrez* I and II reagents; M4 - extraction with water:methanol (60:40) and clarification with *Carrez* I and II; M5 - extraction with water:methanol (70:30) and clarification with *Carrez* I and II; M6 - extraction with water:methanol (80:20) and clarification with *Carrez* I and II. $^{a-e}$ Means with different letters in the same line are significantly different ($p < 0.05$).

The furanic compounds under study are relatively less polar than water, thus, the addition of a small percentage of an organic solvent, particularly methanol in the extraction methodology can increase extraction yield. To test this hypothesis different proportions of methanol:water were assayed: 40:60, 30:70 and 20:80 (M3, M4 and M5 methods, respectively). Results are also shown in Table 6.1 and highlight that high extraction was obtained with M5, which demonstrates the importance of adding methanol to the original extraction procedure to increase extraction of these compounds, particularly furfural.

Figure 6.1 shows a chromatogram of a model cake baked in microwave (1 min and 30 sec) spiked and unspiked with HMF and furfural. The maximum wavelength of the furanic compounds in the spiked samples was confirmed: 284 nm for HMF and 277 nm for furfural, which are in accordance with other authors (Ait-Ameur *et al.*, 2006; Chávez-Servín *et al.*, 2006). The retention times were 4.76 min and 8.18 min, respectively.

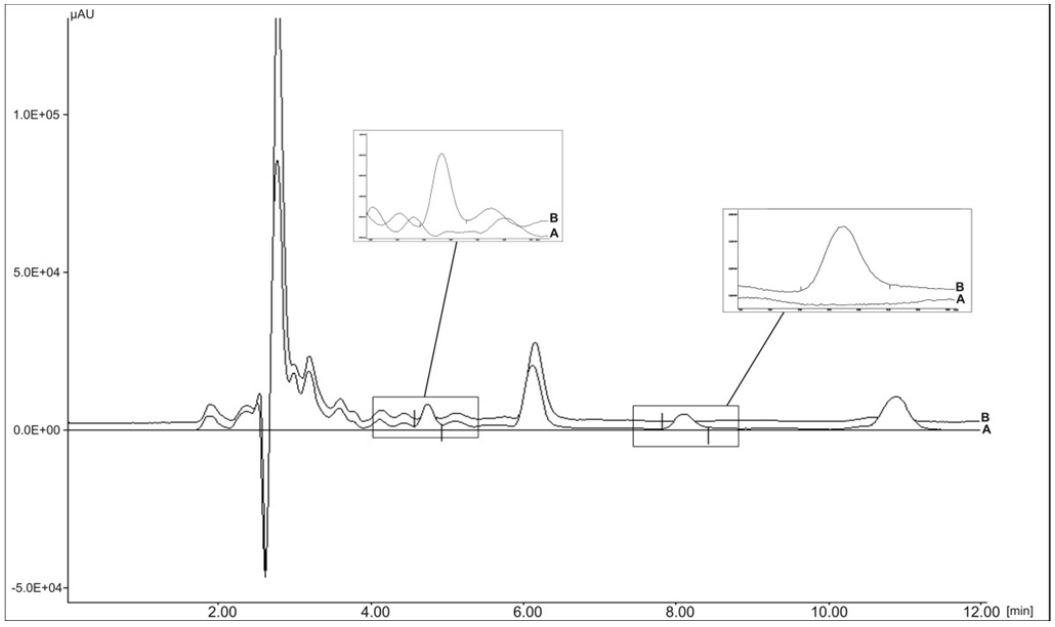



**Figure 6.1:** Chromatograms of microwave baked model cake unspiked (A) and spiked (B) with HMF (4.76 min; 284 nm, 0.789 mg $L^{-1}$) and furfural (8.18 min, 277 nm, 0.804 mg $L^{-1}$).

The compounds under study were not detected in the unspiked sample baked in microwave during 1 min and 30 sec, being an adequate matrix of baked dough free of HMF and furfural. Thus, it was used to study the matrix effect. Comparison was performed between the slopes of the regression lines obtained for pure standard calibration curve and for the sample curve calibration by least-squares analysis, and statistical differences were found ($p$ = 0.95, n = 12 Student's $t$ - test for paired samples), which points out the matrix effect and the choice of use of calibration curve in matrix for quantification of HMF and furfural in baked cakes.

Table 6.2 presents the quality parameters of the method.

**Table 6.2:** Quality parameters of HMF and furfural achieved by HPLC-DAD methodology.

| | | **Furfural** | | | **HMF** | | |
|---|---|---|---|---|---|---|---|
| LOD (mg $kg^{-1}$) | | 0.001 | | | 0.006 | | |
| LOQ (mg $kg^{-1}$) | | 0.004 | | | 0.018 | | |
| Working range (mg $kg^{-1}$) | | 0.004 – 5.000 | | | 0.018 – 10.000 | | |
| Standard addition (mg $kg^{-1}$) | | 5.000 | 0.804 | 0.402 | 5.000 | 0.789 | 0.395 |
| Recovery (%) | | 94.9 | 96.5 | 98.9 | 100.5 | 99.5 | 98.5 |
| RSD % Intra-day | RT | 0.05 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | Area | 2.43 | 4.65 | 0.79 | 2.02 | 0.96 | 1.86 |
| RSD % Inter-day | RT | 3.67 | 1.80 | 3.16 | 2.44 | 1.32 | 2.32 |
| | Area | 2.51 | 3.85 | 7.51 | 1.84 | 1.96 | 3.68 |

LOD: Limits of detection. LOQ: Limits of quantification. RSD: relative standard deviation

Calibration curves with values ranging from 0.004 to 5.000 mg $kg^{-1}$ for furfural and 0.018 to 10.00 mg $kg^{-1}$ for HMF were made, obtaining $r^2$ values higher than 0.99. Additionally, low LOD (0.001 mg $kg^{-1}$ for furfural and 0.006 mg $kg^{-1}$ for HMF) and LOQ values (0.004 mg $kg^{-1}$ for furfural and 0.018 mg $kg^{-1}$ for HMF) were obtained, lower than those found by several authors (Ait-Ameur *et al.* 2006; Ferrer *et al.*, 2005; Andrade *et al.*, 2010). The recovery values ranged between 94.9 % and 98.9 % for furfural and 98.5 % and 100.5 % for HMF. Concerning the intra-day precision, RSD was less than 0.05% for retention time, and less than 4.65% for area. For inter-day precision, RSD was less than 3.67% for retention time and 7.51% for area. These results highlight the good performance of the analytical method.

### *6.3.2. Formation of HMF and furfural in model cakes baked under different conditions*

The content of HMF and furfural in model cakes prepared under different baking conditions are shown in Table 6.3.

**Table 6.3:** HMF and Furfural amounts present in model cakes baked under different conditions. Results are presented as mean (mg $kg^{-1}$) ± standard deviation.

| | Microwave | | | | Convection Oven | | | | Steam Oven | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1min 30 sec | 2 min | 2 min 30 sec | *p* | 20min | 40min | 60min | *p* | 20min | 40min | 60min | *p* |
| **HMF (mg $kg^{-1}$)** | n.d.[a] | 1.36±0.78[a] | 15.58±1.50[b] | 0.001 | 2.72±1.21[a] | 7.18±1.42[a] | 32.79±3.39[b] | 0.002 | 2.83±1.27 | 2.71±1.42 | 2.14±1.09 | 0.861 |
| **Furfural (mg $kg^{-1}$)** | n.d. | n.d. | 1.21±0.70 | 0.088 | n.d. | 0.90±0.71 | 2.30±1.07 | 0.112 | 1.25±0.35 | 1.07±0.61 | 0.92±0.53 | 0.827 |

[a-b] Means with different letters in the same line are significantly different for each oven type ($p < 0.05$). n.d. – not detected.

As can be seen, the baking time influenced the levels of HMF when using microwave and convection oven but not when baking in steam oven. However, furfural content was not affected by the baking time. When dough was baked during 1 min and 30 sec in microwave oven HMF and furfural were not detected. There were also non detected levels of furfural after microwaving during 2 min, although HMF was found (1.366 mg $kg^{-1}$). Baking the model cake in the microwave during 2 min and 30 sec significantly increased HMF levels (15.58 mg $kg^{-1}$) and furfural was quantified (1.228 mg $kg^{-1}$). Microwave-baked products undergo high thermal centre point and low surface temperature which prevents *Maillard* reactions (Decareau, 1992; Hegenbert, 1992). However, increasing microwave baking time a higher moisture loss occur which promotes the arising of *Maillard* compounds inside the cakes, giving origin to the emergence of HMF and furfural compounds. Regarding the results of convection oven cakes, HMF was detected in cakes baked in convection oven during any time, showing a significant increase at 60 min (32.78 mg $kg^{-1}$) in comparison to 20 and 40 min (2.72 and 7.18 mg $kg^{-1}$, respectively). Furfural was not detected when baking during 20 min but it was quantified in model cakes baked during 40 and 60 min at 180 ºC (0.90 and 2.30 mg $kg^{-1}$, respectively), although not significant differences were obtained. Concerning model cakes baked in steam oven, HMF and furfural no significant differences were found in cakes baked during 20, 40 and 60 min.

Additionally, a two-way ANOVA was performed to establish the effect of using the same baking time on model cakes from convection and steam ovens. Microwave baking times were completely different, thus they were not included in this statistical treatment. The effect of baking time and effect of oven type was statistically significant for HMF ($p$ <0.001) $F$ values were 76.017 and 123.557, respectively; whereas no statistic differences were observed on furfural content ($p$ >0.05), $F$ values were 0.001 and 2.483. Significant interaction between baking time and oven type effects was observed only for HMF content. Model cakes from steam oven contained significantly lower HMF content even baked during long periods ($p$ <0.001) than when using convection oven. In literature, some researchers showed that steam injection affects the crust colour, and acrylamide formation of cakes or breads, resulting in a lower acrylamide concentration by almost 50% in comparison with baking without steam (Bråthen & Knutsen, 2005; Ahrné *et al.*, 2007). Baking in a steam oven is an appropriate mitigation strategy to obtain cakes with low content of HMF. Baking in microwave or in convection ovens during short periods had similar effects on HMF and furfural formation.

### *6.3.3. Volatile composition of model cakes baked under different conditions*

Table 6.4 shows the relative percentage of volatile compounds extracted by SPME from model cakes baked under different conditions. Volatile compounds were clustered in different chemical families (furans, aldehydes, hydrocarbons, esters, alcohols, aromatic hydrocarbons, ketones and pyrazines). Some qualitative differences were found between baking methods. Eighteen volatile compounds were extracted from microwaved samples, 22 when using convection oven and 24 from samples baked in steam oven. In addition, most aldehydes were found in cakes baked in convection and steam oven but not in microwaved samples. Convection and steam oven samples presented higher relative percentage of aldehydes, hydrocarbons and pyrazines than microwave samples, with the exception of D-Limonene (characteristic of citrus fruits flavour). Aldehydes are responsible for strong flavours, especially if they are in their aliphatic form, which is the case. Other compounds namely, 7-methyl-3-methylene-1,6-octadiene (essential oil of several plants) have also a strong influence on aroma (Rega *et al.*, 2009). Furan, furfural and pyrazines (pyrazine,2,5-dimethyl) contribute to caramel-like flavour of baked products (Fisher & Scott, 1997). Baking cakes in convection oven is the most usual procedure and leads to the formation of a variety of aroma compounds. These results clearly indicate that samples baked in steam oven present similar volatile profile, whereas samples from microwave oven present a poor volatile profile, mainly volatiles from citrus already present in dough or those resulting from excessive baking time, detected only in model cakes baked during 2 min and 30 sec.

**Table 6.4:** Relative percentage of volatile compounds present in model cakes baked under different conditions. Results are present as mean percentage ± standard deviation (n=3). [a-c] Means with different letters in the same line are significantly different for each oven type ($p < 0.05$). n.d. – not detected.

| | Microwave Oven | | | | Convection Oven | | | | Steam Oven | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Compounds** | **1 min 30 sec** | **2 min** | **2 min 30 sec** | ***p*** | **20 min** | **40 min** | **60 min** | ***p*** | **20 min** | **40 min** | **60 min** | ***p*** |
| **Furans** | | | | | | | | | | | | |
| Furan | n.d.a | 0.02±0.01a | 0.02±0.00a | 0.141 | 0.02±0.00a | 0.02±0.00a | 0.02±0.00a | 0.046 | 0.02±0.00a | 0.02±0.00a | 0.02±0.00a | 0.096 |
| Furfural | n.d.a | n.d.a | 3.48±0.43b | 0.001 | 0.02±0.00a | 0.02±0.00a | 0.02±0.00a | 0.144 | 0.02±0.00a | 0.01±0.00a | 0.04±0.00b | 0.003 |
| Furfuryl alcohol | n.d.a | n.d.a | 0.29±0.02b | <0.001 | n.d. | n.d. | n.d. | _ | n.d. | n.d. | n.d. | _ |
| 2-Acetylfuran | n.d.a | n.d.a | 0.19±0.02b | 0.001 | n.d. | n.d. | n.d. | _ | n.d. | n.d. | n.d. | _ |
| 5-methylfurfural | n.d.a | n.d.a | 0.49±0.04b | <0.001 | n.d. | n.d. | n.d. | _ | n.d. | n.d. | n.d. | _ |
| **Aldehydes** | | | | | | | | | | | | |
| Pentanal | n.d. | n.d. | n.d. | _ | n.d.a | 0.31±0.02b | 0.48±0.01c | <0.001 | 0.28±0.01a | 0.26±0.01a | 0.18±0.00b | 0.001 |
| Hexanal | n.d. | n.d. | n.d. | _ | 1.82±0.02a | 1.97±0.16a | 1.58±0.01a | 0.057 | 0.87±0.03a | 0.94±0.01a | 0.65±0.04b | 0.005 |
| Heptanal | n.d. | n.d. | n.d. | _ | 0.24±0.02a | 0.29±0.03a | 0.32±0.06a | 0.298 | n.d.a | 0.26±0.00b | 0.25±0.01b | <0.001 |
| 2-Heptenal | n.d. | n.d. | n.d. | _ | n.d. | n.d. | n.d. | _ | n.d.a | n.d.a | 0.17±0.01b | <0.001 |
| Octanal | n.d. | n.d. | n.d. | _ | 1.19±0.08a | n.d.b | n.d.b | <0.001 | n.d.a | 1.24±0.00b | 1.41±0.03c | <0.001 |
| Nonanal | 0.09±0.01a | n.d.b | n.d.b | 0.002 | 0.42±0.00a | 0.32±0.13a | 0.34±0.04a | 0.456 | 0.44±0.02a | 0.44±0.02a | 0.74±0.02b | 0.001 |
| 3-7-dimethyloct-6-enal | n.d.a | 0.07±0.00b | 0.08±0.00b | <0.001 | n.d. | n.d. | n.d. | _ | n.d.a | 0.12±0.00b | 0.11±0.00b | <0.001 |
| 3-7-dimethyl-2,6-octadienal | 0.2±0.04a | 0.24±0.00a | 0.29±0.03a | 0.133 | 0.27±0.06a | 0.2±0.13a | 0.18±0.00a | 0.532 | 0.32±0.05a | 0.37±0.01a | 0.34±0.03a | 0.552 |
| **Hydrocarbons** | | | | | | | | | | | | |
| Dichloromethane | n.d.a | 0.53±0.02b | 0.37±0.04c | 0.001 | n.d. | n.d. | n.d. | _ | n.d. | n.d. | n.d. | _ |
| Hexane | 0.75±0.02a | n.d.b | n.d.b | <0.001 | 2.09±0.22a | 2.71±0.39a | 1.52±0.12a | 0.055 | 0.55±0.04a | 1.01±0.04b | 0.37±0.01c | 0.001 |
| 1,3,6-heptatriene, 2,5,5-trimethyl | n.d.a | n.d.a | 0.16±0.01b | <0.001 | n.d. | n.d. | n.d. | _ | n.d. | n.d. | n.d. | _ |
| 7-methyl-3-methylene-1,6-octadiene | n.d. | n.d. | n.d. | _ | 8.03±0.00a | 6.9±1.92a | 6.79±0.16a | 0.538 | 6.69±0.32a | 6.82±0.18a | n.d.b | <0.001 |
| Terpene | 7.56±0.41a | 8.9±0.51a | 8.01±0.70a | 0.188 | 9.26±0.55a, b | 9.56±0.07b | 8.12±0.02a | 0.040 | 9.53±0.21a | 10.22±0.10 a | 11.47±0.21 b | 0.004 |
| D-Limonene | 68.05±1.27a | 75.07±2.31 b | 70.86±1.22a, b | 0.035 | 50.4±0.29a | 51.29±0.37 a | 50.94±0.79 a | 0.367 | 54.87±1.44 a | 54.62±0.26 a | 58.00±0.08 a | 0.048 |
| 1,3,6-Octatriene,3,7-dimethyl | n.d.a | 0.11±0.00b | n.d.a | <0.001 | 0.29±0.01a | 0.23±0.07a | 0.22±0.01a | 0.348 | 0.23±0.01a | 0.29±0.00a, b | 0.34±0.04b | 0.047 |
| 1-methyl-4-(1-methylethylidene)-cyclohexene | 3.51±0.40a | 2.36±0.03b | 1.9±0.04b | 0.013 | 4.44±0.46a | 4.54±0.18a | 5.02±0.40a | 0.366 | 3.72±0.18a | 4.54±0.39a, b | 4.92±0.08b | 0.037 |

**Table 6.4:** *(continued)*

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Esters** | | | | | | | | | | | |
| Propanoic acid methyl ester | n.d. | n.d. | n.d. | _ | n.d. | n.d. | n.d. | _ | n.d.a | n.d.a | 0.07±0.00b | <0.001 |
| Butanoic acid methyl ester | n.d. | n.d. | n.d. | _ | 0.14±0.00a | n.d.b | n.d.b | <0.001 | n.d.a | n.d.a | 0.12±0.01b | <0.001 |
| Propanoic acid linalyl ester | n.d.a | n.d.a | 0.08±0.00b | <0.001 | 0.16±0.00a | 0.13±0.03a | 0.19±0.00a | 0.141 | n.d.a | 0.4±0.02b | 0.27±0.00c | <0.001 |
| **Alcohols** | | | | | | | | | | | | |
| 1-Pentanol | n.d. | n.d. | n.d. | _ | 0.17±0.01a | 0.22±0.02a | 0.19±0.01a | 0.052 | n.d. | n.d. | n.d. | _ |
| 1-Hexanol | n.d. | n.d. | n.d. | _ | 0.3±0.05 | 0.31±0.00 | 0.3±0.03 | 0.931 | 0.23±0.02a | 0.17±0.00b | 0.1±0.00c | 0.004 |
| **Aromatic hydrocarbons** | | | | | | | | | | | | |
| Toluene | n.d.a | 0.38±0.01b | 0.41±0.04b | 0.001 | 1.76±0.08a | 1.62±0.10a | 1.84±0.07a | 0.174 | 0.98±0.02a | 1.07±0.01a | 1.31±0.06b | 0.008 |
| 1-methyl-4-(1-methylethyl)benzene | 19.85±1.29a | 12.33±1.76b | 13.37±0.80b | 0.021 | 19.42±0.01a | 19.24±0.98a | 21.8±0.54a | 0.049 | 21.25±1.00a | 17.21±0.31b | 18.63±0.08b | 0.015 |
| **Ketones** | | | | | | | | | | | | |
| 2-butanone | n.d. | n.d. | n.d. | _ | 0.15±0.00a | n.d.b | n.d.b | <0.001 | n.d.a | n.d.a | 0.28±0.01b | <0.001 |
| **Pyrazines** | | | | | | | | | | | | |
| Pyrazine,2,5-dimethyl | n.d. | n.d. | n.d. | _ | 0.08±0.00a | 0.11±0.01b | 0.15±0.00c | 0.004 | n.d.a | n.d.a | 0.20±0.01b | <0.001 |

## 6.4 Conclusions

This paper reported the optimisation and validation of an extraction method for HMF and furfural from cakes. The solvent mixture methanol:water (30:70) allowed the highest yield. Both baking method and time influenced HMF and furfural levels. The quantity of HMF and furfural increased with the baking time when using microwave and convection oven but not when cooking in steam oven. In addition, convection oven at 180 ºC during 60 min leads to the highest amount of HMF and furfural, while the minor content of these compounds were found in cakes baked in steam oven.

The baking method also influenced the profile of volatile compounds in cakes, with higher relative percentage of aldehydes, hydrocarbons and pyrazines in cakes baked in convection and steam oven in comparison to microwaved cakes. Thus, it can be point out that baking in steam oven might be an appropriate procedure that leads to low levels of HMF and furfural in cakes without modifying the profile of volatile compounds.

# CHAPTER 7

## Assessment of HMF and Furfural in commercial bakery products

## Abstract

Bakery products, such as bread, biscuits and cakes/pastry are widely consumed. A survey was conducted on the presence of HMF and furfural in these products, for this purpose a reliable extraction procedure followed by HPLC was applied. The performance of the method was evaluated in terms of linearity (r always > 0.99); detection limits (0.001 mg $L^{-1}$ for furfural and 0.006 mg $L^{-1}$ for HMF); recovery percentages (98.5-100.5 % for HMF and 94.9-98.9 % for furfural); intraday precision (<4.65 %) and interday precision (<7.51 %). Two batches of a wide variety of products commercially available were analysed (a total of 88 samples). HMF and furfural levels presented high variability between products and batches of the same product. Cake/pastry samples showed the lowest HMF content (2.98 mg $kg^{-1}$fw) while biscuits showed the highest content (7.84 mg $kg^{-1}$fw) ($p$ <0.05). Regarding furfural, bread samples presented the highest furfural content (5.27 mg $kg^{-1}$fw) ($p$ <0.05), cake/pastry and biscuits showed the lowest content (1.86 and 3.00 mg $kg^{-1}$ fw, respectively). Chocolate containing samples presented higher amounts of furfural (>20 mg $kg^{-1}$). These results indicate that special attention should be given to furfural content of bread (due to its daily high consumption) and re-evaluation of dietary exposure.


**Keywords:** Bread, biscuits, cakes, HMF, furfural, HPLC-DAD

## 7.1. Introduction

The largest bakery product market in Europe is bread with a 56 % share of the total bakery products. *Per capita* annual consumption of bread in the EU is around 67 kg. However, different patterns are observed in EU countries. UK and Luxembourg are at the bottom of the list with 50 kg, Ireland and Spain in the middle field with 65 and 67 kg, respectively, Denmark with 74 kg and at the top Germany with 85 kg (CNN, 2000). Biscuits and industrial pastry/cakes although less consumed are also significant. Biscuits are the second largest product with 13 % of the total bakery product market. The three largest product market adding industrial pastry/cakes make up 81 % of the total market.

Bakery products are prepared with dough containing cereal flours, sugar, fat and eventually, eggs, chocolate and dried fruits among others. Bakery industry uses different kinds of organic acids, such as, citric acid or ascorbic acid to stabilize and regulate dough properties and prevent microbiological contaminations as well as baking agents namely, sodium bicarbonate to enhance the leavening (Nanditha & Prabhasankar, 2009).

The chemical reactions, like *Maillard* reactions and caramelization, during baking are essential for final sensorial attributes, like colour surface, texture and flavour, which are the main features influencing consumer´s preference in bakery products (Abdullah, 2008). However, these reactions also led to the formation of harmful compounds, such as furanic compounds (Ait-Ameur *et al.,* 2006; Gökmen *et al.,* 2007; Ciesarová *et al.*, 2009). HMF and furfural are commonly studied as the *Maillard* reaction products and sugar pyrolysis intermediates formed during thermal processing of foods (Capuano *et al.*, 2009; Ferrer *et al.*, 2002; Kroh, 1994; Morales *et al.*, 1992). HMF and furfural are formed, respectively, by the decomposition of hexoses and pentoses during heating. The generation of HMF and furfural is influenced by the concentration and type of sugar, being favoured by lower pH, low water activity and high baking temperatures (Ait-Ameur *et al.*, 2006; Gökmen *et al.*, 2007; Purlis, 2010). These furanic compounds have been evaluated as indicators of the severity of heat treatment or length of storage in several foods including fruit juices (Gökmen & Acar, 1999), ultrahigh-temperature-treated milks (Morales *et al.*, 1992, 1997, Ferrer *et al.*, 2000), breakfast cereals (Rufián-Henares *et al.,* 2006b), honey, wine and other alcoholic beverages, vinegars, coffee, breads and baby cereals (Teixidó *et al.*, 2006, 2011). Although the toxicological relevance of HMF and furfural is not clear, several *in vitro* studies point out that these compounds are suspected to have genotoxic and mutagenic effects which makes its presence undesired in thermally processed foods (Arts *et al.*, 2004; Capuano & Fogliano 2011; Abraham *et al.*, 2011, EFSA, 2005).

Regarding bakery products, in the scientific literature there are works focused on the amounts of HMF and furfural in model systems to study the influence of different ingredients on their formation (Ramírez-Jiménez *et al.*, 2000b, Ait-Ameur *et al.*, 2007, 2008; Gökmen *et al.*, 2007, 2008; Zhang *et al.*, 2012b). However, assessment of these harmful compounds in commercial samples is of major relevance to understand their contribution to daily intake. HMF quantification in commercial breads and biscuits are scarce (Ramírez-Jiménez *et al.*, 2000a; Ait-Ameur *et al.*, 2006; Delgado-Andrade *et al.,* 2009). No recent studies have been found for furfural content in commercial bakery products.

HPLC provides a fast, low cost, reliable and precise technique to analyse simultaneously HMF and furfural in food matrices. Selection of extraction conditions that favour simultaneous extraction of HMF and furfural is a crucial step that can improve reliable quantification.

The main objectives of this work were: i) to analyse the HMF and furfural content in bakery products commercially available, namely, bread, biscuits, and cakes/pastry samples using an effective extraction methodology, followed by high performance liquid chromatography analysis; ii) to investigate the effect of type of bakery product on the amounts of HMF and furfural; iii) to check the relationship between HMF and furfural content and formulation information provided by the manufacturer.

## 7.2. Material and Methods

### *7.2.1 Chemical and Reagents*

The chemical and reagents used were the same as described in Chapter 6; Section 6.2.1. Chemical and Reagents.

### *7.2.2. Sampling*

Forty-four different bakery products were selected for this study, two different batches of each product were analysed, which makes a total of 88 samples. They were clustered in three major types: bread (BR, n= 20), biscuits (BI, n= 24), and cakes/pastry (CA, n= 44). The samples were randomly codified, as described in Table 7.1. Total content of each package was powdered in a commercial crusher (Flama, Model 1705 FL, Portugal), homogenized, stored in flasks at 4 ºC and analysed in two working days.

**Table 7.1:** Code, main ingredients and additives of bread, biscuit and cake/pastry samples purchased for this study.

| Bread Samples | Sugars | Cereals | Chocolate | Dried fruits | Chemical leavenings | Antioxidants |
|---|---|---|---|---|---|---|
| **BR1** | S | W | N | N | N | AAc; LAc |
| **BR2** | S | W; Sunf; Lin; Mz; Soy; R | N | N | N | AAc |
| **BR3** | S | Soy; W | N | N | N | AAc |
| **BR4** | S | W | N | N | N | AAc; CC; CH |
| **BR5** | S | W | N | N | N | AAc; CC; CH |
| **BR6** | S; Glu-fruct syrup | W;O | 12% | N | N | LAc |
| **BR7** | N | W | N | N | N | AAc; CC |
| **BR8** | N | W | N | N | N | AAc; CC |
| **BR9** | N | W | N | N | N | AAc; CC |
| **BR10** | S | W | N | N | N | CC |
| **Biscuit Samples** | **Sugars** | **Cereals** | **Chocolate** | **Dried fruits** | **Chemical leavenings** | **Antioxidants** |
| **BI1** | S; Glu-fruct syrup | W | 17% | N | E331; E500ii; E503ii | AAc; CAc |
| **BI2** | S; Glu-fruct syrup | W | 24.9% | N | E331; E500ii; E503ii | AAc; CAc |
| **BI3** | S | W | N | N | N | AAc; CAc |
| **BI4** | S | W | 16% | N | E450; E500ii | CAc |
| **BI5** | S | W | N | N | E450; E500ii | CAc |
| **BI6** | S | W | N | N | E450; E500ii | CAc |
| **BI7** | S | W | N | Driedfruits | N | N |
| **BI8** | N | W | N | N | N | AAc |
| **BI9** | S | W | N | N | N | CAc |
| **BI10** | S | W | N | N | N | CAc |
| **BI11** | S | W | N | N | E450; E500 | CAc |
| **BI12** | S; Liq caramel | W | N | N | N | N |

**Table 7.1:** *(continued)*

| Cake/pastry Samples | Sugars | Cereals | Chocolate | Dried fruits | Chemical Leavenings | Antioxidants |
|---|---|---|---|---|---|---|
| **CA1** | S; Glu-fruct syrup | W | N | N | E450; E500ii | AAc; CAc |
| **CA2** | Sucrose | W | N | N | N | AAc; CAc |
| **CA3** | S; Glu-fruct syrup | W | N | N | E450; E500ii | AAc; CAc |
| **CA4** | S; Glu-fruct syrup | W | N | N | E450; E500ii; E541 | AAc; CAc |
| **CA5** | S | W | N | N | N | AAc; CAc |
| **CA6** | S; Glu syrup | W | N | N | E460; E500 | AAc; CAc |
| **CA7** | S; Glu-fruct syrup | W | N | N | E450; E500 | AAc; CAc |
| **CA8** | S; Glu-fruct syrup; Glu powder | W | N | N | N | AAc; CAc |
| **CA9** | S; Glu-fruct syrup | W | N | N | E450; E500ii | CAc |
| **CA10** | S; Glu-fruct syrup | W | N | N | E450; E500ii | CAc |
| **CA11** | S | W | N | 15% ALM | N | CAc |
| **CA12** | S; Glu-fruct syrup | W | N | N | E450i; E500i; E500ii | TAc |
| **CA13** | S; Glu-fruct syrup | W | N | N | E450i; E500ii | N |
| **CA14** | S | W | N | N | E450; E500ii; E541 | N |
| **CA15** | S | W | N | N | E450i; E500ii | N |
| **CA16** | S | W | N | N | E450; E500ii; E541 | N |
| **CA17** | S | W | N | N | N | AAc |
| **CA18** | M | W; Se | N | N | N | CAc |
| **CA19** | S; Glu syrup | R | N | N | E341; E450; E500ii | CAc |
| **CA20** | S; Glu-fruct syrup | W | N | N | E450i; E500ii | N |
| **CA21** | S; Glu-fruct syrup | W | 3% | N | E450i; E500ii | N |
| **CA22** | S | W | N | N | E341i; E450i; E500ii | N |

**S:** Sucrose; **Glu syrup:** Glucose syrup; **Glu-fruct syrup:** Glucose-fructose syrup; **Liq. Caramel:** Liquid caramel; **M:**Maltitol; **W:** Wheat; **Se:** Sesame; **H:** Hazelnut;**R:** Rice; **Sunf:** Sunflower; **Lin:** Linseed; **Mz:** Maize, **Soy:** Soybean; **R:** Rice; **O:** Oat; **N:** None; **ALM**: almonds; **AAc:** Ascorbic acid; **CAc:** Citric Acid; **TAc**: Tartaric acid; **LAc:** Lactic acid; **CC**: Calcium carbonate; **CH**: Calcium hydrogenophosphate.

*7.2.3 Extraction method*

The extraction procedure was based on the method proposed by Rufián-Henares *et al.* 2006b with some modifications. Ten g of sample were suspended in 5 mL water:methanol (70:30). The mixture was thoroughly stirred during 1 min and then 0.250 mL *Carrez* I and *Carrez* II solutions were added and centrifuged at 5 000 rpm (4 ºC) during 15 min, recovering the supernatant to a 15 mL flask. Two more consecutive extractions were made with 2 mL of water:methanol (70:30) until collecting 10 mL of supernatant. Two millilitres of this solution was centrifuged at 8000 rpm during 15 min before being analysed.

*7.2.4 HPLC-DAD methodology*

A 20 µL portion of the final extract was injected into the HPLC system using an autosampler (Jasco AS – 2057 Plus, Japan) onto an Ultracarb ODS column (5 µm, 250 mm length, 4.6 mm i.d.). It was used an analytical HPLC unit (Jasco, Tokyo, Japan) equipped with Jasco PU-2080 HPLC pumps. The identity and purity of HMF (284 nm) and furfural (277 nm) were confirmed by DAD (Waters – 2996, USA). The mobile phase was composed of sodium acetate (0.04 M) and methanol (70:30), adjusted to pH=4.0 with acetic acid (99.8%) (Fisher Scientific, Leicestershire, UK). The flow rate was 0.8 mL $min^{-1}$. All analyses were performed in triplicate. Results are expressed as mg $kg^{-1}$ fresh weigh (fw).

*7.2.5 Quality control and criteria*

Calibration curves were constructed for HMF and furfural. LOD and LOQ based on a signal to noise ratio of 3:1 and 10:1, respectively, were determined using standard solutions (n= 5).

The recovery experiments were carried out using three different samples in order to assess the accuracy of the method. Each sample was analysed in triplicate. Intraday precision (n= 3) and interday precision (n= 9) were also assessed.

*7.2.6 pH analyses*

For pH measurement, 1.0 g of sample was mixed with 20 mL of water and vortexed for 2 min. The mixture was held at room temperature (25 ºC) for 30 min to separate solid and

liquid phases. pH was measured after appropriately removing the supernatant layer by using a potentiometer (Micro pH 2001, Crison, Barcelona, Spain).

### *7.2.7 Statistical design*

To ensure data were of normal distribution, standardized skewness and standardized kurtosis values were checked. Analysis of variance was performed to establish the effect of the type of product on HMF and furfural content. Pearson's Correlation coefficient was carried out for establishing correlations between HMF, furfural, pH and nutritional composition in bakery products. PCA was performed to summarise major variation in data. Statistical analyses were carried out using SPSS (v. 20.0).

## 7.3. Results and Discussion

### *7.3.1 Analytical performance of the method*

The analytical methodology used was as described in Chapter 6; Section 6.2.3.3. Quality control and citeria.

HMF and furfural extraction with methanol:water (30:70) instead of water, as suggested by Rufián-Henares *et al.*, (2006b) gave increased extraction yield, specially for furfural content. For example, a sample that presented 18.27 mg $kg^{-1}$ of HMF and 0.5 mg $kg^{-1}$ of furfural when extracted with water showed a content of 25.21 mg $kg^{-1}$ of HMF and 2 mg $kg^{-1}$ of furfural when extracted with methanol:water (30:70). This is not surprising since these furanic compounds are less polar than water, and better extracted with the addition of a small percentage of an organic solvent such as methanol.

The recovery experiments were carried out using three different samples in order to assess the accuracy of the method. Recovery percentages ranged from 98.5 % to 100.5% for HMF and from 94.9 % to 98.9 % for furfural. Regarding intraday precision (n = 3), RSD was less than 0.05% for retention time, and less than 4.65% for area. For interday precision (n = 9), RSD was less than 3.67% for retention time and 7.51% for area which indicated good reproducibility of the method.

### *7.3.2. Quantification of HMF and furfural in commercial bakery products*

Table 7.2 presents the contents of HMF and furfural in the two batches of the forty-four bakery products. High variability was observed concerning HMF and furfural content, between the two batches of the same product, purchased and analysed separately and between different products. This great variability can be related with baking conditions applied to each type of product and with the different composition of the bakery products analysed (Purlis, 2010). It was expected that the two batches of the same product presented similar composition and baking conditions; however, the content of HMF and furfural was significantly different in samples from the two batches, except CA8, BR2 and BR5. Differences in pH were also observed in samples from the two batches of the same product (Table 7.2), highlighting lack of reproducibility in the manufacture of the analysed bakery products.

**Table 7.2:** HMF and furfural content (mg $kg^{-1}$ fresh weigh ± standard deviation (n=3)) and pH in two batches of commercial bread, biscuits and cake/pastry.

| **Bread Samples** | **HMF (mg $kg^{-1}$)** | | *p* | **Furfural (mg $kg^{-1}$)** | | *p* | **pH** | |
|---|---|---|---|---|---|---|---|---|
| | Batch 1 | Batch 2 | | Batch 1 | Batch 2 | | Batch 1 | Batch 2 |
| **BR1** | 2.13±0.01 | 1.44±0.01 | <0.001 | 1.70±0.00 | 2.90±0.01 | <0.001 | 5.47 | 5.24 |
| **BR2** | 8.81±0.01 | 8.93±0.06 | 0.111 | 2.85±0.01 | 2.60±0.00 | 0.001 | 5.88 | 5.97 |
| **BR3** | 7.73±0.04 | 18.34±0.27 | <0.001 | 4.77±0.03 | 11.20±0.05 | <0.001 | 6.44 | 6.04 |
| **BR4** | 7.15±0.01 | 6.01±0.17 | 0.011 | 3.18±0.02 | 4.39±0.12 | 0.005 | 5.38 | 5.23 |
| **BR5** | 3.14±0.01 | 1.60±0.01 | <0.001 | 3.11±0.19 | 3.24±0.01 | 0.432 | 5.46 | 5.41 |
| **BR7** | 5.30±0.01 | 4.37±0.13 | 0.010 | 2.59±0.03 | 9.29±0.27 | 0.001 | 5.96 | 6.06 |
| **BR8** | 0.78±0.00 | 5.34±0.13 | <0.001 | 3.15±0.02 | 9.61±0.14 | <0.001 | 6.53 | 5.99 |
| **BR9** | 0.66±0.00 | 2.47±0.04 | <0.001 | 6.49±0.02 | 18.19±0.15 | <0.001 | 6.98 | 6.63 |
| **BR10** | 0.69±0.06 | 2.33±0.07 | 0.002 | 1.47±0.17 | 4.12±0.03 | 0.002 | 5.03 | 3.54 |
| **Biscuit Samples** | **HMF (mg $kg^{-1}$)** | | *p* | **Furfural (mg $kg^{-1}$)** | | *p* | **pH** | |
| | B1 | B2 | | B1 | B2 | | B1 | B2 |
| **BI3** | 3.84±0.02 | 4.55±0.00 | <0.001 | 2.04±0.11 | 2.82±0.03 | 0.010 | 7.58 | 6.46 |
| **BI5** | 3.03±0.07 | 21.29±0.23 | <0.001 | n.d. | 3.36±0.01 | <0.001 | 7.43 | 7.12 |
| **BI6** | 4.53±0.00 | 5.46±0.04 | 0.001 | 2.05±0.03 | 3.60±0.08 | 0.001 | 6.49 | 6.69 |
| **BI8** | 5.22±0.04 | 3.96±0.04 | 0.001 | 2.20±0.14 | 10.49±0.03 | <0.001 | 6.01 | 5.58 |
| **BI9** | 7.21±0.27 | 68.58±1.06 | <0.001 | 2.31±0.07 | 8.58±0.20 | 0.001 | 6.59 | 6.93 |
| **BI10** | 6.17±0.03 | 82.78±2.09 | <0.001 | 3.05±0.03 | 7.31±0.14 | 0.001 | 5.37 | 4.86 |
| **BI11** | 2.40±0.00 | 1.65±0.01 | <0.001 | 2.69±0.01 | 3.45±0.07 | 0.004 | 7.40 | 6.80 |
| **BI12** | 8.95±0.28 | 26.24±0.18 | <0.001 | 0.74±0.01 | 1.01±0.00 | <0.001 | 5.11 | 5.63 |

**Table 7.2:** *(continued)*

| Cake/pastry Samples | HMF (mg kg$^{-1}$) | | *p* | Furfural (mg kg$^{-1}$) | | *P* | pH | |
|---|---|---|---|---|---|---|---|---|
| | B1 | B2 | | B1 | B2 | | B1 | B2 |
| **CA1** | 3.81±0.50 | 22.75±0.27 | <0.001 | 0.46±0.01 | 1.31±0.04 | 0.001 | 6.50 | 5.32 |
| **CA2** | 6.40±0.01 | 5.35±0.21 | 0.019 | 1.04±0.03 | 1.34±0.01 | 0.004 | 5.80 | 5.61 |
| **CA3** | 1.60±0.02 | 3.06±0.24 | 0.013 | 1.48±0.00 | 2.21±0.04 | 0.001 | 5.90 | 6.09 |
| **CA4** | 3.44±0.01 | 5.45±0.00 | <0.001 | 1.44±0.01 | 2.10±0.06 | 0.004 | 5.57 | 5.93 |
| **CA5** | 1.43±0.04 | 3.63±0.03 | <0.001 | 0.59±0.01 | 0.89±0.03 | 0.005 | 5.86 | 6.15 |
| **CA6** | 1.30±0.10 | 4.11±0.16 | 0.002 | 0.26±0.01 | 1.01±0.02 | <0.001 | 6.21 | 5.92 |
| **CA7** | 0.45±0.00 | 1.50±0.03 | <0.001 | 1.96±0.02 | 2.19±0.07 | 0.046 | 7.15 | 5.84 |
| **CA8** | 3.39±0.00 | 3.21±0.08 | 0.083 | 2.54±0.02 | 3.43±0.03 | 0.001 | 5.41 | 4.79 |
| **CA9** | 4.61±0.01 | 29.21±0.24 | <0.001 | 0.35±0.01 | 1.63±0.02 | <0.001 | 4.38 | 5.63 |
| **CA10** | 4.12±0.01 | 4.91±0.16 | 0.019 | 1.57±0.00 | 2.54±0.03 | <0.001 | 6.66 | 6.87 |
| **CA12** | 1.02±0.01 | 2.78±0.08 | 0.001 | 1.87±0.05 | 2.09±0.04 | 0.045 | 7.47 | 7.25 |
| **CA13** | 1.36±0.01 | 5.43±0.08 | <0.001 | 1.34±0.01 | 2.20±0.03 | 0.001 | 6.65 | 5.99 |
| **CA14** | 1.74±0.02 | 0.06±0.01 | <0.001 | 0.77±0.01 | 1.74±0.03 | <0.001 | 6.37 | 6.10 |
| **CA15** | 0.26±0.00 | 0.55±0.01 | 0.001 | 0.28±0.03 | 1.08±0.03 | 0.001 | 6.10 | 5.55 |
| **CA16** | 2.76±0.16 | 0.12±0.01 | 0.002 | 2.40±0.02 | 2.47±0.01 | 0.040 | 5.72 | 5.72 |
| **CA17** | 1.38±0.00 | 1.48±0.12 | <0.001 | 1.78±0.04 | 7.00±0.09 | 0.001 | 5.53 | 5.57 |
| **CA18** | 9.62±0.14 | 44.28±0.44 | <0.001 | 2.00±0.00 | 2.94±0.01 | <0.001 | 5.69 | 4.38 |
| **CA19** | 0.80±0.02 | 1.74±0.01 | <0.001 | 0.84±0.01 | 2.79±0.05 | <0.001 | 5.69 | 7.06 |
| **CA20** | 4.85±0.02 | 9.55±0.34 | 0.003 | 1.83±0.00 | 2.54±0.08 | 0.007 | 6.62 | 6.64 |
| **CA22** | 0.94±0.01 | 1.32±0.04 | 0.006 | 2.06±0.04 | 2.68±0.07 | 0.007 | 7.22 | 6.57 |

**Table 7.2:** *(continued)*

| Chocolate/ Dried fruit Samples | HMF (mg kg$^{-1}$) | | *p* | Furfural (mg kg$^{-1}$) | | *p* | pH | |
|---|---|---|---|---|---|---|---|---|
| | B1 | B2 | | B1 | B2 | | B1 | B2 |
| **BR6** | 0.76±0.02 | 13.09±0.56 | 0.001 | 55.86±0.09 | 71.02±0.47 | 0.001 | 5.62 | 5.60 |
| **BI1** | n.d. | 2.10±0.000 | <0.001 | 68.33±0.03 | 20.76±0.04 | <0.001 | 5.30 | 4.20 |
| **BI2** | n.d. | 12.36±0.03 | <0.001 | 56.94±1.02 | 51.16±0.07 | 0.016 | 5.15 | 3.60 |
| **BI4** | 6.30±0.04 | 12.39±0.04 | <0.001 | 2.50±0.21 | 6.28±0.24 | 0.004 | 6.91 | 7.15 |
| **BI7** | 6.72±0.14 | 26.06±0.85 | 0.001 | 2.64±0.02 | 3.87±0.02 | <0.001 | 5.51 | 6.37 |
| **CA11** | 0.30±0.00 | 2.11±0.11 | 0.002 | 0.34±0.00 | 1.09±0.01 | <0.001 | 6.07 | 6.33 |
| **CA21** | 0.52±0.04 | 12.44±0.35 | <0.001 | 94.22±3.06 | 116.8±0.30 | 0.009 | 7.18 | 7.09 |

n.d.: not detected. Significant differences $p < 0.05$

The dough formulation, namely, type of cereal and sugar, acidic regulators, antioxidants, chemical leavenings, chocolate and dried fruits (Table 7.1) can influence HMF and furfural content. Most samples were produced only from wheat. The exceptions were CA18 with a mixture of wheat and sesame, CA19 that contained rice, BR2 presented a mixture of wheat, sunflower, linseed, maize, soybean and rice and BR3 composed by a mixture of soy and wheat. All of these samples (except CA19) showed higher amounts of HMF when compared with samples made from wheat. According to the literature (Capuano *et al.*, 2009), rye model systems produced more HMF at all temperature tested. In our study, samples with mixtures of wheat and other cereal-types are the ones that showed higher HMF content. The most common added sugars were sucrose and glucose-fructose syrup. CA18 contained maltitol, a sugar substitute. Although, BI8, BR7, BR8 and BR9 did not contain additional sugars in their formulation, their HMF and furfural content was similar to other samples of the same type.

Regarding chemical leavenings, antioxidants like ascorbic acid and acidity regulators such as citric acid, tartaric acid, lactic acid, calcium carbonate and calcium hydrogenophosphate can influence the final pH of dough, and the formation of HMF and furfural (Mesías-García *et al.*, 2010; Zang et al 2012b). In general, bread presented the lowest pH values, while biscuits presented the highest figures. Bread samples were the only ones containing $Ca^{2+}$ ions acidity regulators that can contribute to the lower pH (Quarta & Anese, 2010) when comparing with the other samples. However, no correlation was found between pH and HMF/furfural content ($p$ >0.05).

Samples CA21 (3% chocolate), BI1 (17% chocolate), BI2 (24.9% chocolate), BI4 (16% chocolate) and BR6 (12% chocolate) contained chocolate in their formulation. Chocolate contain furanic compounds due to the beans manufacture (Akkarachaneeyakorn *et al.*, 2010; Murkovic & Bornik, 2007), thus, it is expected higher HMF and furfural content in these samples. HMF content ranged from not detectable to 13.09 ± 0.56 mg $kg^{-1}$fw, which is within the levels found for other analysed samples, however, furfural content ranged from 20.76 ± 0.04 mg $kg^{-1}$fw to 116.8 ± 0.30 mg $kg^{-1}$fw, except for BI4 whose lower furfural levels can be related with the fact that chocolate is placed only in the cookie centre protected from intense baking temperature. The other samples contained chocolate in the whole dough and presented higher furfural levels due to the high temperatures achieved on the surface during baking that favour *Maillard* reactions. Teixidó *et al.*, 2011 analysed different chocolate samples only for the presence of HMF, accounting with amounts ranging from 42.1 to 164.7 mg $kg^{-1}$. Kroh (1994) demonstrated that at pyrolysis temperature of glucose (300 ºC), HMF is degraded into furfural by decarboxylation, thus this can be a hypothesis for the higher furfural content in the chocolate samples.

Consequently, it is important to analyse not only HMF but also furfural in bakery products, especially those containing chocolate.

In 2006, Murkovic & Pichler showed that dried fruits presented a high HMF content, pointing out the importance of evaluating this type of products. CA11 and BI7 were the only samples that contain dried fruits in their formulation, HMF and furfural amounts were similar to samples without dried fruits.

### *7.3.3. Effect of product type on HMF and furfural content of bread, biscuits and cakes/pastry*

Analysis of variance was performed on data of HMF and furfural in bread, biscuits and cakes/pastry. Samples that contained chocolate or dried fruits were excluded. HMF in bread samples (n= 18) ranged from 0.66 to 18.34 mg $kg^{-1}$fw and furfural amounts were between 1.47 and 18.19 mg $kg^{-1}$fw. Ramírez-Jiménez *et al.*, 2000a, evaluated several types of bread samples (white, toast and snacks), obtaining for white bread HMF values from 0.6 to 2.2 mg $kg^{-1}$ in the crumb, and values between 18.3 and 176.1 mg $kg^{-1}$ in the crust. Although results were on the same order of magnitude, no information was given for content on whole bread samples.

The HMF content in biscuits (n= 16) ranged from 1.65 to 82.78 mg $kg^{-1}$fw and the furfural content were between not detectable and 10.49 mg $kg^{-1}$fw. The values of HMF of our study are in the same range of those obtained by Ait-Ameur *et al.,* (2006) (from 0.5 to 74.6 mg $kg^{-1}$) and by Delgado-Andrade *et al.*, (2009) (from 3.1 to 182.5 mg $kg^{-1}$) in commercial cookies. However, Ait-Ameur *et al.,* (2008) obtained higher levels of HMF (2000 mg $kg^{-1}$) and furfural (200 mg $kg^{-1}$) in model cookies. These results point out the overestimation of HMF and furfural in model systems and the necessity of analysing furfural content in real biscuit samples.

The HMF content in cake/pastry samples (n= 40) ranged from 0.06 to 44.28 mg $kg^{-1}$fw and furfural levels were between 0.26 and 7.00 mg $kg^{-1}$fw. Johnson *et al.,* (1989) found higher HMF levels (between 19.30 and 301.40 mg $kg^{-1}$fw) in the crust of cake samples. However, these results do not represent the whole cake and could give overestimate HMF values, because crust reaches higher temperatures than crumb, giving origin to higher HMF amounts (Ait-Ameur *et al.*, 2006). No studies were found of furfural content of commercial cakes/pastry, but the values found in our study justify its monitoring.

The effect of type of sample was statistically significant for HMF ($p$= 0.008) and furfural ($p$ <0.001), with $F$ values of 5.140 and 14.181, respectively. Cake/pastry samples presented

the lowest HMF content (average of 2.98 mg $kg^{-1}$fw) while biscuits showed the highest content (average of 7.84 mg $kg^{-1}$fw). These results could be related to the nutritional composition of the samples (Table 7.3).

**Table 7.3:** Summary of the energy, protein, carbohydrate, fat, fibre and moisture contents in 100 g of commercial bread, biscuits, cakes/pastry (without chocolate) and as indicated by the manufacturers.

| | | Energy (kcal) | Protein (g) | Carbohydrate (g) | Fat (g) | Fibre (g) | Moisture (g) |
|---|---|---|---|---|---|---|---|
| **Bread samples (n=18)** | **Mean** | 291.7 | 8.722 | 52.07 | 6.222 | 3.267 | 31.04 |
| | **Standard Deviation** | 70.33 | 1.698 | 7.780 | 7.114 | 1.685 | 7.529 |
| | **Minimum** | 221.0 | 7.600 | 39.00 | 1.900 | 1.800 | 20.20 |
| | **Maximum** | 418.0 | 13.00 | 62.80 | 20.00 | 7.400 | 39.80 |
| **Biscuit samples (n=16)** | **Mean** | 450.2 | 6.190 | 69.41 | 15.06 | 2.090 | 5.243 |
| | **Standard Deviation** | 53.65 | 2.845 | 8.439 | 8.298 | 1.127 | 5.521 |
| | **Minimum** | 382.0 | 3.000 | 59.00 | 3.100 | 0.600 | 1.600 |
| | **Maximum** | 515.0 | 13.20 | 87.00 | 26.60 | 4.300 | 17.90 |
| **Cake samples (n=40)** | **Mean** | 376.9 | 5.406 | 53.45 | 16.65 | 2.636 | 22.07 |
| | **Standard Deviation** | 62.83 | 1.824 | 8.523 | 8.363 | 3.178 | 8.096 |
| | **Minimum** | 251.0 | 2.800 | 40.29 | 3.200 | 0.600 | 8.300 |
| | **Maximum** | 484.0 | 8.430 | 75.80 | 32.00 | 13.81 | 42.50 |

Biscuits presented the highest carbohydrate and most of them contain glucose-fructose syrup, the main precursors of furanic compounds (Ait-Ameur *et al.*, 2007). In addition, biscuits showed the lowest moisture content, which could also explained their higher HMF content, as previously has been indicated (Smith *et al.* 2004). Cakes/pastry and biscuits presented similar fat levels. Thus, the lipid content in bakery products would not influence on HMF levels, which is in concordance with Gökhan & Gökmen (2010), who indicated that inclusion of oils in dough formulation is not responsible for a significant occurrence of HMF. Regarding furfural, cake/pastry and biscuits showed lower content (average of 1.86 and 3.00 mg $kg^{-1}$fw, respectively) than bread samples (5.27 mg $kg^{-1}$fw), which can be related to the protein and moisture levels, being higher in bread than in cake/pastry and biscuit samples (Table 7.3), since amino acids are reactive groups in the *Maillard* reactions (Purlis, 2010).

A PCA was performed using as variables nutritional parameters and furanic compounds of bread, biscuits and cake samples (Figure 7.1).

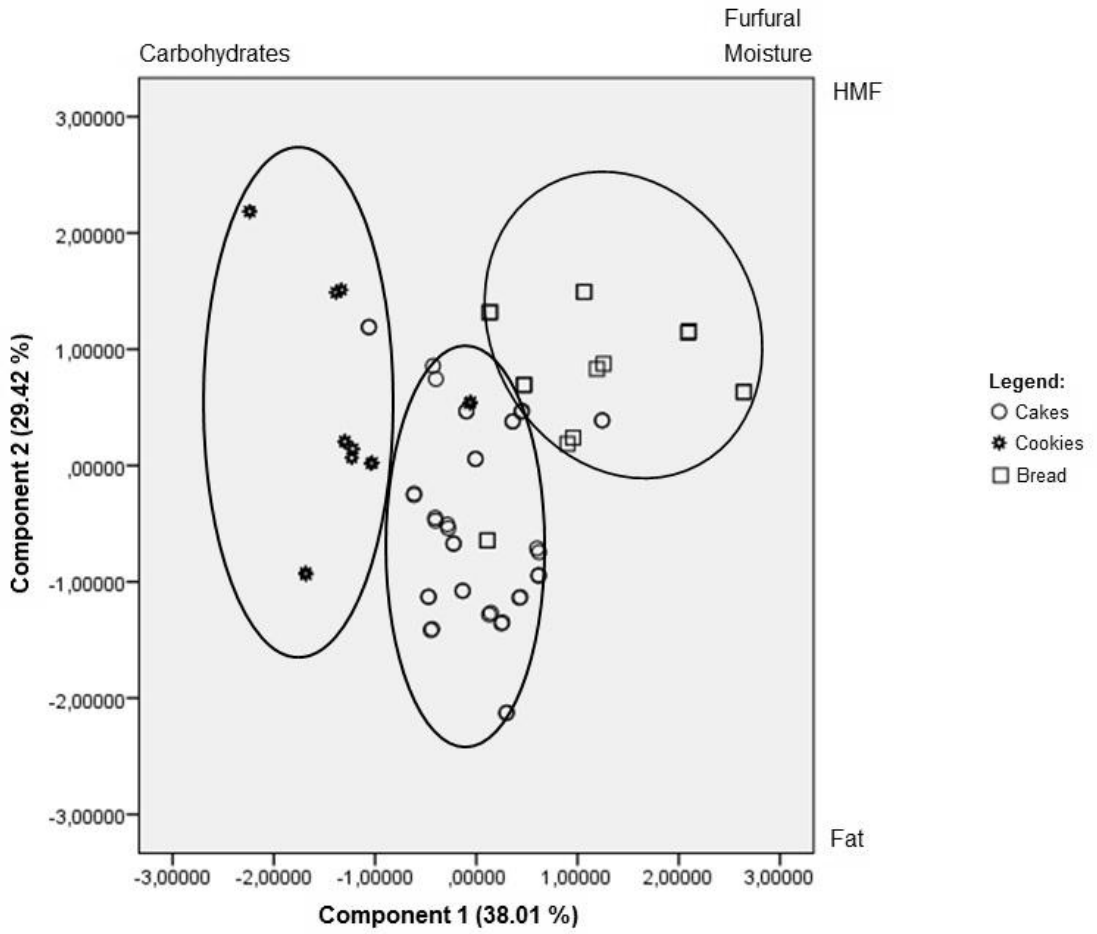



**Figure 7.1:** PCA using nutritional parameters (carbohydrates, fat and moisture) and furanic compounds (HMF and furfural) of bread, biscuits, cakes/pastry, and samples (n= 74)

The Component 1 justified 38.01 % of total variance of results and separate biscuits, cake/pastry, and bread samples. Furfural and moisture were positively correlated with this component showing an increase from biscuits to bread samples, whereas carbohydrates were negatively correlated with this component. Component 2 justified 29.42 % of total variance of results and is related positively with HMF and negatively with fat contents. This component is related with variation of sample composition within each type of bakery product. Pearson's correlation showed a highly significant negative correlation between HMF and moisture ($p$= -0.224), indicating that HMF increased when the moisture decreased.

## 7.4. Conclusions

An efficient extraction procedure was applied and enabled reliable quantification of HMF and furfural. The high variability of HMF and furfural between samples and batches points out that the dough formulations as well as the different baking processes are of major concern. Regarding pH values, it was not possible to obtain a correlation with HMF and furfural content. However, it was possible to conclude that HMF and furfural content was influenced by the type of product. Biscuit samples presented low moisture, higher sugar content and higher HMF content while bread samples showed higher moisture, higher protein content and higher furfural amounts. Moreover, from all analysed samples the ones that showed higher amounts of furfural were samples containing chocolate in their formulation, especially if the chocolate is on the surface. Thus, the control of certain ingredients in bakery products is of major relevance. The results of the present study contribute to the challenge of developing a reliable database for HMF and furfural contents in bakery products, and highlight the importance of lowering furfural content in highly consumed bakery products, and re-evaluation of dietary exposure.

# PART V

# CHAPTER 8

## Conclusions and Future Prospects

## 8.1 Conclusions

The work described in this thesis was conducted to understand the composition of furanic compounds in food products that are described as potential contributors to daily intake, and search for mitigation strategies that will reduce its content maintaining overall product characteristics. The matrices under study were coffee, coated deep-fried and bakery products. Optimisation and validation of reliable extraction procedures and chromatographic methodologies for screening and quantification of volatile and less volatile furanic compounds in the selected matrices were required to perform this study. Analyses were always performed by simulating usual preparation and consumption for each product. The main conclusions of the work presented in this thesis are organised according the specific goals established.

Concerning the optimisation of SPME sampling parameters for GC-MS analysis of volatile compounds, especially furan and furanic compounds in coffee, the first part of the work was the selection of most appropriate conditions for screening of coffee volatiles. The performance of three fibres was tested. CAR/PDMS fibre was chosen due to its higher extraction efficiency of coffee volatile compounds when compared with PDMS and PA fibres. HS-SPME-GC-MS analyses of ground coffee were performed using 1.5 g of sample, whereas analyses of *espresso* samples were carried out using 5 mL of sample prepared directly to 15 mL vials containing 1.5 g of NaCl. These sample preparation procedures used the sum of volatiles GC peak area (TIC) in the same order of magnitude and reproducible results were obtained in both cases. Volatiles were clustered in the following chemical classes: furans, pyrroles, pyridines, pyrazines, ketones, hydrocarbons, aldehydes and expressed as relative percentage of total peak area. Furans constituted the most abundant chemical family in ground and *espresso Arabica* coffee, representing between 30 and 50% of relative percentage of total peak area. Afterwards, this methodology was validated for simultaneous quantification of major furanic compounds and furan in different types of *espresso* coffees prepared from hermetically closed capsules. SIM of furan, furfural, furfuryl alcohol, 5-methylfurfural and furfuryl acetate was used for quantification purposes. $d_4$-Furan was applied as internal standard (IS). Calibration curves were performed by headspace analyses of a blank matrix spiked with the analytes under study and IS in water, since roasted coffee free of furanic compounds is not available. Furthermore, the same extraction conditions applied to *espresso* coffee samples were used. High sensitivity was achieved with low LOD and LOQ. The parameters of the calibration curves (slope and intercept) and their respective errors indicated good linearity of the method. The accuracy of the method was evaluated by

recovery studies performed in *espresso* coffee. Recovery percentages for the compounds under study ranged between 97.98 % and 101.9 %. Run-to-run and day-to-day precision was lower than 5 and 11%, respectively.

A HS-SPME-GC-MS methodology for quantification of furan, furfural, furfuryl alcohol, 2-pentylfuran, and a HPLC-DAD methodology for quantification of HMF in coated deep-fried samples were validated. Samples were processed and handled as usually consumed and simulating the eating process. Parameters affecting the efficiency of extraction conditions of both procedures were selected by RSM, using a $2^3$ full-factorial CCD. Consistency between predicted and experimented values was observed for the two methods and quality parameters of each method indicated good linearity and low LOD and LOQ. The quantification of volatile furanic compounds by HS-SPME-GC-MS was carried out by external calibration curve and by standard addition method. In general, no significant differences were observed between the results obtained by the two methods; however, lower standard deviation was found using external calibration method. Run-to-run and day-to-day precision depended on the compound but it was lower than 11 and 13%, respectively. Standard addition method was preferred for the analyses of HMF by HPLC-DAD due to matrix effect phenomenon. Adequate precision was achieved, RSD <5% for run-to-run and <16% for day-to-day. Percentage of recovery of HMF was 94 %.

Concerning model cakes and different types of bakery products, analyses of furanic compounds included mainly quantification of HMF and furfural. Screening of volatile compounds by HS-SPME-GC-MS in model cakes discouraged further analyses of volatile furanic compounds in commercial bakery products due to its negligible content. This is not surprising since bakery products are generally consumed after variable periods of air exposition that contribute to loss of highly volatile furanic compounds. Consequently, for bakery products, the analyses of less volatile furanic compounds turned out to be more valuable. An extraction procedure was validated to perform reliable quantification of HMF and furfural, since these two compounds are considered chemical markers of severe heat treatment or inadequate storage conditions. Three methodologies described in the literature for other food matrices were tested. Modifications of the most efficient procedure for extraction of HMF and furfural were carried out. Higher extraction yield was obtained using water:methanol (70:30, v/v) and clarification with *Carrez* I and II reagents. Regarding to HPLC-DAD methodology good performance of the analytical method was obtained with good linearity, low LOD and LOQ values, as well as an excellent precision. Run-to-run and day-to-day precision was lower than 5 and 8%, respectively for both compounds. The recovery values ranged between 94.9% and 98.9% for furfural and 98.5% and 100.5% for HMF.

Assessment of furans and other volatile compounds profile in ground *Arabica* coffee roasted at different speeds and their respective *espresso* brews were evaluated by HS-SPME-GC-MS. Three different blends of *Arabica* coffee were roasted separately at slow, medium and fast speed, in an industrial semifluidized bed roaster and a medium roasting degree according to Roast Colour Classification System was always obtained. Analyses of ground samples and respective *espresso* beverages indicated that the general profile of volatile compounds was similar, with furans being the major class. However, quantitative differences were observed. Multivariate statistic treatment of the results indicated that relative percentages of furans and pyrroles were higher in *espresso* samples, whereas relative percentages of pyrazines and ketones were higher in ground samples. The roasting speed did not influenced total relative percentage of furans class in ground and *espresso* samples; however, furfural and 5-methylfurfural increased with the roasting speed indicating the importance of the roasting parameters for reduction of specific furanic compounds in *espresso* coffee.

Nowadays, the consumption of *espresso* coffee from hermetically closed capsules is increasing, due to the spread of *espresso* coffee machines of easy access, which enables this particular coffee brew consumption at lower price and with higher appreciated organoleptic characteristics. Different types of hermetically closed coffee capsules are commercially available, including 100 % *Coffee Arabica*, blends of *C. Arabica* and *C. Robusta* subjected to different roast degrees and a series of artificially aromatised coffees. Thus, quantification of furan, furfural, furfuryl alcohol, 5-methylfurfural and furfuryl acetate in the commercially available types of *espresso* coffees from hermetically closed capsules were considered of major relevance in order to realise the contribution of these products on daily intake. Quantitative differences were observed in content of furanic compounds of non-aromatised and artificially aromatised samples. Major furanic compound in Coffee *Arabica* and blends of *C. Arabica* and *C. Robusta* were, in decreasing order, furfural (35.1 – 70.2 μg $mL^{-1}$), followed by furfuryl alcohol (31.2 – 57.7 μg $mL^{-1}$), 5-methylfurfural (23.8 – 44.7 μg $mL^{-1}$), furfuryl acetate (13.3 – 25.8 μg $mL^{-1}$) and furan (0.22 – 0.24 μg $mL^{-1}$). In artificially aromatised samples the major furanic compound was furfuryl alcohol (48.8 – 69.9 μg $mL^{-1}$), followed by furfural (38.7 – 44.6 μg $mL^{-1}$), 5-methylfurfural (21.4 – 24.8 μg $mL^{-1}$), furfuryl acetate (17.1 – 17.9 μg $mL^{-1}$) and furan (0.23 μg $mL^{-1}$). The furan content is in accordance with literature data concerning *espresso* coffee composition, however significantly higher contents of other furanic compounds were observed, though it should be highlighted that no studies were found for quantification of these compounds in coffee.

Coated deep-fried products represent a susceptible food matrix for formation of furanic compounds due to the high temperatures that the bread coat suffers during frying. The appreciated consumption of such products, immediately after cooking, can also contribute to high furan intake, although they can also be consumed cool or reheated in microwave. The content of furan, furfural, furfuryl alcohol and 2-penthylfuran in coated deep-fried products was 5.59±1.43, 0.27±0.07, 10.48±1.18, 1.77±0.12 μg $g^{-1}$. It should be highlighted that the furan levels found were in the range considered by EFSA for other products, such as coffee, as presenting high furan content. Thus, coated deep-fried products should also be included in the list. Additionally, cooking and handling conditions can exert an important effect on volatile and furanic compounds contents in coated deep-fried products; thus, the impact of different parameters was pertinently evaluated.

The furanic compounds content was evaluated under different conditions: coated deep-frying fish at different combinations of time and temperature; use of different oils and baking in dry-oven, followed by microwave heating, after a certain period of time. Deep-frying in sunflower oil showed the highest furan content (5.51 μg $g^{-1}$ sample), when compared with olive oil deep-frying or dry-oven baking, although differences were not significant for the latter. Concerning other furanic compounds, the highest levels were found in deep-frying with olive oil products (with a total content of 30.36 μg $g^{-1}$ sample), and the minor content was found for baking in the electric oven (with a total content of 9.99 μg $g^{-1}$ sample) and reheating in microwave after deep-frying in sunflower oil (with a total content of 8.15 μg $g^{-1}$ sample). Total furanic compounds levels significantly raised as both time and temperature increased, and this trend is also followed by the individual compounds, except for 2-pentylfuran, which was not influenced by the cooking temperature. Thus, the highest value of furanic compounds was found when samples were deep-fried at 200 ºC during 6 min (38.83 μg $g^{-1}$ sample) and the lowest value at 160 ºC for 2 min (10.77 μg $g^{-1}$ sample). No studies were found for quantification of furanic compounds in coated deep-fried products, thus comparison with literature is not possible.

Furfural and HMF are known as food quality markers, generated as a result of excessive heating in a wide range of carbohydrate-rich foods. Thus, understanding the effect of baking method and time conditions on HMF and furfural content of model cakes was of major relevance to search for mitigation strategies. The quantity of HMF and furfural increased significantly with baking time when using microwave and convection oven but not with steam oven. No significant differences were observed on HMF and furfural content of cakes baked in steam oven during 20, 40 and 60 min. Model cakes from steam oven contained a significantly lower HMF content, even when baked during long periods.

Baking in microwave or in convection ovens during short periods led to the formation of low amounts of HMF and furfural.

Commercial bakery products, such as bread, biscuits and cakes/pastry are widely consumed. Thus, a survey on the presence of HMF and furfural in the former of products available was conduted to investigate the effect of type of bakery product and the amounts of these compounds. Moreover, their content was compared with the information provided by the manufacturer concerning product formulation. It was possible to identify products that can contribute to a high daily intake of HMF and furfural. High variability was observed regarding HMF and furfural content in the different samples analysed and within two batches of the same products, which points out that dough formulations as well as the different baking processes have great influence on their daily intake. Biscuits showed the highest HMF content (average of 7.84 mg $kg^{-1}$ fw) and cake/pastry provided the lowest HMF content (average of 2.97 mg $kg^{-1}$ fw). Regarding furfural, bread samples presented the highest content (5.27 mg $kg^{-1}$ fw), while cake/pastry and biscuits showed lower amount (average of 1.855 and 3.000 mg $kg^{-1}$ fw, respectively). These results are probably related with the nutritional composition of samples. Biscuit samples presented the highest HMF and sugar content and the lowest moisture degree, while bread samples showed higher furfural, moisture and protein amounts. From all analysed samples, the ones containing chocolate in their formulation showed higher furfural levels, especially when chocolate was on the product surface and consequently exposed to high temperatures during baking. Thus, the results of the present study may contribute to the challenge of developing a reliable database for HMF and furfural contents in bakery products, highlighting the importance of reducing these furanic compounds content in highly consumed bakery products such as bread, and for a re-evaluation of dietary exposure.

The pursue of cooking/baking mitigation strategies to reduce furanic compounds content in homemade processed products, such as coated deep-fried and baked products, is of great relevance for lowering the intake of furanic compounds. However, keeping the overall characteristics that are appreciated by consumers is also important. Consequently, experiments related with the search of mitigation strategies for the reduction of furanic compounds included the evaluation of their impact on volatile profile, analysed by HS-SMPE-GC/MS, which includes analyses of aldehydes, alcohols, pyrazines and pyridines, compounds of major relevance on organoleptic characteristics.

Concerning coated deep-fried products, as aforementioned, the generation of furanic compounds can be minimized by adjusting the cooking method and conditions, such as using an electric oven or deep-frying in sunflower oil at 160 ºC during 4 min, or waiting 10

min after cooking. However, though these conditions showed a furanic compounds level reduction, they also diminished the content of volatile compounds related to the aroma and flavour of fried food, which can influence consumers' acceptability.

The baking method influenced the volatile profile of model cakes. Higher relative percentages were found for aldehydes, hydrocarbons and pyrazines in cakes baked in convection and steam oven in comparison to microwaved samples. Baking in microwave during short periods led to the formation of low amounts of HMF and furfural. Nevertheless, once these cakes revealed a poor volatile profile concerning aldehydes, hydrocarbons and pyrazines, these characteristics can be relevant for low consumers' satisfactoriness. Cakes baked in steam oven contained significantly lower HMF content, even when baked during long periods. Thus, it can be concluded that baking in steam oven could be an appropriate mitigation strategy, without modifying the profile of aroma and flavour compounds.

## 8.2 Future Prospects

Heat processing is one of the most used food processes, giving origin to diverse chemical reactions and led to modifications on the initial raw ingredients, leading to harmful products like furanic compounds, whose study is of major importance. Furan is the only furanic compound that has been reported as possible carcinogenic compound (classified into 2B group, by IARC). Nevertheless, HMF and furfural are present in higher quantities, of several orders of magnitude, than furan. *In vitro* and *in vivo* studies should be performed in order to verify the real impact of furanic compounds and/or other related compounds in human health.

In protein-rich diets, the perceived benefits of processing are often unrelated to nutritional concerns. However, the traditional processing methods result in protein denaturation, changing their conformation and consequently revealing reactive groups not available when the protein remains in its native fold. These exposed groups can react between them as well as with other food components, such as sugars and vitamins. The literature data on the chemical modification of amino acids during food processing is dominated by reactions of lysine. Despite being lysine an essential amino acid, as well as the first nutritional limited one, it is also the most chemically reactive, with its ε-amino group particularly vulnerable to damage. As a result, it is important to understand the role of furanic compounds present in heat-processed foods with proteins and the essential amino acid lysine, the blockage and/or the availability of these important food nutrients and possible neoformed compounds.

# PART VI

# CHAPTER 9

## References

# 9. References

Abdullah MZ. Quality evaluation of bakery products. In: Sun D-W. Editors. Computer Vision Technology for Food Quality Evaluation. Massachusetts: Academic Press, Burlington; 2008. p. 481–522.

Abraham K, Gürtler R, Berg K, Heinemeyer G, Lampen A, Appel KE. Toxicology and risk assessment of 5-Hydroxymethylfurfural in food. Mol Nutr Food Res 2011 May; 55 (5): 667–78.

Adams TB, Doull J, Goodman JI, Munro IC, Newberne P, Portoghese PS, *et al.* The FEMA GRAS assessment of furfural used as a flavour ingredient. Food Chem Toxicol 1997 Aug; 35 (8): 739–51.

Adahchour M, Van Stee LLP, Beens J, Vreuls RJJ, Batenburg MA, Brinkman UA Th. Comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometric detection for the trace analysis of flavour compounds in food. J Chromatogr A 2003 Nov; 1019 (1-2): 157-72.

Agila A, Barringer S. Effect of Roasting Conditions on Color and Volatile Profile Including HMF Level in Sweet Almonds (Prunus dulcis). J Food Sci 2012 Mar; 77 (4): C461–68.

Ahrné L, Andersson CG, Floberg P, Rosén J, Lingnert H. Effect of crust temperature and water content on acrylamide formation during baking of white bread: steam and falling temperature baking. LWT - Food Sci Technol 2007 40 Dec; (10): 1708–15.

Aida TM, Sato Y, Watanabe M, Tajima K, Nonaka T, Hattori H, *et al.* Dehydration of d-glucose in high temperature water at pressures up to 80 MPa. J Supercrit Fluid 2007 Apr; 40 (3): 381–88.

Ait-Ameur L, Trystam G, Birlouez-Aragon I. Accumulation of 5-HMF in cookies during the baking process: validation of an extraction method. Food Chem 2006; 98 (4): 790–96.

Ait-Ameur L, Mathieu O, Lalanne V, Trystram G, Birlouez-Aragon I. Comparison of the effects of sucrose and hexose on furfural formation and browning in cookies baked at different temperatures. Food Chem 2007; 101 (4): 1424-33.

Ait-Ameur L, Rega B, Giampaoli P, Trystram G, Aragon-Birlouez I. The fate of furfurals and other volatile markers during the baking process of a model cookie. Food Chem 2008 Dec; 111 (3): 758–63.

Akiyama M, Murakami K, Hirano Y, Ikeda M, Iwatsuki K, Wada A, *et al.* Characterization of Headspace Aroma Compounds of Freshly Brewed Arabica Coffees and Studies on a Characteristic Aroma Compound of Ethiopian Coffee. J Food Sci 2008 Jun: 73 (5): C335-46.

Akkarachaneeyakorn S, Laguerre JC, Tattiyakul J, Neugnot B, Boivin P, Morales FJ, *et al.* Optimization of combined microwave-hot air roasting of malt based on energy consumption and neo-formed contaminants content. J Food Sci 2010 May; 75 (4): E201-7.

Akpinar K, Karakas B, Akça H, Certel M. Determination of HMF in roasted flour/oil mixtures and effect of solvent used in the extraction procedure. Food Chem 2011 Oct; 128 (3): 790-4.

Albalá-Hurtado S, Veciana-Nogués MT, Izquierdo-Pulido M, Vidal-Carou MC. Determination of Free and Total Furfural Compounds in Infant Milk Formulas by High-Performance Liquid Chromatography. J Agr Food Chem 1997 Jun; 45 (6): 2128–33.

Alcázar A, Jurado JM, Pablos F, González AG, Martín MJ. HPLC determination of 2-furaldehyde and 5-hydroxymethyl-2-furaldehyde in alcoholic beverages. Microchem J 2006 Jan; 82(1): 22–8.

Altaki M, Santos F, Galceran T. Analysis of furan in foods by headspace solid-phase microextraction–gas chromatography–ion trap mass spectrometry. J Chromatogr A 2007 Mar; 1146 (1): 103-9.

Altaki M, Santos F, Galceran T. Automated headspace solid-phase microextraction versus headspace for the analysis of furan in foods by gas chromatography–mass spectrometry. Talanta 2009 Jun; 78 (4-5): 1315-20.

Altaki M, Santos F, Galceran T. Occurrence of furan in coffee from Spanish market: Contribution of brewing and roasting. Food Chem 2011 Jun; 126 (4): 1527–32.

Anam OO, Dart RK. Influence of metal ions on hydroxymethylfurfural formation in honey. Anal Proc - Anal Commun 1995 Jan; 32 (12): 515-7.

Andrade CD, Seiquer I, Haro A, Castellano R, Navarro MP. Development of the *Maillard* reaction in foods cooked by different techniques. Intake of Maillard-derived compounds. Food Chem 2010 Sep; 122 (1): 145–53.

Andueza S, Maeztu L, Pascual L, Ibáñez C, Pena MP, Cid C. Influence of extraction temperature on the final quality of espresso coffee. J Sci Food Agric 2003 Feb; 83 (3): 240–48.

Andueza S, Vila MA, Pena MP, Cid C. Influence of coffee/water ratio on the final quality of espresso coffee. J Sci Food Agric 2007 87 Mar; (4): 586–92.

Anese M, Suman M. Mitigation strategies of furan and 5-hydroxymethylfurfural in food. Food Res Int 2013 Apr; 51 (1): 257-64.

Ansorena D, Gimeno O, Astiasaran I, Bello J. Analysis of volatile compounds by GC–MS of a dry fermented sausage: chorizo de Pamplona. Food Res Int 2001; 34 (1): 67–75.

Arisseto AP, Vicente E, Ueno MS, Tfouni SA, Toledo MC. Furan levels in coffee as influenced by species, roast degree, and brewing procedures. J Agric Food Chem 2011 Apr; 59 (7): 3118-24.

Arribas-Lorenzo G, Morales FJ. Estimation of dietary intake of 5-hydroxymethylfurfural and related substances from coffee to Spanish population. Food Chem Toxicol 2010 Feb; 48 (2): 644–49.

Arts JHE, Muijser H, Appel MJ, Kuper CF, Bessems JGM, Woutersen RA. Subacute (28-day) toxicity of furfural in Fischer 344 rats: A comparison of the oral and inhalation route. Food Chem Toxicol 2004 Sep; 42 (9): 1389–99.

Baltes W, Bochmann G. Model reactions on roast aroma formation - II. Mass spectrometric identification of furans and furanones from the reaction of serine and threonine with sucrose under the conditions of coffee roasting. Z Lebensm Unters For 1987 Mar; 184 (3): 179–86.

Bastida S, Sánchez-Muniz FJ. Thermal oxidation of olive oil, sunflower oil and a mix of both oils during forty discontinuous domestic fryings of different foods. Food Sci Tech Int 2001 Feb; 7: 15-23.

Becalski A, Hayward S, Krakalovich T, Pelletier L, Roscoe V, Vavasour E. Development of an analytical method and survey of foods for furan, 2-methylfuran and 3-methylfuran with estimated exposure. Food Addit Cont A 2010 Jun; 27 (6): 764-75.

Becalski A, Seaman S. Furan precursors in food: a model study and development of a simple headspace method for determination of furan. J AOAC Int 2005 Jan-Feb; 88 (1): 102–6.

Belitz H D, Grosch W. Química de los Alimentos. 2nd ed. Zaragoza: Acribia; 1997.

Berg HE, Van Boekel MAJS. Degradation of lactose during heating of milk. I. Reaction pathways. Netherlands Milk and Dairy Journal. 1994; 48: 157-75.

Bicchi C, Ruosi M, Cagliero C, Cordero C, Liberto E, Rubiolo P, *et al.* Quantitative analysis of volatiles from solid matrices of vegetable origin by high concentration capacity headspace techniques: Determination of furan in roasted coffee. J Chromatogr A 2011 Feb; 1218 (6): 753-62.

Bianchi F, Careri M, Mangia A, Musci M. Development and validation of a solid phase microextraction- gas chromatography-mass spectrometry method for the determination of furan in baby-food. J Chromatogr A 2006 Jan; 1102 (1-2): 268–72.

Bråthen 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 Oct; 92 (4): 693–700.

Budavari S, O'Neil MJ, Smith A, Heckelman PE. The Merck Index. An Encyclopedia of Chemicals, Drugs and Biologicals. 30th ed. Merck & Co., Inc.: New Jersey; 2001.

Canas S, Belchoir AP, Spranger MI, Bruno-De-Sousa R. High-performance liquid chromatography method for analysis of phenolic acids, phenolic aldehydes, and furanic derivatives in brandies. Development and validation. J Sep Sci 2003 May; 26 (6-7): 496–502.

Capuano E, Ferrigno A, Acampa I, Ait-Ameur L, Fogliano V. Characterization of Maillard reaction in bread crisps. Eur Food Res Technol 2008 Dec; 228 (2): 311-9.

Capuano E, Ferrigno A, Acampa I, Serpen A, Açar ÖÇ, Gökmen 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 Nov; 42 (9): 1295-302.

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 May; 44 (4): 793-810.

Cárdenas-Ruiz J, Guerra-Hernandez E, García-Villanova B. Furosine is a useful indicator in pre-baked breads. J Sci Food Agr 2004 Mar; 84 (4); 366-70.

Cerny C, Grosch W. Evaluation of potent odorants in roasted beef by aroma extract dilution analysis. Z Lebensm Unters Forsch 1992 Apr; 194 (4): 322–5.

CEFS, (1998). Council of Europe, Committee of Experts on Flavouring Substances, RD 4.2/4-42, Detailed and publication datasheet on furfural, Brussels, 20-24 April 1998.

Chambel P, Oliveira MB, Andrade PB, Seabra RM, Ferreira MA. Development of an HPLC/Diode-Array Detector Method for Simultaneous Determination of 5-HMF, Furfural, 5-O-Caffeoylquinic Acid and Caffeine in Coffee. J Liq Chrom Relat Tech 1997; 20 (18): 2949-57.

Chauhan B, Gupta R. Application of statistical experimental design for optimization of alkaline protease production from Bacillus sp RGR-14. Process Biochem 2004 Oct; 39 (12): 2115–22.

Chávez-Servín JL, Castellote AI, López-Sabater MC. Analysis of potential and free furfural compounds in milk-based formulae by high-performance liquid chromatography: Evolution during storage. J Chromatogr A 2005 May; 1076 (1-2): 133-40.

Chávez-Servín JL, Castellote AI, López-Sabater MC. Evolution of potential and free furfural compounds in milk-based infant formula during storage. Food Res Int 2006 Jun; 39 (5): 536–43.

Chevallier S, Della Valle G, Colonna P, Broyart B, Trystram G. Structural and chemical modifications of short dough during baking. J Cereal Sci 2002; 35 (1): 1 – 10.

Ciesarová Z, Kukurová K, Bednáriková A, Morales FJ. Effect of heat treatment and dough formulation on the formation of Maillard reaction products in fine bakery products – benefits and weak points. J Food Nut Res 2009; 48 (1): 20-30.

Čížková H, Ševčík R, Rajchl A, Voldřich M. Nutritional quality of commercial fruit baby food. Czech J Food Sci 2009; 27 (Special issue): S134-7.

CNN (2000) http://archives.cnn.com/2000/FOOD/news/11/21/germany.bread.reut/ (accessed in 28/01/2013).

Cocchi M, Durante C, Lambertini P, Manzini S, Marchetti A, Sighinolfi S, *et al.* Evolution of 5-(hydroxymethyl)furfural and furfural in the production chain of the aged vinegar Aceto Balsamico Tradizionale di Modena. Food Chem 2011 Feb; 124 (3): 822–32.

Costa M, Viegas O, Melo A, Petisca C, Pinho O, Ferreira IMPLVO. Heterocyclic aromatic amine formation in barbecued sardines (sardina pilchardus) and atlantic salmon (salmo salar). J Agric Food Chem. 2009 Apr; 57(8): 3173-9.

Crews C, Castle L. A review of the occurrence, formation and analysis of furan in heat-processed foods. Trends Food Sci Tech 2007 Jul; 18 (7): 365–72.

Crews C, Roberts D, Lauryssen S, Kramer G. Survey of furan in foods and coffees from five European Union countries. Food Addit Contam B 2009 Nov; 2 (2): 95-8.

Davidek T, Davidek J. Chemistry of the Maillard Reactions in foods. In: Tomasik P. Chemical and Functional properties of food sacharides. US: CRC Press LLC; 2004. p. 291 – 314.

De La Iglesia F, Lázaro F, Puchades R, Maquieira A. Automatic determination of 5-hydroxymethylfurfural (5-HMF) by a flow injection method. Food Chem 1997 Oct; 60 (2): 245–50.

Debnath S, Bhatb KK, Rastogic NK. Effect of pre-drying on kinetics of moisture loss and oil uptake during deep fat frying of chickpea flour based snack food. Food Sci Technol-Leb 2003 Feb; 36 (1): 91–8.

Decareau RV. Microwave Foods: New Product Development. Connecticut: Food and Nutrition Press, Inc.; 1992.

Del Campo G, Berregi I, Caracena R, Zuriarrain J. Quantitative determination of caffeine, formic acid, trigonelline and 5-(hydroxymethyl)furfural in soluble coffees by 1H NMR spectrometry. Talanta 2010 Apr; 81 (1-2): 367–71.

Delgado-Andrade C, Rufián-Henares JA, Morales FJ. Hydroxymethylfurfural in commercial biscuits marketed in Spain. J Food Nutr Res 2009; 48 (1): 14–9.

Delgado-Andrade C, Seiquer I, Navarro MP, Morales FJ. Maillard reaction indicators in diets usually consumed by adolescent population. Mol Nutr Food Res 2007 Mar 51 (3): 341–51.

Delgado-Andrade C, Seiquer I, Navarro MP, Morales FJ. Estimation of hydroxymethylfurfural availability in breakfast cereals. Studies in Caco-2 cells. Food Chem Toxicol 2008 May; 46 (5): 1600–7.

Di Pede C, Viegi G, Taddeucci R, Landuccie C, Settmi L, Paggiaro PO. Biological monitoring of work exposure to furfural (abstract). Arch Environ Health 1991 Aug; 46 (2): 125–6.

Dobarganes C, Márquez-Ruiz G, Velasco J. Interactions between fat and food during deep-frying. Eur J Lipid Sci Technol 2000 Sep; 102 (8-9): 521–8.

Domingo JL. Influence o cooking processes on the concentrations of toxic metals and various organic environmental pollutants in food: a review of the published literature. Crit Rev Food Sci Nutr 2011 Jan; 51 (1): 29-37.

Doxastakis G, Papageorgiou M, Mandalou D, Irakli M, Papalamprou E, D'Agostina A, *et al.* Technological properties and non-enzymatic browning of white lupin protein enriched spaghetti. Food Chem 2007; 101 (1): 57–64.

Driffield M, Chan D, Macarthur R, MacDonald S, Brereton P, Wood R. Single laboratory validation of a method for the determination of hydroxymethylfurfural in honey by using solid-phase extraction cleanup and liquid chromatography. J AOAC Int 2005 Jan-Feb; 88 (1): 121-7.

Dunn RT, Klaassen CD. Tissue-specific expression of rat sulfotransferase messenger RNAs. Drug Metab Dispos 1998 Jun; 26 (6): 598–604.

Durmaz G, Gökmen V. Determination of 5-hydroxymethyl-2-furfural and 2-furfural in oils as indicators of heat pre-treatment. Food Chem 2010 Dec; 123 (3): 912–16.

Dutra S. Portugueses bebem café em casa: O ano das cápsulas. Distribuição Hoje. 2010 (http://www.distribuicaohoje.com/ResourcesUser/ICM_IFEDISTRIBUICAOHOJE_STG/Imagens/Mercados/Dossiers/DH_388_Out10_Dossier.pdf, accessed in 03/02/2013).

Echavarría AP, Pagán J, Ibarz A. Melanoidins Formed by Maillard Reaction in Food and Their Biological Activity. Food Eng Rev 2012 Dec; 4 (4): 203–23.

EFSA – European Food Safety Agency. Report of the Scientific Panel on Contaminants in the Food Chain on provisional findings on furan in food. EFSA J 2004a Dec; 137: 1 – 20.

EFSA - European Food Safety Agency. Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food on a request from the Commission related to Furfural and Furfural Diethylacetal. EFSA J 2004b Jun; 67: 1–27.

EFSA - European Food Safety Agency. Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) related to Flavouring Group Evaluation 13 (FGE.13); Furfuryl and furan derivatives with and without additional side-chain substituent's and heteroatoms from chemical group 14. 2005. In: EFSA Scientific Documents [online]. [accessed January 2013]. <http://www.efsa.europa.eu/en/scdocs/scdoc/215.htm>.

EFSA - European Food Safety Agency. Results on the monitoring of furan levels in food. EFSA Scientific Report 2009a Jun; 304: 1–23.

EFSA - European Food Safety Agency. Scientific Opinion of the Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food on a request from Commission on Flavouring Group Evaluation 60 (FGE.60). Consideration of furfuryl alcohol and related flavouring substances evaluated by JECFA (55th meeting) structurally related to Furfuryl and furan derivatives with and without additional side chain substituents and heteroatoms evaluated by EFSA in FGE.13 (2005). EFSA J 2009b; ON-752, 1-2.

EFSA - European Food Safety Agency. Furan in heat processed food products including home cooked food products and ready-to-eat products. Scientific report submitted to EFSA. EFSA-Q-2009-00846. 2009c Dec; 49 pp.

EFSA - European Food Safety Agency. Update of the monitoring of furan levels in food. EFSA Journal 2010 Jul; 8 (7): 1702.

EFSA - European Food Safety Agency. Update on furan levels in food from monitoring years 2004-2010 and exposure assessment. EFSA J 2011a Sep; 9 (9): 2347.

EFSA - European Food Safety Agency. Scientific Opinion on Flavouring Group Evaluation 218, Revision 1 (FGE.218Rev1): alpha,beta-Unsaturated aldehydes and precursors from subgroup 4.2 of FGE.19: Furfural derivatives. EFSA J 2011b Mar; 9 (3): 1840 (30 pp.).

Elmore SJ, Mottram DS, Enser M, Wood JD. Effect of the polyunsaturated fatty acid composition of beef muscle on the profile of aroma volatiles. J Agric Food Chem 1999 Apr; 47 (4): 1619-25.

Es-Safi N-E, Cheynier V, Moutounet M. Study of the reactions between (+)-catechin and furfural derivatives in the presence or absence of anthocyanins and their implication in food color change. J Agr Food Chem 2000 Nov; 48 (12): 5946-54.

Estevinho LM, Feás X, Seijas JA, Vázquez-Tato MP. Organic honey from Trás-Os-Montes region (Portugal): Chemical, palynological, microbiological and bioactive compounds characterization. Food Chem Toxicol 2012 Feb; 50 (2): 258–64.

Fallico B, Arena E, Zappalà M. Roasting of hazelnuts. Role of oil in colour development and hydroxymethylfurfural formation. Food Chem 2003 Jun; 81 (4): 569–73.

Fallico B, Zappalà M, Arena E, Verzera A. Effects of conditioning on HMF content in unifloral honeys. Food Chem 2004 Apr; 85 (2): 305–13.

Fan X, Huang L, Sokorai KJB. Factors affecting thermally induced furan formation. J Agr Food Chem 2008 Sep; 56 (20): 9490–4.

Fang G, Lv Y, Sheng W, Liu B, Wang X, Wang S. Development of an enzyme-linked immunosorbent assay for the determination of 5-hydroxymethyl-2-furfural in food. Anal Bioanal Chem 2012 Jan; 401 (10): 3367–73.

FDA – Food and Drug Adinistration Center for Food Safety & Applied Nutrition, Silver Spring, MD. Furan in foods-Thermal treatment (2004). Available in: http://www.fda.gov/Food/FoodSafety/FoodContaminantsAdulteration/ChemicalContaminants/Furan/ucm078469.htm (accessed in 18/01/2013)

FDA - Food Drug and Administration. Determination of furan in foods. (2006). Available in: http://www.fda.gov/Food/FoodSafety/FoodContaminantsAdulteration/ChemicalContaminants/Furan/ucm078400.htm (acccessed in 03/02/2013).

Flek J, Sedivic V. The absorption, metabolism and excretion of furfural in man. Int Arch Occup Environ Health 1978; 41 (3): 159–68.

Fernández-Artigas P, Guerra-Hernández E, García-Villanova B. Browning indicators in model systems and baby cereals. J Agr Food Chem 1999 May; 47 (7): 2872–8.

Ferreira IMPLVO, Pinho O, Sampaio P. Volatile fraction of DOP Castelo Branco cheese: Influence of breed. Food Chem 2009 Feb; 112 (4): 1053-9.

Ferrer E, Alegría A, Courtois G, Farré R. High-performance liquid chromatographic determination of Maillard compounds in store-brand and name-brand ultra-high-temperature-treated cows' milk. J Chromatogr A 2000 88 Jun; (1-2): 599-606.

Ferrer E, Alegria A, Farré R, Abellán P, Romero F. High-performance liquid chromatographic determination of furfural compounds in infant formulas: Changes during heat treatment and storage. J Chromatogr A 2002 Feb; 947 (1): 85–95.

Ferrer E, Alegría A, Farré R, Abellá P, Romero F. High-performance liquid chromatographic determination of furfural compounds in infant formulas during full shelf-life. Food Chem 2005 Mar; 89 (4): 639–45.

Fisher C, Scott TR. Food flavours: Biology and chemistry. Cambridge: The Royal Society of Chemistry; 1997.

Flament I. Coffee Flavor Chemistry. Great Britain: John Wiley & Sons Inc.; 2001.

Foo Wong Y, Makahleh A, Al Azzam KM, Yahaya N, Saad B, Amrah Sulaiman S. Micellar electrokinetic chromatography method for the simultaneous determination of furanic compounds in honey and vegetable oils. Talanta 2012 Aug; 97: 23-31.

Friedemann TE, Keegan PK, Witt NF. Determination of furan aldehydes. Reaction with aniline in acetic and hydrochloric acid solutions. Anal Biochem 1964 Jul; 8 (3): 300-11.

Garza S, Ibarz A, Pagán J, Giner J. Non-enzymatic browning in peach puree during heating. Food Res Int 1999 Jun; 32 (5): 335-43.

Gaspar EMSM, Lopes JF. Simple gas chromatographic method for furfural analysis. J Chromatogr A 2009 Abr; 1216 (14): 2762–7.

Gaspar EMSM, Lucena AFF. Improved HPLC methodology for food control - furfurals and patulin as markers of quality. Food Chem 2009 Jun; 114 (4): 1576–82.

Ghafoor K, Choi YH, Jeon JY, Jo IH. Optimization of Ultrasound-Assisted Extraction of Phenolic Compounds, Antioxidants, and Anthocyanins from Grape (Vitis vinifera) Seeds. J Agric Food Chem 2009 Apr; 57 (11): 4988–94.

Giri L, Osako K, Ohshima T. Identification and characterisation of headspace volatiles of fish miso, a Japanese fish meat based fermented paste, with special emphasis on effect of fish species and meat washing. Food Chem 2010 May; 120 (2): 621-31.

Giordano L, Calabrese R, Davoli E, Rotilio D. Quantitative analysis of 2-furfural and 5-methylfurfural in different Italian vinegars by headspace solid-phase microextraction coupled to gas chromatography-mass spectrometry using isotope dilution. J Chromatogr A 2003 Oct; 1017 (1-2): 141–9.

Gökhan D, Gökmen V. Determination of 5-hydroxymethyl-2-furfural and 2-furfural in oils as indicators of heat pre-treatment. Food Chem 2010 Dec; 123 (3): 912-6.

Gökmen V, Şenyuva HZ. Improved method for the determination of hydroxymethylfurfural in baby foods using liquid chromatography-mass spectrometry. J Agr Food Chem 2006 Mar; 54 (8): 2845–9.

Gökmen V, Acar J. Simultaneous determination of 5-hydroxymethylfurfural and patulin in apple juice by reversed-phase liquid chromatography. J Chromatogr A 1999 Jun; 847 (1-2): 69–74.

Gökmen V, Açar ÖC, Köksel H, Açar J. Effects of dough formula and baking conditions on acrylamide and hydroxymethylfurfural formation in cookies. Food Chem 2007; 104 (3): 1136-42.

Gökmen V, Açar ÖC, Serpen A, Morales FJ. Effect of leavening agents and sugars on the formation of hydroxymethylfurfural in cookies during baking. Eur Food Res Technol 2008 Mar; 226 (5): 1031-7.

Goldberg DM, Hoffman B, Yang J, Soleas GJ. Phenolic constituents, furans, and total antioxidant status of distilled spirits. J Agr Food Chem 1999 Sep; 47 (10): 3978–85.

Goldmann T, Périsset A, Scanlan F, Stadler RH. Rapid determination of furan in heated foodstuffs by isotope dilution solid phase micro-extraction-gas chromatography-mass spectrometry (SPME-GC-MS). Analyst 2005 Apr; 130 (6): 878–83.

Goldsworthy TL, Goodwin R, Burnett RM, King P, El-Sourady H, Moser G, Foley J, Maronpot RR. Dose–response relationships between furan induced cytotoxicity and liver cancer. Presented at Society of Toxicologic Pathology (STP) 2001. Annual Conference, Orlando, Florida. Available from: http://www.fda.gov/OHRMS/DOCKETS/ac/04/briefing/4045b2_08_STP%20abstract2.pdf/.

Gonzalez-Rios O, Suarez-Quiroza ML, Boulanger R, Barel M, Guyot B, Guiraud J-P, *et al.* Impact of ‘‘ecological’’ post-harvest processing on coffee aroma: II. Roasted coffee. J Food Comp Anal 2007 May; 20 (3-4): 297–307.

Guidurus M, Turtoi M, Boskou G, Nuculita P, Stan V. Nutritional and health aspects related to frying (I). Rom Biotech Lett 2010; 15 (6): 5675-82.

Hasnip C, Crews C, Castle L. Some factors affecting the formation of furan in heated foods. Food Addit and Contam 2006 Mar; 23 (3): 219–27.

Hegenbert S. Microwave quality: Coming of age. Food Prod Des 1992; 17: 29–52.

Ho IP, Yoo SJ, Tefera S. Determination of furan levels in coffee using automated solid-phase microextraction and gas chromatography/mass spectrometry. J AOAC Int 2005 Mar-Apr; 88 (2): 574–6.

Hodge JE. Dehydrated foods: Chemistry of browning reactions in model systems. J Agr Food Chem 1953 Oct; 1 (15): 928–43.

Hovell AMC, Pereira EJ, Arruda NP, Rezende CM. Evaluation of alignment methods and data pretreatments on the determination of the most important peaks for the discrimination of coffee varieties Arabica and Robusta using gas chromatography–mass spectroscopy. Anal Chim Acta 2010 Sep; 678 (2): 160-8.

Husøy T, Haugen M, Murkovic M, Jöbstl D, Stølen H, Bjellaas T, *et al.* Dietary exposure to 5-hydroxymethylfurfural from Norwegian food and correlations with urine metabolites of short term exposure. Food Chem Toxicol 2008 Dec; 46 (12): 3697–702.

IARC - International Agency for Research on Cancer. Dry cleaning, some chlorinated solvents and other industrial chemicals. In: Monographs on the evaluation of carcinogenic risks to humans 1995; 63: 394–407.

Illy A, Viani R. Espresso Coffee: The Chemistry of Quality. Academic Press Limited, London; 1995.

IOFI - International Organization of the Flavor Industry. European inquiry on volume of use. Private communication to the Flavor and Extract Manufacturers' Association of the United States, Washington DC, United States; 1995.

Isleroglu H, Kemerli T, Sakin-Yilmazer M, Guven G, Ozdestan O, Uren A, Kaymak-Ertekin F. Effect of steam baking on acrylamide formation and browning kinetics of cookies. J Food Sci 2012 Oct; 77 (10): E257-63.

Jaganathan J, Dugar SM. Authentication of straight whiskey by determination of the ratio of furfural to 5-hydroxymethyl-2-furaldehyde. J AOAC Int 1999 Jul-Aug; 82 (4): 997–1001.

Janzowski C, Glaab V, Samimi E, Schlatter J, Eisenbrand G. 5-Hydroxymethylfurfural: Assessment of mutagenicity, DNA-damaging potential and reactivity towards cellular glutathione. Food Chem Toxicol 2000 Sep; 38 (9): 801–9.

JECFA - Joint WHO/FAO Expert Committee on Food Additives. Safety evaluation of certain food additives and contaminants. WHO Food Additives Series. 2001, 46. (http://www.inchem.org/documents/jecfa/jecmono/v46je01.htm acessed in 22/01/2013).

JECFA – Joint WHO/FAO Expert Committee on Food Additives. Opinion of the Scientific Committee on Food on furfural and furfural diethylacetal (expressed on 2 December 2002). Available in: http://ec.europa.eu/food/fs/sc/scf/out160_en.pdf. Accessed in 18/01/2013.

JECFA - Joint FAO/WHO Food Standards Programme. Discussion paper on furan. Codex Committee on Contaminants in Foods 2011 9: 1-17.

Johnson JM, Harris CH, Barbeau WE. Effects of high-fructose corn syrup replacement for sucrose on browning starch gelatinization and sensory characteristics of cakes. Cereal Chem 1989; 66 (3): 155-7.

Kanjahn D, Jarms U, Maier HG. Hydroxymethylfurfural and furfural in coffee and related beverages: I. Evaluation of the method. Deut Lebensm–Rundsch 1996 Oct; 92 (10): 328–31.

Kaseleht K, Leitner E. Optimization of the automated SPME method for the analysis of volatile compounds in traditional Estonian food (Kama and Kvass) using GC/MS. Austrian Food Chemistry Conference. 28–30 May 2008, WKO, Eisenstadt, Austria. pp. 384-9.

Keeney M, Bassette R. Detection of intermediate compounds in the early stages of browning reaction in milk products. J Dairy Sci 1959 Jun; 42 (6): 945–60.

Kim T, Kim S, Lee K-G. Analysis of furan in heat-processed foods consumed in Korea using solid phase microextraction–gas chromatography/mass spectrometry (SPME–GC/MS). Food Chem 2010 Dec; 123 (4): 1328–33.

Kim T-K, Lee Y-K, Park YS, Lee K-G. Effect of cooking or handling conditions on the furan levels of processed foods. Food Add Cont A 2009 Jun; 26 (6): 767-75.

Kirit AB, Erdogdu F, Ozdemir Y. Accumulation of 5-Hydroxymethyl-2-Furfural during toasting of White Bread Slices. J Food Process Eng 2012. DOI: 10.1111/j.1745-4530.2012.00677.x

Korhonová M, Hron K, Klimcíková D, Müller L, Bednár P, Barták P. Coffee aroma-statistical analysis of compositional data. Talanta 2009 Dec; 80 (2): 710-5.

Kroh LW. Caramelisation in food and beverages. Food Chem 1994; 51 (4): 373-9.

Kuballa T, Stier S, Strichow N. Furan concentrations in coffee and coffee beverages. Deut Lebensm–Rundsch 2005; 101 (6): 229 – 235.

La Pera L, Liberatore A, Avellone G, Fanara S, Dugo G, Agozzino P. Analysis of furan in coffee of different provenance by head-space solid phase microextraction gas chromatography-mass spectrometry: effect of brewing procedures. Food Addit and Contam A 2009 Jun; 26 (6): 786–92.

Languerre JC, Pascale GW, David M, Onillon E, Ait-Ameur L, Birlouez-Aragon I. The impact of microwave heating of infant formula model on neo-formed contaminant formation, nutrient degradation and spore destruction. J Food Eng 2011 Dec; 107 (2): 208–13.

Le Man H, Behera SK, Park HS. Optimization of operational parameters for ethanol production from Korean food waste leachate. Int J Environ Sci Tech 2010; 7 (1): 157-64.

Le TT, Bhandari B, Holland JW, Deeth HC. Maillard reaction and protein cross-linking in relation to the solubility of milk powders. J Agr Food Chem 2011 Oct; 59 (23): 12473–9.

Leardi R. Experimental design in chemistry: A tutorial. Anal Chim Acta 2009 Oct; 652 (1-2): 161–72.

Lee HS, Nagy S. Relative reactivities of sugars in the formation of 5- hydroxymethyl furfural in sugar-catalyst model systems. J Food Process Pres 1990 May; 14 (3): 171-8.

Liardon R, Ott U. Application of multivariate statistics for the classification of coffee headspace profiles. Lebensm Technol 1984 Feb; 17 (1): 32–8.

Limacher A, Kerler J, Conde-Petit B, Blank I. Formation of furan and methylfuran from ascorbic acid in model systems and food. Food Add Cont 2007; 24 (S1): 122–35.

Limacher A, Kerler J, Davide T, Schmalzried F, Blank I. Formation of furan and methylfuran by Maillard-type reactions in model systems and food. J Agric Food Chem 2008 May; 56 (10): 3639–47.

Lineback DR, Stadler RH. Introduction to food process toxicants. In: Stadler RH, Lineback DR, Editors. Process-Induced Food Toxicants: Ocurrence, formation, mitigation, and health risks. New Jersey: Wiley & Sons, Inc.; 2009. p. 3-19.

Lo Coco F, Valentini C, Novelli V, Ceccon L. Liquid chromatographic determination of 2-furaldehyde in beer. Anal Chim Acta 1995 Apr; 306 (1): 57–64.

Lo Coco F, Novelli V, Valentini C, Ceccon L. High-Performance Liquid Chromatographic Determination of 2-Furaldehyde and 5-Hydroxymethyl-2-furaldehyde in Fruit Juices. J Chromatogr Sci 1997 Dec; 35 (12): 578–83.

Lo Coco F, Valentini C, Novelli V, Ceccon L. High-performance liquid chromatographic determination of 2-furaldehyde and 5-hydroxymethyl-2-furaldehyde in honey. J Chromatogr A 1996 Oct; 749 (1-2): 95–102.

López R, Aznar M, Cacho J, Ferreira V. Determination of minor and trace volatile compounds in wine by solid-phase extraction and gas chromatography with mass spectrometric detection. J Chromatogr A 2002 Aug; 966 (1-2): 167-77.

López-Darias J, Anderson JL, Pino V, Afonso AM. Developing qualitative extraction profiles of coffee aromas utilizing polymeric ionic liquid sorbent coatings in headspace solid-phase microextraction gas chromatography-mass spectrometry. Anal Bioanal Chem 2011 Nov; 401 (9): 2965-76.

López-Galilea I, Fournier N, Cid C, Guichard E. Changes in Headspace Volatile Concentrations of Coffee Brews Caused by the Roasting Process and the Brewing Procedure. J Agric Food Chem 2006 Nov; 54 (22): 8560-6.

Louarme L, Billaud C. Evaluation of ascorbic acid and sugar degradation products during fruit dessert processing under conventional or ohmic heating treatment. LWT - Food Sci Technol 2012 Dec; 49 (2): 184–7.

Luijkx GCA, Van Rantwijk F, Van Bekkum H. Hydrothermal formation of 1,2,4-benzenetriol from 5-hydroxymethyl-2-furaldehyde and D-fructose. Carbohyd Res 1993 Apr; 242: 131–9.

Maarse H, Visscher CA, Willimsens LC, Nijssen LM, Boelens MH. Volatile Components in Food: Qualitative and Quantitative Data. 7th ed., Vol. III. Zeist, Centraal Instituut voor Voedingsonderzoek TNO; 1994.

Maeztu L, Sanz C, Andueza S, Pena MP, Bello J, Cid C. Characterization of Espresso Coffee Aroma by Static Headspace GC-MS and Sensory Flavor Profile. J Agric Food Chem 2001 Nov; 49 (11); 5437-44.

Maga JA. Furans in foods. CRC Cr Rev Food Sci 1979; 11 (4): 355–400.

Mansilha C, Melo A, Rebelo H, Ferreira IMPLVO, Pinho O, Domingues V, Pinho C, Gameiro P. Quantification of endocrine disruptors and pesticides in water by gas chromatography-tandem mass spectrometry. Method validation using weighted linear regression schemes. J Chromatogr A 2010 Oct; 1217 (43): 6681 91.

Märk J, Pollien P, Lindinger C, Blank I, Mark T. Quantitation of furan and methylfuran formed in different precursor systems by proton transfer reaction mass spectrometry. J Agric Food Chem 2006 Apr; 54 (7): 2786–93.

Martins SIFS, Jongen WMF, Van Boekel MAJS. A review of Maillard reaction in food and implications to kinetic modelling. Trends Food Sci Tech 2000 Sep; 11 (9-10): 364–73.

Martysiak-Zurowska D, Borowicz A. A comparison of spectrophotometric Winkler method and HPLC technique for determination of 5-hydroxymethylfurfural in natural honey. Chem Anal - Warsaw 2009; 54 (5): 939-47.

Mayer F, Czerny M, Grosch W. Influence of provenance and roast degree on the composition of potent odorants in Arabica coffees. Eur Food Res Technol 1999 Aug; 209 (3-4): 242-50.

Melo A, Viegas O, Petisca C, Pinho O, Ferreira IMPLVO. Effect of beer/red wine marinades on the formation of heterocyclic aromatic amines in pan-fried beef. J Agric Food Chem. 2008 Nov; 56 (22): 10625-32.

Merlo AB, Vetere V, Ruggera JF, Casella ML. Bimetallic PtSn catalyst for the selective hydrogenation of furfural to furfuryl alcohol in liquid-phase. Catal Commun 2009 Jul; 10 (13): 1665–9.

Mesías M, Seiquer I, Navarro MP. Consumption of highly processed foods: Effects on bioavailability and status of zinc and copper in adolescents. Food Res Int 2012 Jan; 45 (1): 184–90.

Mesías-García M, Guerra-Hernández E, García-Villanova B. Determination of furan precursors and some thermal damage markers in baby foods: Ascorbic acid, dehydroascorbic acid, hydroxymethylfurfural and furfural. J Agr Food Chem 2010 May; 58 (10): 6027–32.

Miller JN, Miller JC, in: Statistics and Chemometrics for Analytical Chemistry. 5th Ed, England; Pearson Education Limited, 2005.

Miranda JM, Martínez B, Pérez B, Antón X, Vázquez BI, Fente CA, *et al.* The effects of industrial pre-frying and domestic cooking methods on the nutritional compositions and fatty acid profiles of two different frozen breaded foods. LWT - Food Sci Technol 2010 Oct; 43 (8): 1271-6.

Monien BH, Engst W, Barknowitz G, Seidel A, Glatt H. Mutagenicity of 5-Hydroxymethylfurfural in V79 cells expressing human SULT1A1: Identification and mass spectrometric quantification of DNA adducts formed. Chem Res Toxicol 2012 Jul; 25 (7): 1484–92.

Monien BH, Frank H, Seidel A, Glatt H. Conversion of the common food constituent 5-hydroxymethylfurfural into a mutagenic and carcinogenic sulfuric acid ester in the mouse in vivo. Chem Res Toxicol 2009 Jun; 22 (6): 1123-8.

Monien BH, Herrmann K, Florian S, Glatt H. Metabolic activation of furfuryl alcohol: Formation of 2-methylfuranyl DNA adducts in Salmonella typhimurium strains expressing human sulfotransferase 1A1 and in FVB/N mice. Carcinogenesis 2011 Oct; 32 (10): 1533-39.

Montgomery DC. Design and Analysis of Experiments. 7th ed. New York: John Wiley & Sons; 2000.

Moon JK, Shibamoto T. Role of Roasting Conditions in the Profile of Volatile Flavor Chemicals Formed from Coffee Beans. J Agric Food Chem 2009 Jun; 57 (13): 5823–31.

Morales FJ. Hydroxymethylfurfural (HMF) and related compounds. In: Stadler RH, Lineback DR, Editors. Process-Induced Food Toxicants: Ocurrence, formation, mitigation, and health risks. New Jersey: Wiley & Sons, Inc.; 2009. p. 135-74.

Morales FJ, Jiménez-Pérez S. Hydroxymethylfurfural determination in infant milk - based formulas by micellar electrokinetic capillary chromatography. Food Chem 2001; 72 (4): 525–31.

Morales FJ, Romero C, Jimenez-Perez S. An enhanced liquid chromatographic method for 5-hydroxymethylfurfural determination in UHT milk. Chromatographia 1992; 33 (1-2): 45-8.

Morales FJ, Romero C, Jimenez-Perez S. Evaluation of heat-induced changes in Spanish commercial milk: Hydroxymethylfurfural and available lysine content. Int J Food Sci Tech 1996 Oct; 31 (5): 411-8.

Morales FJ, Romero C, Jiménez-Pérez S. Chromatographic determination of bound hydroxymethylfurfural as an index of milk protein glycosylation. J Agric Food Chem 1997 May; 45 (5): 1570-3.

Moreira RG, Sun X, Chen Y. Factors affecting oil uptake in tortilla chips in deep-fat frying. J Food Eng 1997 Mar; 31 (4): 485-98.

Moro S, Chipman JK, Wegener J-W, Hamberger C, Dekant W, Mally A. Furan in heat-treated foods: Formation, exposure, toxicity, and aspects of risk assessment. Mol Nutr Food Res 2012 Aug; 56 (8): 1197–211.

Mottram DS. Meat. In: H. Maarse editors. Volatile Compounds in Foods and Beverages. New York. Marcel Dekker, Inc., 1991. p. 107–77.

Mottram DS. Flavour formation in meat and meat products: a review. Food Chem 1998 Aug; 62 (4): 415-24.

Murkovic M, Bornik M-A. Formation of 5-hydroxymethyl-2-furfural (HMF) and 5-hydroxymethyl-2-furoic acid during roasting of coffee. Mol Nutr Food Res 2007 Apr; 51 (4): 390–94.

Murkovic M, Pichler N. Analysis of 5 - hydroxymethylfurfural in coffee, dried fruits and urine. Mol Nutr Food Res 2006 Sep; 50: 842–6.

Nanditha B, Prabhasankar P. Antioxidants in bakery products: a review. Crit Rev Food Sci Nut 2009 Jan; 49 (1): 1-27.

Nikolov PY, Yaylayan VA. Thermal decomposition of 5-(hydroxymethyl)-2-furaldehyde (HMF) and its further transformations in the presence of glycine. J Agr Food Chem 2011 Sep; 59 (18): 10104–13.

Nomeir AA, Silveira DM, McComish MF, Chadwick M. Comparative metabolism and disposition of furfural and furfuryl alcohol in rats. Drug Metab Dispos 1992; 20(2): 198–204.

Nawar WW. Volatile components of the frying process. Grasas y Aceites 1998; 49 (3-4): 271-4.

Nursten H. The Maillard Reaction: Chemistry, Biochemistry and Implications. Cambridge: The Royal Society of Chemistry; 2005.

Paine JB, Pithawalla YB, Naworal JD. Carbohydrate pyrolysis mechanisms from isotopic labeling. Part 4. The pyrolysis of d-glucose: The formation of furans. J Anal Appl Pyrol 2008 Sep; 83 (1): 37–63.

Parent RA. Furfural. In: Wexler P, editors. Encyclopedia of Toxycology. Elsevier Inc.; 2005. p. 394-97.

Parker JK. The kinetics of thermal generation of flavour. J Sci Food Agr 2013 Jan; 93 (2): 197-208.

Parliament TH, Stahl HB. What makes the coffee smell so good? Chem Tech 1995; 25 (8): 38-47.

Pereira V, Albuquerque FM, Ferreira AC, Cacho J, Marques JC. Evolution of 5-hydroxymethylfurfural (HMF) and furfural (F) in fortified wines submitted to overheating conditions. Food Res Int 2011 Jan 44 (1): 71–6.

Perelló G, Martí-Cid R, Castell V, Llobet JM, Domingo JL. Concentrations of polybrominated diphenylethers, hexachlorobenzene and polycyclic aromatic hydrocarbons in various food stuffs before and after cooking. Food Chem Toxicol 2009 Apr; 47 (4): 709-15.

Perelló G, Martí-Cid R, Castell V, Llobet JM, Domingo JL. Influence of various cooking processes on the concentrations of PCDD/PCDFs, PCBs and PCDEs in foods. Food Control 2010 Feb 21 (2): 178-85.

Perez Locas C, Yaylayan VA. Origin and mechanistic pathways of formation of the parent furan - A food toxicant. J Agr Food Chem 2004 Nov; 52 (22): 6830–36.

Pérez-Palacios T, Petisca C, Pinho O, Ferreira IMPLVO. Headspace solid-phase Microextraction of Volatile and furanic Compounds in Coated Fish Sticks: Effect of the Extraction Temperature. World Academy of Science, Engeneering and Technology 2012a Sep; 69: 1077.

Pérez-Palacios T, Petisca C, Melo A, Ferreira IMPLVO. Quantification of furanic compounds in coated deep-fried products simulating normal preparation and consumption: optimisation of HS-SPME analytical conditions by response surface methodology. Food Chem 2012b Dec; 135 (3): 1337-43.

Pérez-Palacios T, Petisca C, Melo A, Ferreira IMPLVO. Quantification of 5-Hydroxymethylfurfural in Coated Deep-Fried Products: Optimization of the Extraction Procedure by Using Statistical Design. Food Anal Method 2013 Feb; 6 (1): 10–6.

Pérez RA, Sánchez-Brunete C, Calvo RM, Tadeo JL. Analysis of volatiles from Spanish honeys by solid-phase microextraction and gas chromatography-mass spectrometry. J Agr Food Chem 2002 Apr; 50 (9): 2633-7.

Peterson LA, Naruko KC, Predecki DP. A reactive metabolite of furan, cis-2-butene-1,4-dial, is mutagenic in the Ames assay. Chem Res Toxicol 2000 Jul; 13 (7): 531–4.

Peterson LA, Cummings ME, Chan JY, Vu CC, Matter BA. Identification of a cis-2-butene-1,4-dial-derived glutathione conjugate in the urine of furan-treated rats. Chem Res Toxicol 2006 Sep; 19 (9): 1138–41.

Petisca C, Pérez-Palacios T, Farah A, Pinho O, Ferreira IMPLVO. Furans and other volatile compounds in ground roasted and espresso coffee using headspace solid-phase microextraction: Effect of roasting speed. Food Bioprod Process 2012, in press.

Piazza L, Masi P. Development of crispness in cookies during baking in an industrial oven. Cereal Chem 1997 Mar; 74 (2): 135-40.

Pinho C, Melo A, Mansilha C, Ferreira I. Optimization of Conditions for Anthocyanin Hydrolysis from Red Wine Using Response Surface Methodology (RSM). J Agric Food Chem 2011 Dec; 59 (1): 50–5.

Pinho O, Peres C, Ferreira IMPLVO. Solid-phase microextraction of volatile compounds in "Terrincho" ewe cheese: Comparison of different fibers. J Chromatogr A 2003 Sep; 1011 (1-2): 1-9.

Pinho O, Mendes E, Alves MM, Ferreira IMPLVO. Chemical, physical and sensorial characteristics of “Terrincho” ewe cheese: changes during ripening and intravarietal comparison. J Dairy Sci 2004 Feb; 87 (2): 249-57.

Pinho O, Ferreira IMPLVO, Santos LHMLM. Method Optimization by Solid-Phase Microextraction in Combination with GC/MS for Analysis of Beer Volatile Fraction. J Chromatogr A 2006 Jul; 1121 (2): 145-53.

Pokorny J. Changes in nutrients at frying temperatures. In: Boskou D, Elmadfa, I. Editors. Frying of Food. Lancaster: Technomic Publishing Co., 1999. p. 69-103.

Purlis E. Browning development in bakery products – A review. J Food Eng 2010 Aug; 99 (3): 239 –49.

Quarta B, Anese M. The effects of salts on acrylamide and 5-hydroxymethylfurfural formation in glucose – asparagine model solutions and biscuits. J Food Nut Res 2010 49; (2): 69-77.

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 Sep; 122 (1): 98–104.

Quiles JL, Ramírez-Tortosa M, Gómez A, Huertas JR, Mataix J. Role of vitamin E and phenolic compounds in the antioxidant capacity, measured by ESR, of virgin olive, olive and sunflower oils after frying. Food Chem 2002 Apr; 76 (4): 461–8.

Rada-Mendoza M, Luz Sanz M, Olano A, Villamiel M. Formation of hydroxymethylfurfural and furosine during the storage of jams and fruit – based infant foods. Food Chem 2004 May; 85 (4): 605–9.

Rada-Mendoza M, Olano A, Villamiel M. Determination of hydroxymethylfurfural in commercial jams and in fruit-based infant foods. Food Chem 2002 Dec; 79 (4): 513–6.

Ramírez R, Estévez M, Morcuende D, Cava R. Effect of the type of frying culinary fat on volatile compounds isolated in fried pork loin chops by using SPME GC-MS. J Agric Food Chem 2004 Dec; 52 (25): 7637-43.

Ramírez-Jiménez A, Guerra-Hernández E, García-Villanova B. Browning indicators in bread. J Agr Food Chem 2000a; 48 (9): 4176–81.

Ramírez-Jiménez A, García-Villanova B, Guerra-Hernández E. Hydroxymethylfurfural and methylfurfural content of selected bakery products. Food Res Int 2000b; 33 (10): 833–8.

Ramírez-Jiménez A, García-Villanova B, Guerra-Hernández E. Effect of toasting time on the browning of sliced bread. J Sci Food Agr 2001; 81 (5): 513–8.

Ramírez-Jiménez A, Guerra-Hernández E, García-Villanova B. Evolution of non-enzymatic browning during storage of infant rice cereal. Food Chem 2003 Nov; 83 (2): 219–25.

Ramos B, Pinho O, Ferreira IMPLVO. Volatile profile and sensory characteristics of creams used as fillers of bakery products: Home made egg creams vs. industrial creams. Ital J Food Sci 2009; 21 (3): 255-68.

Rega B, Guerard A, Delarue J, Maire M, Giampaoli P. On-line dynamic HS-SPME for monitoring endogenous aroma compounds released during the baking of a model cake. Food Chem 2009 Jan; 112 (1): 9–17.

Rendic S, Guengerich FP. Contributions of Human Enzymes in Carcinogen Metabolism. Chem Res Toxicol 2012 Apr; 25 (7): 1316–83.

Rizelio VM, Gonzaga LV, Da Silva Campelo Borges G, Micke GA, Fett R, Costa ACO. Development of a fast MECK method for determination of 5-HMF in honey samples. Food Chem 2012 Aug; 133 (4): 1640–5.

Roberts D, Crews C, Grundy H, Mills C, Matthews W. Effect of consumer cooking on furan in convenience foods. Food Add Cont A 2008 Jan; 25 (1): 25-31.

Rocha S, Maetzu L, Barros A, Cid C, Coimbra MA. Screening and distinction of coffee brews based on headspace solid phase microextraction/gas chromatography/principal component analysis. J Sci Food Agric 2004 Jan; 84 (1): 43–51.

Romero A, Cuesta C, Sanchez-Muniz FJ. Cyclic fatty acid monomers and thermoxidative alteration compounds formed during frying of frozen foods in extra virgin olive oil. J Am Oil Chem Soc 2000; 77 (11): 1169-73.

Rufián-Henares JA, De la Cueva SP. Assessment of hydroxymethylfurfural intake in the Spanish diet. Food Addit and Contam A 2008; 25 (11): 1306–12.

Rufián-Henares JA, Delgado-Andrade C, Morales FJ. Analysis of heat - damage indices in breakfast cereals: influence of composition. J Cereal Sci 2006a Jan; 43 (1): 63–6.

Rufián-Henares JA, Delgado-Andrade C, Morales FJ. Application of a fast high-performance liquid chromatography method for simultaneous determination of furanic compounds and glucosylisomaltol in breakfast cereals. J AOAC Int 2006b Jan-Feb; 89 (1): 161–5.

Rufián-Henares JA, Delgado-Andrade C, Morales FJ. Assessing the Maillard reaction development during the toasting process of common flours employed by the cereal products industry. Food Chem 2009 May; 114 (1): 93–9.

Rufián-Henares JA, Garcia-Villanova B, Guerra-Hernandez E. Determination of furfural compounds in enteral formula. J Liq Chromatogr RT 2001 Jan; 24 (19): 3049-61.

Sablani SS, Marcotte M, Baik OD, Castaigne F. Modeling of simultaneous heat and water transport in the baking process. LWT – Food Sci Technol 1998 Apr; 31 (3): 201–9.

Sanz C, Ansorena D, Bello J, Cid C. Optimizing Headspace Temperature and Time Sampling for Identification of Volatile Compounds in Ground Roasted Arabica Coffee. J Agric Food Chem 2001 Mar; 49 (3): 1364-9.

Selmanoğlu G, Karacaoğlu E, Kiliç A, Koçkaya EA, Akay MT. Toxicity of food contaminant furan on liver and kidney of growing male rats. Environ Toxicol 2012 Oct; 27 (10): 613–22.

Semmelroch P, Grosch W. Analysis of roasted coffee Powders and Brews by Gas Chromatography-Olfactometry of Headspace samples. Lebensm Technol 1995; 28 (3): 310-3.

Semmelroch P, Grosch W. Studies on Character Impact Odorants of coffee brews. Lebensm Technol 1996 Feb; 44 (2): 537-43.

Sesidoni A, Peressini D, Pollini CM. Study of the Maillard reaction in model systems under conditions related to the industrial process of pasta thermal VHT treatment. J Sci Food Agric 1999 Feb; 79 (2): 317–22.

Senyuva HZ, Goekmen V. Analysis of furan in foods. Is headspace sampling a fit-for-purpose technique? Food Addit and Contam 2005 Dec; 22 (12): 1198–202.

Smith JP, Daifas DP, El-Khoury W, Koukoutsis J, El-Khoury A. Shelf Life and Safety Concerns of Bakery Products-A Review. Crit Rev Food Sci Nut 2004; 44 (1): 19-55.

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 and Contam 2006 Dec; 23(12): 1276-82.

Sujatha PS. Monitoring cytotoxic potentials of furfuryl alcohol and 2-furyl methyl ketone in mice. Food Chem Toxicol 2008 Jan; 46 (1): 286-92.

Sumnu G. A review on microwave baking of foods. Int J Food Sci Technol 2001; 36 (2): 117 – 127.

Swasti YR, Murkovic M. In: Proceedings of 1st international congress on cocoa, coffee and tea. Novara, Italy, 2011. p. 278- 82.

Tang J, Jin QZ, Shen G-H, Ho C-T. Isolation and identification of volatile compounds from fried chicken. J Agric Food Chem 1983 Nov; 31 (6): 1287–92.

Teixidó E, Santos FJ, Puignou L, Galceran MT. Analysis of 5-hydroxymethylfurfural in foods by gas chromatography-mass spectrometry. J Chromatogr A. 2006 Nov; 1135 (1): 85-90

Teixidó E, Núñez O, Santos FJ, Galceran MT. 5-Hydroxymethylfurfural content in foodstuffs determined by micellar electrokinetic chromatography. Food Chem 2011 Jun; 126 (4): 1902–8.

Teubner W, Meinl W, Florian S, Kretzschmar M, Glatt H. Identification and localization of soluble sulfotransferases in the human gastrointestinal tract. Biochem J 2007 Jun; 404 (2): 207–15.

The HEATOX Project (2007). http://heatox.org/ (Acessed in January, 2013)

Theobald A, Müller A, Anklam E. Determination of 5 - hydroxymethylfurfural in vinegar samples by HPLC. J Agr Food Chem 1998 May; 46 (5): 1850–4.

Timón ML, Carrapiso AI, Jurado A, Lagemaat J. A study of the aroma of fried bacon and fried pork loin. J Sci Food Agric 2004 Jun; 84 (8): 825-31.

Toci AT, Farah A. Volatile compounds as potential defective coffee beans' markers. Food Chem 2008 Jun; 108 (3): 1133–41.

Tosi E, Ciappini M, Ré E, Lucero H. Honey thermal treatment effects on hydroxymethylfurfural content. Food Chem 2002; 77 (1): 71–4.

Tsai S-W, Kao K-Y. Determination of furfural in beers, vinegars and infant formulas by solid-phase microextraction and gas chromatography/mass spectrometry. Int J Environ An Ch 2012 Jan; 92 (1): 76-84.

Van Boekel M. Effect of heating on Maillard reactions in milk. Food Chem 1998 Aug; 62 (4): 403–14.

Van Boekel M, Fogliano V, Pellegrini N, Stanton C, Scholz G, Lalljie S, *et al.* A review on the beneficial aspects of food processing. Mol Nutr Food Res 2010 Sep; 54 (9): 1215–47.

Van Lancker F, Adams A, Owczarek A, De Meulenaer B, De Kimpe N. Impact of various food ingredients on the retention of furan in foods. Mol Nutr Food Res 2009 Dec; 53 (12): 1505-11.

Van Lancker F, Adams A, De Kimpe N. Chemical modifications of peptides and their impact on food properties. Chem Rev 2011 Dec; 111 (12): 7876–903.

Verendel JJ, Church TL, Andersson PG. Catalytic one-pot production of small organics from polysaccharides. Synthesis 2011 Abr; 11: 1649–77.

Vhangani LN, Van Wyk J. Antioxidant activity of Maillard reaction products (MRPs) derived from fructose-lysine and ribose-lysine model systems. Food Chem 2013 Abr; 137 (1-4): 92–8.

Viegas O, Novo P, Pinho O, Ferreira IMPLVO. A comparison of the extraction procedures and quantification methods for the chromatographic determination of polycyclic aromatic hydrocarbons in charcoal grilled meat and fish. Talanta. 2012a Jan; 88: 677-83.

Viegas O, Novo P, Pinto E, Pinho O, Ferreira IMPLVO. Effect of charcoal types and grilling conditions on formation of heterocyclic aromatic amines (HAs) and polycyclic aromatic hydrocarbons (PAHs) in grilled muscle foods. Food Chem Toxicol 2012b Jun; 50(6): 2128-34.

Vranová J, Bednáridová A, Ciesarová Z. In-house validation of a simple headspace gas chromatographymass spectrometry method for determination of furan levels in food. J Food Nutr Res 2007; 46 (3): 123-7.

Vranová J, Ciesarová Z. Furan in food - A review. Czech J Food Sci 2009; 27 (1): 1-10.

Vorlová L, Borkovcová I, Kalábová K, Večerek V. Hydroxymethylfurfural contents in foodstuffs determined by HPLC method. J Food Nutr Res 2006; 45 (1): 34–8.

Wagner R, Grosch W. Key odorants of French fries. J Am Oil Chem Soc 1998; 75 (10): 1385–92.

Walker S, Seetharaman K, Goldstein A. Characterizing physicochemical changes of cookies baked in a commercial oven. Food Res Int 2012 Aug; 48 (1): 249 – 56.

Wang H-Y, Qian H, Yao W-R. Melanoidins produced by the Maillard reaction: Structure and biological activity. Food Chem 2011 Oct; 128 (3): 573–84.

Wang J, Schnute WC. Simultaneous quantitation of 2-acetyl-4-tetrahydroxybutylimidazole, 2- and 4-methylimidazoles, and 5-hydroxymethylfurfural in beverages by ultrahigh-performance liquid chromatography-tandem mass spectrometry. J Agr Food Chem 2012 Feb; 60 (4): 917-21.

Wardencki W, Michulec M, Curyło J. A review of theoretical and practical aspects of solid-phase microextraction in food analysis. Int J Food Sci Technol 2004 Aug; 39 (7): 703-17.

Wegener JW, Lopez-Sanchez P. Furan levels in fruit and vegetables juices, nutrition drinks and bakery products. Anal Chim Acta 2010 Jul; 672 (1-2): 55–60.

Wenzl T, Lachenmeier D, Gökmen V. Analysis of heat-induced contaminants (acrylamide, chloropropanols and furan) in carbohydrate-rich food. Anal Bioanal Chem 2007 Sep; 389 (1): 119-37.

WHO (1999). Safety Evaluation of Certain Food Additives. WHO Food Additives Series: 42. (http://www.inchem.org/documents/jecfa/jecmono/v042je03.htm). Accessed in 22/01/2013.

WHO Evaluation of certain contaminants in food. Technical Report Series, 959; 2011.

Wilson DM, Goldsworthy TL, Popp JA, Butterworth BE. Evaluation of genotoxicity, pathological lesions, and cell proliferation in livers of rats and mice treated with furan. Environ Mol Mutagen 1992; 19 (3): 209–22.

Yu A-N, Sun BG, Tian DT, Qu WY. Analysis of volatile compounds in traditional smoke-cured bacon (CSCB) with different fiber coatings using SPME. Food Chem 2008 Sep; 110 (1): 233-8.

Yuan J-P, Chen F. Separation and identification of furanic compounds in fruit juices and drinks by high - performance liquid chromatography photodiode array detection. J Agr Food Chem 1998 Apr; 46 (4): 1286-91.

Yuan J-P, Chen F. Simultaneous separation and determination of sugars, ascorbic acid and furanic compounds by HPLC - Dual detection. Food Chem 1999 Feb; 64 (3): 423-7.

Zhang Y, Song Y, Hu X, Liao X, Ni Y, Li Q. Determination of 5-hydroxymethylfurfural and furfural in soft beverages by HPLC. Adv Mat Res 2012a; 550-553: 1959–66.

Zhang Y, Song Y, Hu X, Liao K, Ni Y, Li Q. Effects of sugars in batter formula and baking conditions on 5-hydroxymethylfurfural and furfural formation in sponge cake models. Food Res Int 2012b Nov; 49 (1): 439-45.

Zhang Z, Pawliszyn J. Headspace solid-phase microextraction. Anal Chem 1993 Jul; 65 (14): 1843–52.

Zoller O, Sager F, Reinhard H. Furan in food: Headspace method and product survey. Food Addit and Contam 2007; 24 (S1): 91–107.