UNIVERSITAT POLITÈCNICA DE VALÈNCIA

INSTITUTO UNIVERSITARIO DE INGENIERÍA DE ALIMENTOS PARA EL DESARROLLO

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Evaluation of acrylamide in foods and development of some strategies for its reduction

DOCTORAL THESIS

Mariola Sansano Tomás

Supervisors:
Dra. Ana Mª Andrés Grau
Dra. Ana Belén Heredia Gutiérrez

June 2017

AGRADECIMIENTOS,

A mis directoras, Ana Andrés y Ana Heredia por confiar en mí y transmitirme vuestros conocimientos y forma de trabajar tan rigurosa y modélica.

A mi familia, y ahí te incluyo a ti Fernan, por vuestro apoyo incondicional, por enseñarme a esforzarme y no rendirme y por contagiar la actitud positiva para afrontar y resolver los problemas.

A mi prima Mª José, por meterme el gusanillo del mundo de la ciencia.

A mis compañeros de laboratorio y de planta Victoria, Ruth M., Susana, Laura C., Salva, Irene, Eva, Ester, Andrea, Carolina, Quim, Raúl, Bruno, Sara, Laura L., Ruth, Virginia que hemos pasado tanto juntos, por las risas, los buenos momentos además de los consejos y apoyo. A Victoria y Ester, por el tiempo que hemos compartido en Cesena, hicisteis que todo fuera más fácil y divertido con vosotras allí.

Al grupo del Prof. Marco Dalla Rosa (Ula, Fede, Virginia, Cinzia, Silvia, Nicolò Santina, Pietro) por la acogida en mi estancia en Cesena.

A los "tesinandos", Juan Alberto, Miriam, Viviana, Sandra, Isabel y Miriam, ha resultado una grata experiencia trabajar conjuntamente. Con algunos tengo la suerte de conservar una gran amistad, a pesar de la distancia.

A Marisol, eres toda una fuente de conocimiento… y de paciencia…

A Susana y Anna, nos conocemos desde hace más de diez años y he teniendo la suerte de teneros trabajando cerca. Una terapia inmejorable nuestras conversaciones de camino al trabajo.

Dra. Ana Mª Andrés Grau, Catedrática de Universidad perteneciente al Departamento de Tecnología de Alimentos y Directora del Instituto Universitario de Ingeniería de Alimentos para el Desarrollo de la Universidad Politécnica de Valencia,

Dra. Ana Belén Heredia Gutiérrez, Profesora perteneciente al Departamento de Tecnología de Alimentos de la Universidad Politécnica de Valencia,

CONSIDERAN: que la memoria titulada ***Evaluation of acrylamide in foods and development of some strategies for its reduction*** que presenta ***D.ª Mariola Sansano Tomás***, para aspirar al grado de Doctora de la Universidad Politécnica de Valencia, y que ha sido realizada bajo su dirección en el Instituto Universitario de Ingeniería de Alimentos para el Desarrollo de la Universidad Politécnica de Valencia, reúne las condiciones adecuadas para constituir sus tesis doctoral, por lo que **AUTORIZAN** a la interesada para su presentación.

Valencia, Mayo de 2017

Fdo.: Ana Mª Andrés Grau Fdo.: Ana Belén Heredia Gutiérrez

ABSTRACT

Although the presence of acrylamide in foods was detected a decade ago, public concern about this issue, and in general about food health, seems to have increased in recent years. Acrylamide is a toxic human carcinogen present mainly in foods from plant origin and subjected to transformation processes in which temperatures above 120 °C are reached, such as frying and baking. Foods that mostly contribute to the intake of acrylamide are: French fries and chips, coffee and coffee substitute, biscuits, bread, pastries, battered and breaded products, breakfast cereals..., being children and adolescents the most exposed population. From a chemical point of view, acrylamide is formed mainly from the reaction, during thermal processing, between asparagine and reducing sugars, as an intermediate product of the Maillard reaction.

In the last decade, both health authorities and the scientific community have made great efforts in scientifically establishing limits of toxicity as well as exploring strategies aimed at reducing acrylamide formation. The purpose of this doctoral thesis is framed in this last sense. On the one hand, our work has been focused on searching new strategies for acrylamide mitigation, in two different types of food: fried potatoes and battered products. The effect of an emerging frying technique, named air frying, as well as pre-frying treatments, were studied in fried potatoes. In the case of batters however, the strategy consisted in modifying their composition by adding a hydrocolloid, chitosan, after having tested its potential in model systems. Furthermore, the effect of chitosan addition on the physical properties of the batter formulations and on the quality of the final product was evaluated.

Finally, acrylamide bioaccessibility of the food products with the highest acrylamide content was addressed. These food products were subjected to an in vitro gastrointestinal simulation in order to study how acrylamide content progressed throughout the digestion process.

Results showed that air fried potatoes as well as chitosan addition to batter formulations reduced the formation of acrylamide by about 90 and 60 %,

respectively. The modification of the characteristics of chitosan (molecular weight and deacetylation degree) conditioned its ability to reduce the formation of acrylamide. Furthermore, the incorporation of chitosan to batters reduced the absorption of fat during frying. Chitosan increased viscosity and consistency of batters but, the color and texture of the fried products were not significantly altered.

The total replacement of wheat flour by rice flour allowed to obtain gluten-free batters with similar viscosity, when adding chitosan. This new formulation could be adjusted to gluten-free battered products.

The simulation of *in vitro* digestion showed a significant increment of acrylamide after the gastric stage in a wide range of foods. However, acrylamide bioaccessibility (after the intestinal stage) was reduced until pre-digestion levels or even lower in chips and French fries.

RESUMEN

Si bien es verdad que la presencia de acrilamida en alimentos se detectó hace más de una década, la preocupación por parte del consumidor, no sólo por este tema si en la alimentación saludable en general, parece haberse incrementado en los últimos años. La acrilamida es un tóxico posible carcinógeno humano presente principalmente en alimentos derivados de materias primas de origen vegetal y sometidos a procesos de transformación en los que se alcanzan temperaturas por encima de 120 ºC como la fritura y horneado. Los alimentos que contribuyen mayoritariamente a la ingesta de acrilamida son: patatas fritas (tipo *French fries* y *chips*), café y substituto de café, galletas, pan, bollería, rebozados y empanados, cereales de desayuno…, siendo la exposición al tóxico superior por parte de población infantil y adolescente. Desde un punto de vista químico, la acrilamida se forma principalmente a partir de la reacción entre la asparagina y azúcares reductores durante el procesado térmico, como producto intermedio de las reacciones de Maillard.

En la última década, tanto las autoridades sanitarias como la comunidad científica han puesto un gran empeño en establecer científicamente los límites de toxicidad y en la búsqueda de estrategias orientadas a reducir su formación. El cometido de esta tesis doctoral se enmarca en este último sentido. Por un lado, se ha trabajado en la búsqueda de nuevas estrategias para la reducción de acrilamida, en dos tipos de alimentos distintos: patatas fritas y masas de productos rebozados. En patatas fritas se estudió el efecto de una técnica emergente de fritura, concretamente la fritura por aire caliente, junto con tratamientos previos a la fritura. En masas de rebozados, en cambio, se optó por modificar la composición de estos, adicionando un hidrocoloide, el quitosano, después de haber ensayado su potencial en sistemas modelo. Además, se evaluó el efecto de la incorporación de quitosano sobre las propiedades físicas de la masa de rebozado y sobre la calidad del producto final.

Por último y no por ello menos importante, se abordó la problemática de la acrilamida sobre la salud humana desde el punto de vista de su bioaccesibilidad.

Para ello, se seleccionaron los productos con mayor contenido en acrilamida dietética y se sometieron a una simulación gastrointestinal *in vitro* con el fin de conocer los cambios experimentados por el tóxico durante este proceso y determinar su bioaccesibilidad.

Los resultados mostraron que la fritura de patatas por aire caliente, así como la incorporación de quitosano a las masas de rebozado reducían la formación de acrilamida alrededor de un 90 y 60 %, respectivamente. La modificación de las características del quitosano (en cuanto a masa molecular y grado de desacetilación) condicionó su capacidad de reducir la formación de acrilamida. La incorporación de quitosano redujo la absorción de grasa durante la fritura de las masas. Si bien aumentó la viscosidad y consistencia de éstas, no se vio alterado significativamente el color y la textura del producto final frito.

La substitución total de harina de trigo por harina de arroz permitió obtener masas de rebozado sin gluten con semejante viscosidad, al adicionar quitosano. Esta nueva formulación podría ser adaptada a productos rebozados sin gluten.

La simulación de digestión *in vitro* mostró aumentos significativos de acrilamida tras la fase gástrica en una amplia gama de alimentos. Sin embargo, la bioaccesibildad de acrilamida (tras la fase intestinal) se redujo hasta niveles previos a la digestión o incluso menores, en patatas fritas tipo *chips* y tipo *French fries*.

RESUM

Si bé és veritat que la presència d'acrilamida en aliments es va detectar fa més d'una dècada, la preocupació per part del consumidor, no només per aquest tema si no en l'alimentació saludable en general, sembla haver-se incrementat en els últims anys. L'acrilamida és un tòxic possible carcinogen per a l'èsser humà present principalment en aliments derivats de matèries primeres d'origen vegetal i sotmesos a processos de transformació en els quals s'assoleixen temperatures majors a 120 ºC, com el procés de fregida o al forn. Els aliments que contribueixen majoritàriament a la ingesta d'acrilamida són: creïlles fregides (tipus *French fries* i *chips*), cafè i substitut de cafè, galetes, pa, brioixeria, arrebossats i empanats, cereals de desdejuni..., sent l'exposició al tòxic superior per part de població infantil i adolescent. Des d'un punt de vista químic, l'acrilamida es forma principalment a partir de la reacció entre l'asparagina i sucres reductors durant el processat tèrmic, com producte intermedi de les reaccions de Maillard.

En l'última dècada, tant les autoritats sanitàries com la comunitat científica han posat un gran esforç a establir científicament els límits de toxicitat i en la recerca d'estratègies orientades a reduir la seva formació. La comesa d'aquesta tesi doctoral s'emmarca en aquest últim sentit. D'una banda, s'ha treballat en la recerca de noves estratègies per a la reducció d'acrilamida, en dos tipus d'aliments diferents: creïlles fregides i masses de productes arrebossats. En creïlles fregides es va estudiar l'efecte d'una tècnica emergent de fregit, concretament el fregit per aire calent, juntament amb tractaments previs al fregit. En masses d'arrebossats, en canvi, es va optar per modificar la composició d'aquests, addicionant un hidrocol·loide, el quitosà, després d'haver assajat el seu potencial en sistemes model. A més, es va avaluar l'efecte de la incorporació de quitosà sobre les propietats físiques de la massa d'arrebossat i sobre la qualitat del producte final.

Finalment i no per això menys important, es va abordar la problemàtica de l'acrilamida sobre la salut humana des del punt de vista de la seva

bioaccesibilidad. Per a això, es van seleccionar els productes amb major contingut en acrilamida dietètica i es van sotmetre a una simulació gastrointestinal *in vitro* per tal de conèixer els canvis experimentats pel tòxic durant aquest procés i determinar la seva bioaccesibilidad.

Els resultats van mostrar que el fregit de creïlles per aire calent, així com la incorporació de quitosà a les masses d'arrebossat reduïen la formació d'acrilamida al voltant d'un 90 i 60%, respectivament. La modificació de les característiques del quitosà (quant a massa molecular i grau de desacetilación) va condicionar la seva capacitat de reduir la formació d'acrilamida. La incorporació de quitosà va reduir l'absorció de greix durant el fregit de les masses. Si bé va augmentar la viscositat i consistència d'aquestes, no es va veure alterat significativament el color i la textura del producte final fregit.

La substitució total de farina de blat per farina d'arròs va permetre obtenir masses d'arrebossat sense gluten amb semblant viscositat, en addicionar quitosà. Aquesta nova formulació podria ser adaptada a productes arrebossats sense gluten.

La simulació de digestió in vitro va mostrar augments significatius d'acrilamida després de la fase gàstrica en una àmplia gamma d'aliments. No obstant això, la bioaccesibildad d'acrilamida (després de la fase intestinal) es va reduir fins a nivells previs a la digestió o fins i tot menors, en creïlles fregides tipus *chips* i tipus *French fries*.

PREFACE

Justification of the study

Acrylamide is a widely studied compound, certified by the International Agency for Research on Cancer (IARC) as a compound with a high carcinogenic potential. Until the last decade, the main known route of exposure to this toxic compound was tobacco or occupational exposure. It is from 2002 when its presence in food was discovered and therefore it was created an alarm on the foods of risk at worldwide level. Hence, a lot of efforts to seek measures to reduce or eliminate acrylamide in processed foods have been done during the last 15 years.

Current society in developed countries has a responsible conscience towards consumption in general; in terms of food, there is a tendency towards adaptation to a changing lifestyle, increasing awareness about health and environment. It is observed a more rational choice of food, without forgetting that food is consumed by the pleasant sensation that causes. The search for healthy foods, with lower content of sugars, fat or salt is an example of this new tendency. In addition, the current population is more informed about how to process food in the most appropriate way to avoid the formation of toxic compounds such as acrylamide, acrolein, polycyclic aromatic compounds... As for acrylamide, it should not be forgotten that the composition of the standard diet of the child population, makes it the most exposed age group, therefore, acrylamide monitoring and control has to be carried out. In fact, this is one of the objectives that sanitary authorities, at world-wide level (FAO *Food and Agriculture Organization*, WHO *World Health Organization*, EFSA *European Food Safety Authority*...), want to achieve, to make further efforts on developing and implementing acrylamide mitigation methods in foods of major importance of dietary exposure.

Dissertation outline

This Doctoral Thesis is structured in 5 sections: *Introduction*, *Objectives*, *Methodology*, *Results* and *Concluding Remarks*. The *Introduction* is focused in the general information related to acrylamide, concretely its dietary intake, the main routes of formation, consequent health risk, methods of analysis and strategies to reduce its formation. The *Objectives* include the specific aims related to the different Papers, corresponding to the five publications that are discussed in this thesis (*Results*). Finally, the *Concluding Remarks* show some recommendations for future research.

Dissemination of the results

- International journals

Published:

Sansano, M., Juan-Borrás, M., Escriche, I., Andrés, A., & Heredia, A. (2015). Effect of Pretreatments and Air-Frying, a Novel Technology, on Acrylamide Generation in Fried Potatoes. *Journal of Food Science*, 80 (5), 120–128.

Sansano, M., Castelló, M. L., Heredia, A., & Andrés, A. (2016). Protective effect of chitosan on acrylamide formation in model and batter systems. *Food Hydrocolloids*, 60, 1–6.

Sansano, M., Castelló, M. L., Heredia, A., & Andrés, A. (2017). Acrylamide formation and quality properties of chitosan based batter formulations. *Food Hydrocolloids,* 66, 1-7.

Sansano, M., Heredia, A., Peinado, I., & Andrés, A. (2017). Dietary acrylamide: What happens during digestion. *Food Chemistry*, *237*, 58–64.

Submitted:

Sansano, M., Heredia, A., Glicerina, V., Balestra, F., Romani, S.; Andrés, A. Influence of chitosan on thermal, microstructural and rheological properties of rice and wheat flours- based batters. Submitted to LWT-Food Science & Technology on May 2017.

-International conferences

Poster - Sansano, M., Juan-Borrás, M., Escriche, I., Andrés, A., & Heredia, A.2014. Evaluación de los cambios composicionales y la formación de acrilamida durante la fritura de patatas por microondas. 9 Congreso Iberoamericano de Ingeniería de Alimentos, CIBIA, 13-16 January, Valencia, Spain

Poster - Sansano, M., Gimeno, M., Heredia, A., & Andrés, A. 2014. Functionality of chitosan for inhibiting acrylamide formation in model systems. III International Conference of Food Innovation, 20-23 October, Concordia, Entre Ríos, Argentina.

Poster - Sansano, M., Aguilera, V., Heredia, A., & Andrés, A. 2014. Incorporation of chitosan in battered formulations as a strategy to reduce acrylamide formation. III International Conference of Food Innovation, 20-23 October, Concordia, Entre Ríos, Argentina.

Poster- Sansano, M, Peinado, I., Heredia, A., & Andrés, A. 2017. Effect of gastric digestion of different food products on acrylamide content. 5th International Conference on Food Digestion. 4-6 April 2017 Rennes, France.

-Awards

First prize in the 'IX Certamen de investigación Valencia IDEA'. Valencia, Spain. Incorporación de quitosano a la formulación de rebozados saludables. Sansano, M., Colino, S., Heredia, A., & Andrés, A. 2015.

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1. INTRODUCTION

1. INTRODUCTION

1.1. Acrylamide

Acrylamide (CH_2=CH–$CONH_2$) is a colorless and crystalline powder chemical compound, soluble in water and with a low molecular weight (71g/mol). It is a neurotoxic and probably carcinogenic compound (IARC, 1994). Although acrylamide only has been related to the manufacturing industries of products such as adhesives, fibers, drugs, paper or textiles and with tobacco consumption, (Smith & Oehme, 1991), Swedish researchers discovered in 2002 high amounts in certain foods (Tareke, Rydberg, Karlsson, Eriksson, & Tornqvist, 2002). Further studies revealed the presence of acrylamide in foodstuff, mainly based on carbohydrates subjected to high temperatures (>120ºC) as a consequence of Maillard reactions (Mottram, Wedzicha, & Dodson, 2002; Stadler et al., 2002; Tareke et al., 2002). The main precursors of acrylamide are the amino acid asparagine and the reducing sugars (especially fructose and glucose) however, other acrylamide formation routes have been proposed (Eriksson, 2005).

1.2. Dietary intake

Among all the products likely to present acrylamide, fried potatoes are remarkable because potatoes have a high content of asparagine (about 62 mmol / g dry basis) and reducing sugars, the last being the limiting factor (Amrein et al., 2003). In addition, acrylamide is present in a wide range of products commonly consumed as bakery products (bread, biscuits, pastries), coffee and substitutes, battered and breaded products, breakfast cereals... (Europe, 2013). The european intake of acrylamide from 2007 to 2009 was analyzed in different population groups, and the results are described in table 1.1.

Table 1.1. *Acrylamide intake ranges (µg/kg of body weight per day) corresponding to adults, adolescents, children and toddler, and main food contributors to acrylamide exposure. Adapted from EFSA, (2011)*

Population group	Acrylamide intake (µg/kg of bw per day)	Major contributors to overall exposure
Adults (>18 years)	0.31-1.1	Fried potatoes, coffee and soft bread
Adolescents (11-17 years)	0.43-1.4	Fried potatoes, soft bread and biscuits or potato crisps
Children (3-10 years)	0.7-2.05	Fried potatoes, soft bread and unspecified bread biscuits
Toddler (1-3 years)	1.2-2.4	Fried potatoes, soft bread and unspecified bread biscuits

1.3. Routes of acrylamide formation

The reaction of asparagine (which provides the carbonaceous structure) and a carbonyl compound (reducing sugars) during heating, causes dehydration and results in the formation of the Schiff base by corresponding N-glycosylation and decarboxylation reactions (Figure 1.1). Subsequent reactions of hydrolysis, decarboxylation or deamination lead to the formation of intermediate compounds of different degrees of reactivity that can finally trigger in the formation of acrylamide (Medeiros Vinci, Mestdagh, & De Meulenaer, 2012; Yaylayan, Wnorowski, & Perez Locas, 2003). Studies based on model systems use asparagine and fructose and/or glucose to start acrylamide formation process, as they are considered the main precursors.

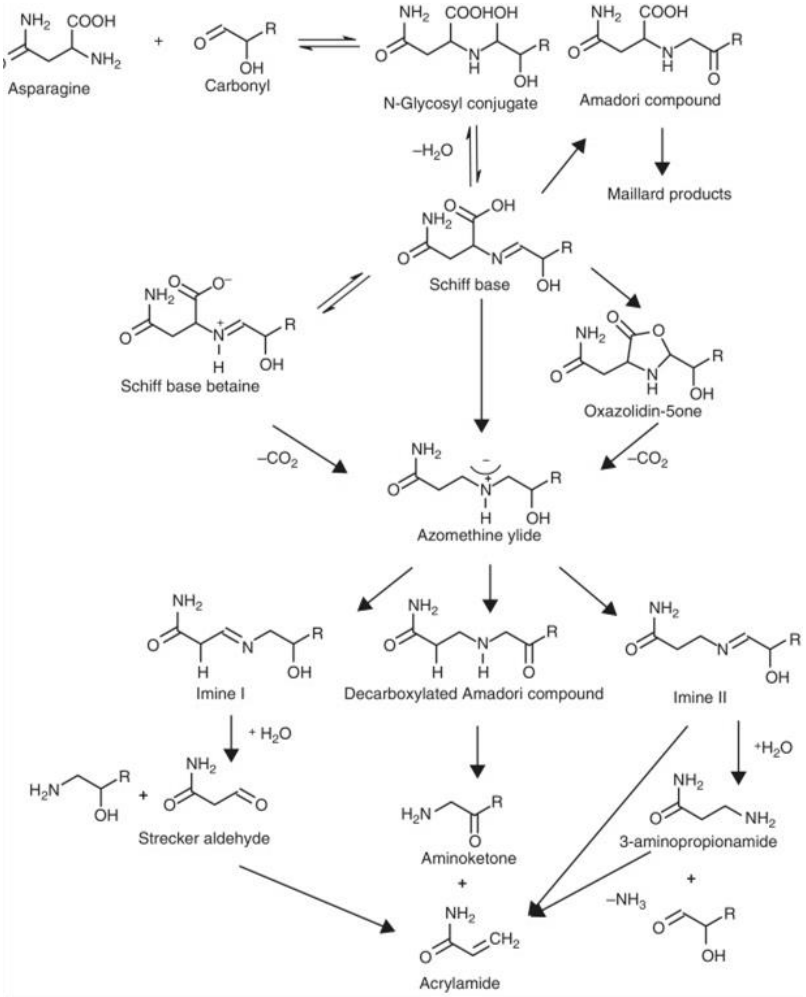

***Figure 1.1.**. Main route of acrylamide formation from asparagine and reducing sugars during heating (adapted from Mogol & Gökmen, 2016).*

Other pathways of acrylamide formation have been proposed by different authors and are schematically presented in Figure 1.2 (Eriksson, 2005; Stadler et al., 2003; Yaylayan, Perez Locas, Wnorowski, & O'Brien, 2005).

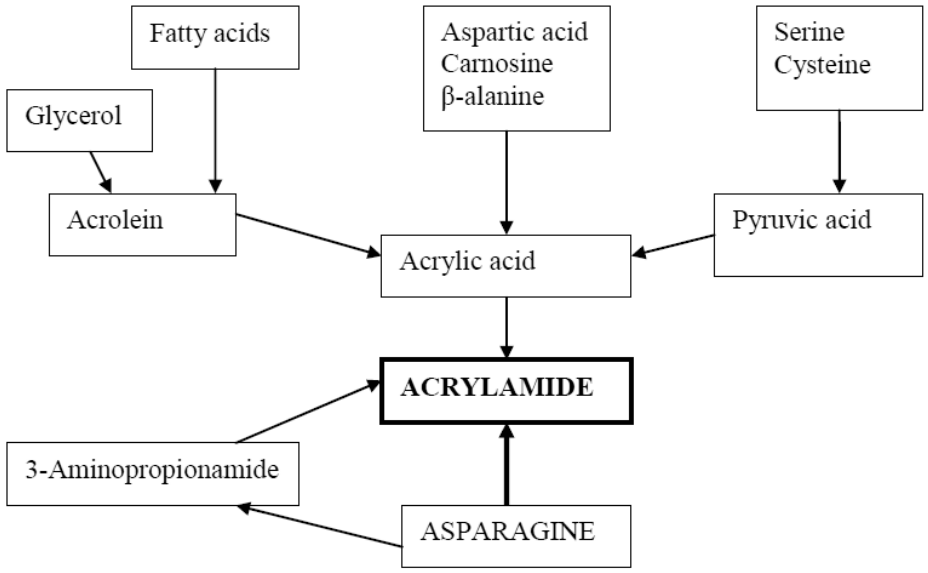

Figure 1.2. *Alternative ways of acrylamide formation (adapted from Eriksson, 2005)*

In products rich in fat, the formation of acrylamide can also be triggered by other pathways. In particular, acrolein, from oxidative degradation of lipids, or glycerol, causes the formation of acrylic acid, which can react with ammonia and form acrylamide (Yaylayan et al., 2005). Other compounds which may result in the formation of acrylic acid (β-alanine, aspartic acid, carnosine, serine or cysteine) are thus precursors of acrylamide (Eriksson, 2005).

1.4. Health risks

-Metabolism

According to data results of animals exposure analysis, acrylamide, after its oral intake, is quickly absorbed from the gastrointestinal tract and widely distributed to all tissues (FAO/WHO, 2005). Acrylamide is metabolized to a reactive compound, called glycidamide, or is conjugated with glutathione. It have been seen a quick elimination of acrylamide and their metabolites in urine, mainly

as mercapturic acids conjugates of acrylamide and glycidamide (FAO/WHO, 2005).

-*Carcinogenesis*

Acrylamide was classified by IARC (International Agency for Research on Cancer) as a Group 2A probable carcinogen for humans. In rodents, acrylamide is related to multi-organ cancer, but in humans, dietary acrylamide might increase the risk of endometrial and ovarian cancers, especially among high acrylamide consumers, myeloma and follicular lymphoma in men, and esophageal cancer, specially related to obese people (Mendel Friedman, 2015).

-*Genotoxicity*

The genotoxic character of acrylamide is derived from a metabolite called glycidamide. This compound is an epoxide that is formed in the organism, metabolized by cytochrome P450 monooxygenase CYP2E1 (Figure 1.3) (Blank, 2005).

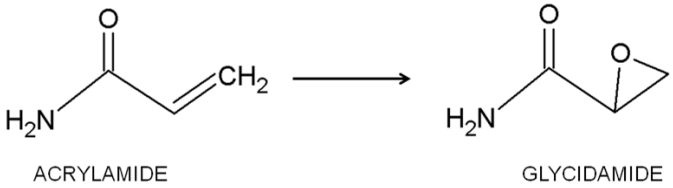

Figure 1.3. *Reaction of acrylamide to the epoxide metabolite glycidamide by cytochrome P450–mediated epoxidation (adapted from Blank, 2005)*

The high reactivity of the glycidamide makes its quantification difficult, since it quickly forms adducts mainly with gluthatione, hemoglobin, mercapturic acid, and DNA (Granvogl, Koehler, Latzer, & Schieberle, 2008). Glycidamide may be metabolized to glyceramide (by epoxide hydrolase or by conjugation to glutathione), or it may react with proteins, including hemoglobin, or with deoxyribonucleic acid (DNA) (FAO/WHO, 2011). The reactions of glycidamide with DNA and hemoglobin are the responsible of the genotoxic and clastogenic character (FAO/WHO, 2005).

- Neurotoxicity

Neurotoxic character of acrylamide is based on its ability to form adducts with thiol (-SH) and amino (-NH_2) groups in the amino acids cysteine and lysine, which are part of neuron residues (Mendel Friedman, 2015). Recent research has sought to mitigate the neurotoxic effects of acrylamide with compounds from foods or plants such as supplementation with leaf extract from the *Bacopa monnieri* plant, or bioactive compounds such as crysin (a flavonoid), curcumin or geraniol, eugenol, linalool , soy sauce, Vitamin E or fish oil (Mendel Friedman, 2015).

-Tolerable dose of acrylamide

EFSA's scientists did not set a tolerable daily intake (TDI) of acrylamide in food, however, they stablished the dose range within which acrylamide is likely to cause a measurable tumor incidence or other potential adverse effects (neurological, pre- and post-natal development and male reproduction). The lower limit of this range is called the Benchmark Dose Lower Confidence Limit ($BMDL_{10}$). For tumors, a $BMDL_{10}$ of 0.17mg/kg bw per day was selected and for other effects, (concretely neurological effects) a $BMDL_{10}$ of 0.43 mg/kg bw per day was set (EFSA, 2015). EFSA's Scientific Committee stated a Margin of Exposure (MOE) for the acrylamide cancer effects range from 425 to 50 for average adult consumers and high consuming toddlers, respectively, meaning a high public health concern (EFSA, 2015).

1.5. Methods of acrylamide analysis

The methods of acrylamide determination require a high sensitivity, since some foods contain levels around parts per billion (µg/kg), while others, such as coffee or potato chips reach levels of parts per million (mg/kg). The main analytical techniques are liquid chromatography and gas chromatography coupled to mass spectrometry (LC/MS and CG/MS, respectively). Both techniques are validated by interlaboratory studies, and their usability has different limitations. The LC/MS method is simpler and requires less time than

CG/MS, but requires an accurate methodology for sample extraction and purification (Wenzl et al., 2006). Generally the equipment used in liquid chromatography uses a triple quadrupole (MS/MS), because it improves the selectivity in the identification and quantification of acrylamide. Specifically, the MRM (Multiple Reaction Monitoring) method is used, in which a product ion is monitored from a precursor ion and some of the following transitions are determined: m/z 72→55 (used for quantification), 72→27, 72→54, 72→44. The main limitation of the GC/MS method is that a derivatization step is needed, generally by bromination, to increase acrylamide volatility. Similar results of acrylamide by both techniques were obtained from an study of a European inter-laboratory, showing higher performance and precision by LC than GC, in bakery products and potato chips (Wenzl et al., 2006). The addition of an internal standard, such as $^{13}C_3$-acrylamide (isotopically labeled acrylamide), is recommended to ensure reproducibility, especially when different food matrix are analyzed (Eriksson & Karlsson, 2006).

Other analytical methods with less sensitivity have been used, like HPLC with ultraviolet detector, however, its use is not recommended because they have not been effective in products with low levels of acrylamide (Lineback, Coughlin, & Stadler, 2012). In addition, novel and promising methods have been developed, such as capillary electrophoresis, or bioanalytical methods as enzyme-linked immunosorbent assays (ELISAs) or the use of electrochemical biosensors (Oracz, Nebesny, & Żyżelewicz, 2011).

The complexity of food matrices means a laborious and complex sample preparation for the extraction of substance. The type of solvent used and the steps of extraction procedure are two key factors to take into account for a correct extraction of acrylamide, because, depending on the food matrix, the acrylamide content might be underestimated by 14-30% (Gökmen, Morales, Ataç, Serpen, & Arribas-Lorenzo, 2009).

The preparation of the sample for the extraction of acrylamide consists on the elimination of interferences and the purification of the sample, and the following

steps are commonly carried out: fat removal (with a non-polar organic solvent), solid-liquid extraction, precipitation of proteins, purification by solid phase extraction (SPE) and dissolution in an suitable buffered solution (Oracz et al., 2011). A new version of the classic SPE is the so-called dispersive solid phase extraction, commonly called QuEChERs or dSPE (dispersive Solid Phase Extraction). This technique consists on mixing the sample solution with specific salts and subsequently centrifuged, rather than traversing the liquid sample through a cartridge (Oracz et al., 2011).

1.6. Strategies to reduce acrylamide formation

FoodDrinkEurope is the European food industry confederation which purpose is promote manufacturers interests in the areas of food safety and science, nutrition and health, consumer trust and choice, competitiveness, and environmental sustainability. Concretely, they published an Acrylamide Toolbox which includes guides, in constant update, whose purpose is helping food manufacturers to reduce acrylamide content in their products (Europe, 2013). These guides include the latest scientific advances in this subject and classifies the strategies depending on the foodstuff: potato-food products (including French fries and chips), cereal based products (breads, biscuits, breakfast cereals…), coffee, roast grains and substitutes and infant foods. In general, these recommendations establish levels of action independently of the food group, and strategies can be classified according to the process stage: at the agronomic level; modifying the recipe or size (and therefore the surface/volume ratio); controlling the thermal process or the attributes of the final product. The composition in acrylamide precursors and the conditions of the thermal process are the most important factors that determine the final acrylamide content.

- Importance of raw material

Limiting the content of reducing sugars and asparagine is a common goal in all food groups. In potato products, despite its high asparagine content (30-60% of the total free amino acids) which is difficult to control, results essential to limit

the levels of reducing sugars, for example, by selecting an appropriate potato variety performing good agricultural practices, or controlling the storage and the degree of ripening (Biedermann-Brem et al., 2003).

The lack of nitrogen in the soil is a factor to consider due to its negative impact on the formation of asparagine. In addition, potato storage at low temperatures (<4-6ºC) favors the accumulation of reducing sugars so it is recommended to storing potatoes at 8-12ºC (Ohara-Takada et al., 2005; Viklund, Olsson, Sjöholm, & Skog, 2010).

Cereal products (bread, breakfast cereals, biscuits or batters) have different levels of acrylamide, mainly due to different asparagine content, which can be modified by selecting the suitable cereal varieties or by changing the ingredients of the formula. For example, replacing ammonium bicarbonate with sodium bicarbonate in gingerbread formulation, reduces the formation of acrylamide (Amrein, Schönbächler, Escher, & Amadò, 2004).

-Effect of processing conditions

The effect of thermal process variables, temperature and time, as well as sample size on acrylamide formation has been extensively studied (Ahrné, Andersson, Floberg, Rosén, & Lingnert, 2007; Bråthen & Knutsen, 2005; Gökmen & Palazoğlu, 2009). In fried products, since acrylamide is mainly generated on the surface, the content will also increase if the ratio of surface to volume increases. That is why the same potato batch, will trigger more acrylamide content in potato chips than French fries because of its larger surface/volume ratio when undergoing the same frying conditions (Gökmen & Palazoğlu, 2009). During baking, the time and temperature of the treatment are closely linked to the final acrylamide content (Ahrné et al., 2007; Elmore, Koutsidis, Dodson, Mottram, & Wedzicha, 2005; Mustafa, Andersson, Rosén, Kamal-Eldin, & Åman, 2005).

Deep-oil frying has been modified to obtain products with lower oil and acrylamide content. Vacuum frying is an example, it implies vacuum pressure

and low temperature because they trigger a reduction of oil and water boiling points (Garayo & Moreira, 2002; Granda, Moreira, & Tichy, 2004). Concretely, an acrylamide reduction of 94% was observed in potato chips, and besides, the quality of oil was maintained due to the low temperature required in the process (Granda et al., 2004).

-Treatments before frying

Blanching is a basic step in the production of French fries, to improve the color and texture of the final product and also could reduce, in some cases, the oil uptake (Califano & Calvelo, 1988).

Blanching step consists of submerging the raw potatoes in hot water (at 70-100°C) for a short time (5-10 minutes). This process provokes heat and matter exchanges that favor the leaching out of reducing sugars and asparagine (Pedreschi, Kaack, & Granby, 2004; Pedreschi, Moyano, Kaack, & Granby, 2005). The formation of acrylamide can be reduced around 91-97% with long blanching treatments (70 min at 50ºC or 40 min at 70ºC) in potato chips at 190ºC (Pedreschi et al., 2004).

Irradiation of potatoes before storage could inhibit sprouting and thus, the increase of reducing sugars content (reduction of 10.7%) and acrylamide (reduction of 8.7%) (Mulla et al., 2011).

Fermentation is a basic step in the bread production in which, levels of acrylamide precursors (mainly asparagine) are reduced, especially when fermentation times are extended (Baardseth et al., 2004; Claus, Mongili, Weisz, Schieber, & Carle, 2008; Fredriksson, Tallving, Rosén, & Åman, 2004).

-Effects of additives addition

Certain chemical agents have been proved to reduce acrylamide content, blocking or interfering different stages in the Maillard reactions. Generally, the different additives are added to an aqueous solution where the potatoes are soaked, or are directly incorporated in the formulation as in cookie doughs, breads or batters.

The use of asparaginase, an enzyme that hydrolyzes asparagine in aspartic acid (Figure 1.4), has been widely tested as a useful technique for reducing the final content of acrylamide in French fries, potato cake, or gingerbread (Amrein et al., 2004; Ciesarova, Kiss, & Boegl, 2006; Hendriksen, Kornbrust, Østergaard, & Stringer, 2009; Pedreschi, Kaack, & Granby, 2008; Pedreschi, Mariotti, Granby, & Risum, 2011). Pedreschi et al., (2011) evaluated the combination of blanching and the use of asparaginase, obtaining reductions of acrylamide up to 90%. In addition to the respective leaching of the acrylamide precursors, the modification of the microstructure during blanching (gelatinization of starch, rupture of the cell walls...) favors the diffusion of the asparagine by improving enzyme-substrate binding.

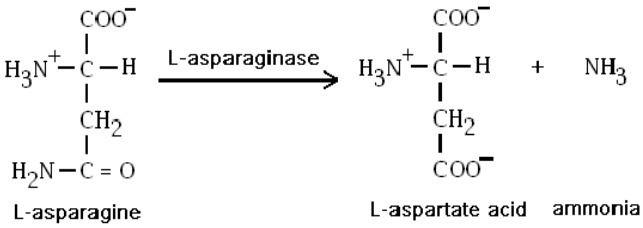

Figure 1.4. *Catalytic reaction of L-asparaginase enzyme. (Singh & Srivastava, 2012)*

Amino acids have been used as chemical agents that compete with asparagine in the union with reducing sugars, concretely, glycine, alanine, lysine, glutamine and glutamic acid added in model systems, reduced 42-70% of the acrylamide content (Rydberg et al., 2003). Kim, Hwang, & Lee, (2005) confirmed the use of lysine, glycine and cysteine in fried snacks (wheat-flour and potato basis).

In baked food products, the use of sodium hydrogencarbonate ($NaHCO_3$) instead of ammonium hydrogencarbonate (NH_4HCO_3) could reduce significantly acrylamide content, for instance in gingerbread, it was reduced up to 60% acrylamide formation (Amrein et al., 2004).

Another way of blocking the reactions that trigger the formation of acrylamide is the use of organic acids, since Maillard reaction is pH dependent (Jung, Choi, & Ju, 2003; Low et al., 2006). Jung et al., (2003) studied the effect of citric acid on model systems, baked corn flakes (by adding 0.1% and 0.2% in the formulation) and French fries (by dipping in 1 and 2% of citric acid solutions before frying). Results showed high reduction levels in acrylamide formation (>70%), probably by protonation of asparagine amino group ($-NH_3^+$) what might block its union with reducing sugars. Although this chemical seems to be very advantageous in terms of its potential reduction of acrylamide, its use could cause undesirable sensory repercussions. Jung et al., (2003) did not observe marked sensory disturbances, except a lighter color of samples of baked corn chips and French fries dipped in 1% of citric acid, however, pretreated potatoes at 2% showed a light undesirable taste and texture. Amrein et al., (2004) studied the addition of citric acid in gingerbread. Acrylamide reductions of 73 and 97% where achieved (at 0.5 and 1% of citric acid) but, samples had a clearly acidic taste and its leavening was not sufficient, probably due to the protonation of NH_3 which reduced gas volume during baking.

The effect of mono and divalent cations on acrylamide formation has been extensively studied in model systems and food products (Gökmen & Şenyuva, 2007; Lindsay & Jang, 2005; Pedreschi, Granby, & Risum, 2010; Tomoda, Hanaoka, Yasuda, Takayama, & Hiwatashi, 2003). The mechanism for the reduction of acrylamide raised by these authors is based on the ability of these cations to interact with asparagine and therefore avoid the linkage with carbonyl groups. It should be noted that the addition of salts to the formulation of products that which require a fermentation step (bakery products and pastries), might influence the development of yeasts and thus, a correct fermentation (Voelker, 2005).

Other strategies focused on blocking Maillard reactions are based on the use of reactive compounds, mainly antioxidants, as plant extracts, spices or pure substances (Morales, Capuano, & Fogliano, 2008; Zhang, Chen, Zhang, Wu, &

Zhang, 2007). There is great controversy regarding the results obtained. Some authors have observed important reductions in acrylamide, but the addition of other compounds such as butylhydroxytoluene (BHT), sesamol or vitamin E to meat, favored the formation of acrylamide, probably because they protect acrylamide molecule against free radical-initiated reactions (Tareke, 2003). Phenolic antioxidants from cranberry and oregano extracts did not reduce acrylamide in potato slices, but chickpea flour, rich in proteins reduced acrylamide formation (Vattem & Shetty, 2003). Taurine addition in potato chips reduced acrylamide content, probably because its free amino group would compete with asparagine for reducing sugars linkage (Shin et al., 2010).

Hydrosoluble vitamins as biotin, pyridoxine, ascorbic acid, and pyridoxamine presented an effective inhibitory effect of the generation of acrylamide in model systems (> 50%), possibly due to its antioxidant character. However, the fat-soluble vitamins did not significantly reduce the formation of acrylamide. The immersion of potatoes in 1% nicotinic acid (for 1 hour) was the most effective vitamin in acrylamide mitigation, up to 51% in potato chips (Zeng et al., 2009).

The use of hydrocolloids in fried products has been widely used in order to obtain fried products with lower fat content. Zeng et al.,(2010) concluded that pectin and alginic acid in model systems and crackers were promising inhibitors of acrylamide formation. Immersion of potatoes in 1 and 5% alginic acid solutions for 5 hours resulted in 30 and 60% acrylamide reductions, respectively, in French fries.

Some strategies aimed in reducing acrylamide formation independently of frying conditions and raw material composition are summarized in Table 1.2. Those strategies would be a previous step to the frying process that might be included in the industrial scale to achieve the purpose of mitigating acrylamide in the final product.

***Table 1.2**. Summary of some strategies to reduce acrylamide in different food products.*

STRATEGY	FOOD PRODUCT	MECHANISM	ACRYLAMIDE REDUCTION (%)	REFERENCES
BLANCHING	Potato chips** and French fries*	Leaching of reducing sugars and asparagine	From 49 to 97%, depending on blanching conditions	Pedreschi et al., (2004, 2005)** Viklund et al., (2010)*
BLANCHING +ADDITIVE	Chips	Blanching reduces precursors and favors the interaction of additives (modifies potato microstructure)	+ asparaginase: ~90% [A] +NaCl: ~41% [B]	Pedreschi et al., (2011) [A] Pedreschi et al., 2010) [B]
FERMENTATION	Bread	Lactic bacteria and yeasts use reducing sugars an asparagine	75% in crisp bread [C] ~55% in bread rolls [D] 87% in whole grain wheat bread and 77% in rye bran bread [E]	Baardseth et al., (2004) [C] Claus et al., (2008) [D] Fredriksson et al., (2004) [E]
ASPARAGINASE	Gingerbread French fries Chips	Enzymatic degradation of asparagine	55% in gingerbread [F] >30% in French fries [G] 17% in chips [H]	Amrein et al., (2004) [F] Pedreschi et al., (2008 [G], 2011 [H]).
AMINO ACIDS	Potato model systems Fried snacks Gingerbread	Competence with asparagine	30-70% in potato model systems [I] 42-80% in fried snacks [J] 14-70% in gingerbread [K]	Low et al., (2006) [I] Kim, Hwang, & Lee, (2005) [J] Amrein et al., (2004) [K]

***Table 1.2**. Continues*

STRATEGY	FOOD PRODUCT	MECHANISM	ACRYLAMIDE REDUCTION (%)	REFERENCES
ACIDS (citric acid)	Model systems French fries Baked corn chips Gingerbread	Protonation of the amino group of asparagine	~20% in model systems[L] >73% and >49% in French fries and baked corn chips, respectively [M] >73% in gingerbread[N]	Low et al., (2006) [L] Jung et al., (2003) [M] Amrein et al., (2004) [N]
CATIONS: Na^{+}, Ca^{2+}; Fe^{3*}	French fries Chips	Interaction with asparagine	40-58% with NaCl and 79-95% with $CaCl_2$ in F. fries [O] 59 and 79% in chips with $FeCl_3$ and $CaCl_2$, respectively [P]	Gökmen & Şenyuva, (2007) [O] Lindsay & Jang, (2005) [P]
ANTIOXIDANTS Plants extracts (bamboo leaves, AOB) and vitamins	French fries Chips Model systems	Unknown mechanism	74.1% and 76.1% in chips and F. fries (at 0.1% and 0.01% AOB (w/w)[Q] 51% and 34% in F. fries (nicotinic acid and pyridoxal, respectively) and 75-80% in model systems [R]	Zhang et al., (2007) [Q] Zeng et al., (2009) [R]
HYDROCOLOIDS Pectin Alginic acid	Model systems Crackers French fries	Improved water retention might avoid surface dehydration and thus, may hinder acrylamide formation	50-65% in model systems 30-50% in crackers 30-60% in French fries	Zeng et al., (2010)

As seen above, there is an important number of strategies to reduce acrylamide in the literature, but it is remarkable that some of them have been evaluated only in model systems, and not in the real product, where possible matrix effects have not been considered. In addition, the explored strategies must guarantee the preservation of the organoleptic and nutritional quality of the final product, and this is not an easy attribute to achieve in some of the techniques proposed to reduce acrylamide. Acid, bitter off-flavours, brittle textures or unpleasant odor must appear when citric acid, calcium salts or cysteine are used as pretreatments (Medeiros Vinci et al., 2012).

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2. OBJECTIVES AND EXPERIMENTAL PLAN

2. OBJECTIVES AND EXPERIMENTAL PLAN

2.1. Objectives

The general objective of this doctoral thesis is the evaluation of acrylamide in foods and the development of some strategies for its reduction. In order to achieve this objective, the following specific objectives were defined in three blocks:

i. Influence of pre-frying treatments and frying process variables on the formation of acrylamide in fried potatoes.

- To evaluate the effect of pre-frying treatments on removing of reducing sugars (precursors of acrylamide formation) in potatoes before frying.

- To set up and to validate the analytical method for the determination of acrylamide in fried potatoes.

- To study acrylamide formation during air-frying process using deep oil frying as the reference frying method. In parallel, to evaluate the color changes and their potential relation to acrylamide content in fried potatoes obtained by the two frying methods.

- To compare the effect of the different pre-treatments on the formation of acrylamide and color development of air fried potatoes and deep-oil fried potatoes.

ii. Functionality of chitosan as ingredient to mitigate acrylamide formation in batter formulations.

- To evaluate the impact of different concentrations of chitosan (0, 0.5 and 1%) on the formation of acrylamide in model systems (solutions of reducing sugars and asparagine).

- To analyze the influence of the main factors affecting the formation of acrylamide in model systems: type of reducing sugars, temperature of the reaction and pH of the solution, as well as the influence of adding chitosan on the acrylamide content during heating.

- To evaluate the impact of chitosan addition to batter formulation on the formation of acrylamide during frying.

- To study the influence of chitosan characteristics (molecular weight and the degree of deacetylation) on the formation of acrylamide in model systems.

- To evaluate the effect chitosan addition to batter formulations on physicochemical properties of raw batters (rheological behavior and water retention capacity) and fried batters (oil absorption, color and texture).

- To analyze the influence of rice and wheat flour combinations on the rheological and thermal properties of chitosan based batter systems.

iii. Evolution of acrylamide content during gastrointestinal digestion of different thermally processed foods.

- To determine the acrylamide content after in vitro gastric digestion of a selection of food products.

- To analyze changes on the acrylamide content on French fries and chips during the two main digestion steps (gastric and intestinal).

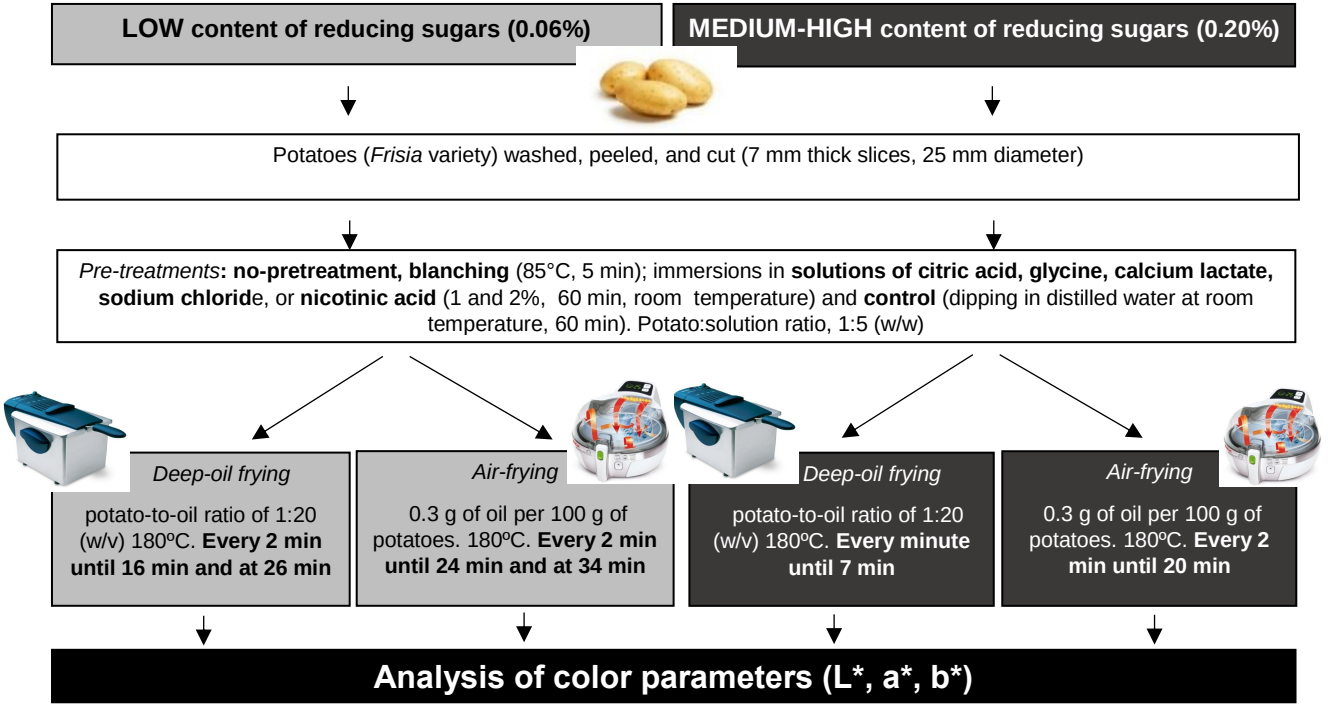
-To study the influence of air-frying and blanching followed by deep-oil frying on the evolution of acrylamide content at the beginning and after the gastric and intestinal digestion of French fries.

2.2. Experimental plan

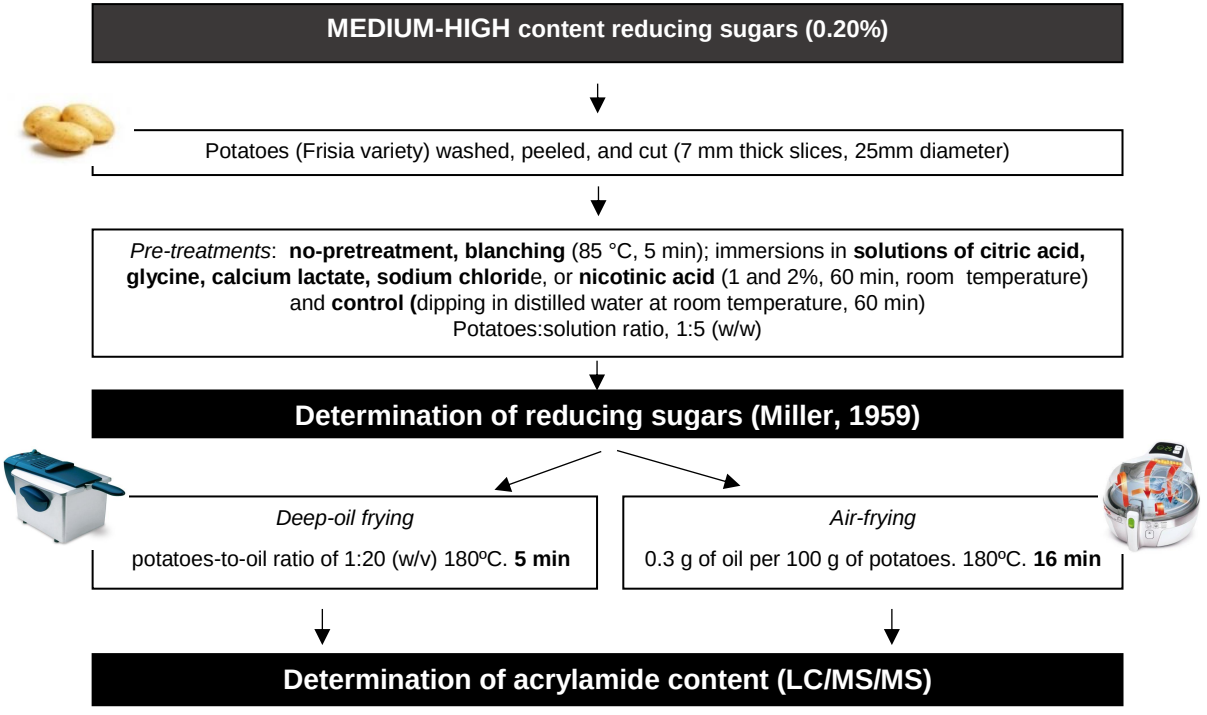
In order to accomplish the specific objectives previously exposed, the following experimental plan was carried out. It is structured according to the correspondent blocks as in the objectives section. Furthermore, bibliography in relation to the topic was periodically reviewed. Statistical techniques were used for data processing in all cases.

The different processes and determinations corresponding to each block are schematically shown below:

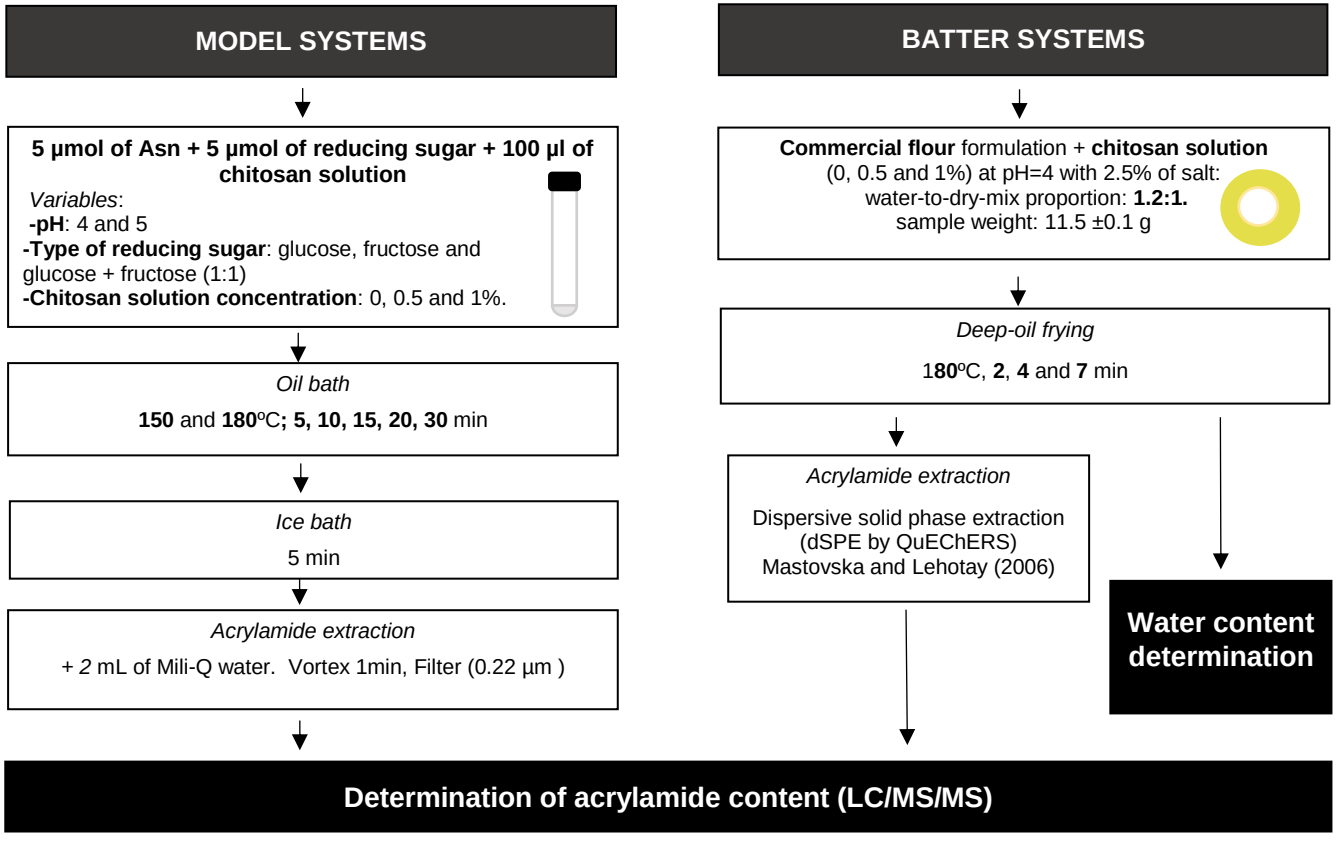
i. Influence of pre-frying treatments and frying process variables on the formation of acrylamide in fried potatoes. Paper I (1st Part)



i. Influence of pre-frying treatments and frying process variables on the formation of acrylamide in fried potatoes. Paper I (2nd Part)

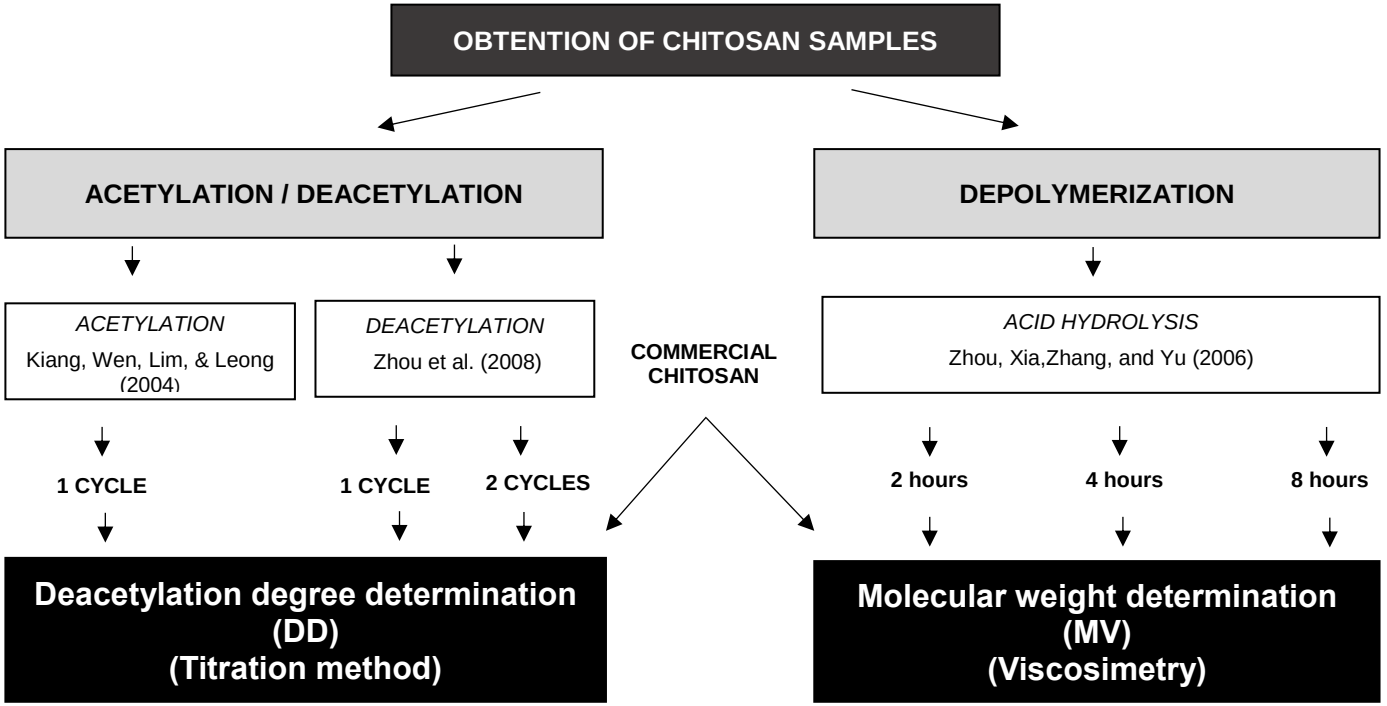


ii. Functionality of chitosan as ingredient to mitigate acrylamide formation in batter formulations. Paper II



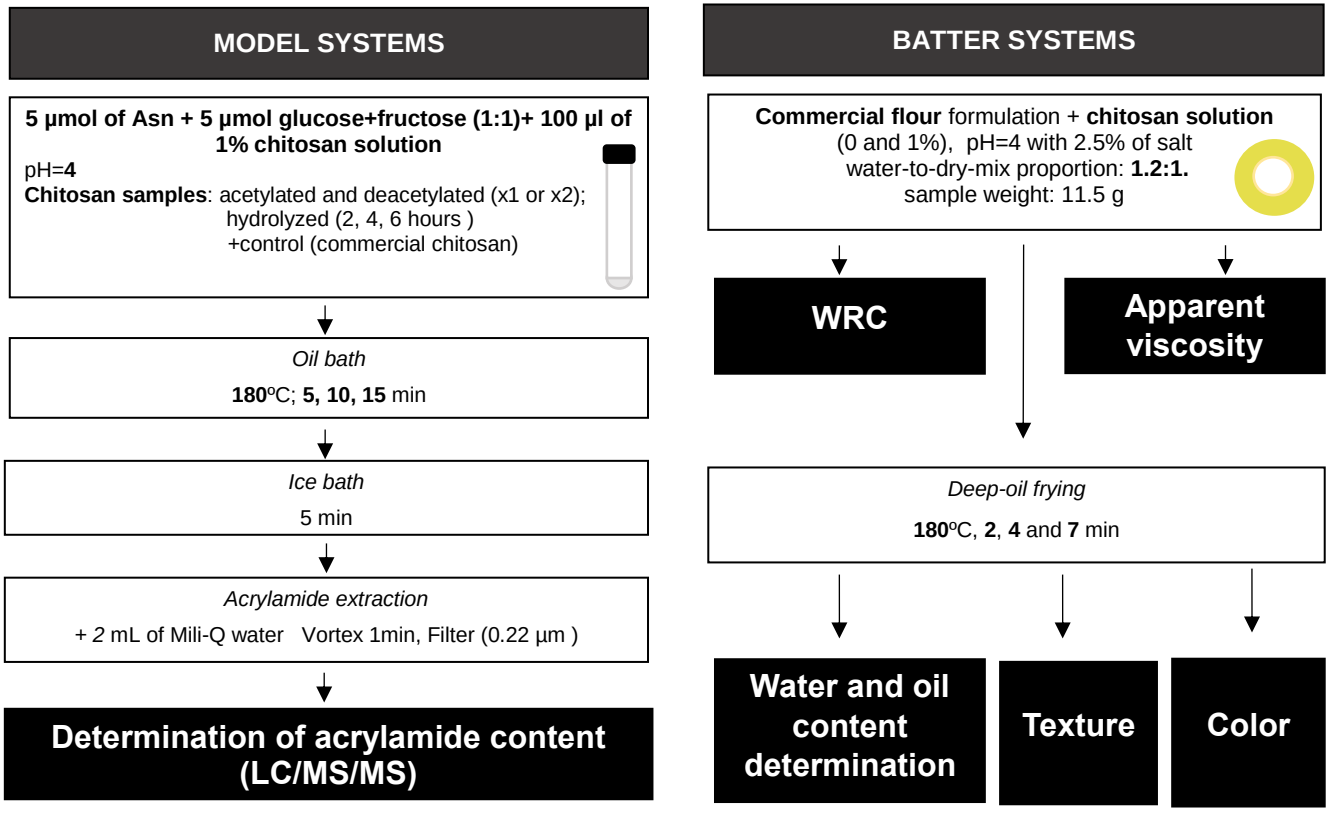
ii. Functionality of chitosan as ingredient to mitigate acrylamide formation in batter formulations.

Paper III (1st Part)



ii. Functionality of chitosan as ingredient to mitigate acrylamide formation in batter formulations.

Paper III (2nd Part)



ii. Functionality of chitosan as ingredient to mitigate acrylamide formation in batter formulations. Paper IV

BATTER SYSTEMS

Formulations:
Rice and wheat flour combinations (100:0; 70:30; 30:70; 0:100)
+ **chitosan** (0, 0.25, 0.5 and 1%),
2.5% of salt; 3.1% of sodium bicarbonate
Water-to-dry-mix proportion: **1.2:1.**

FLOW BEHAVIOR

Strain/stress control rheometer
Fitted to Herschel-Buckley model

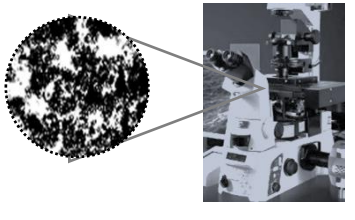
$$\tau = \tau_0 + K \cdot \gamma^n$$

THERMAL PROPERTIES

-Gelatinization enthalpy and temperature
-Glass transition
-Melting temperature and enthalpy
-Unfrozen water content

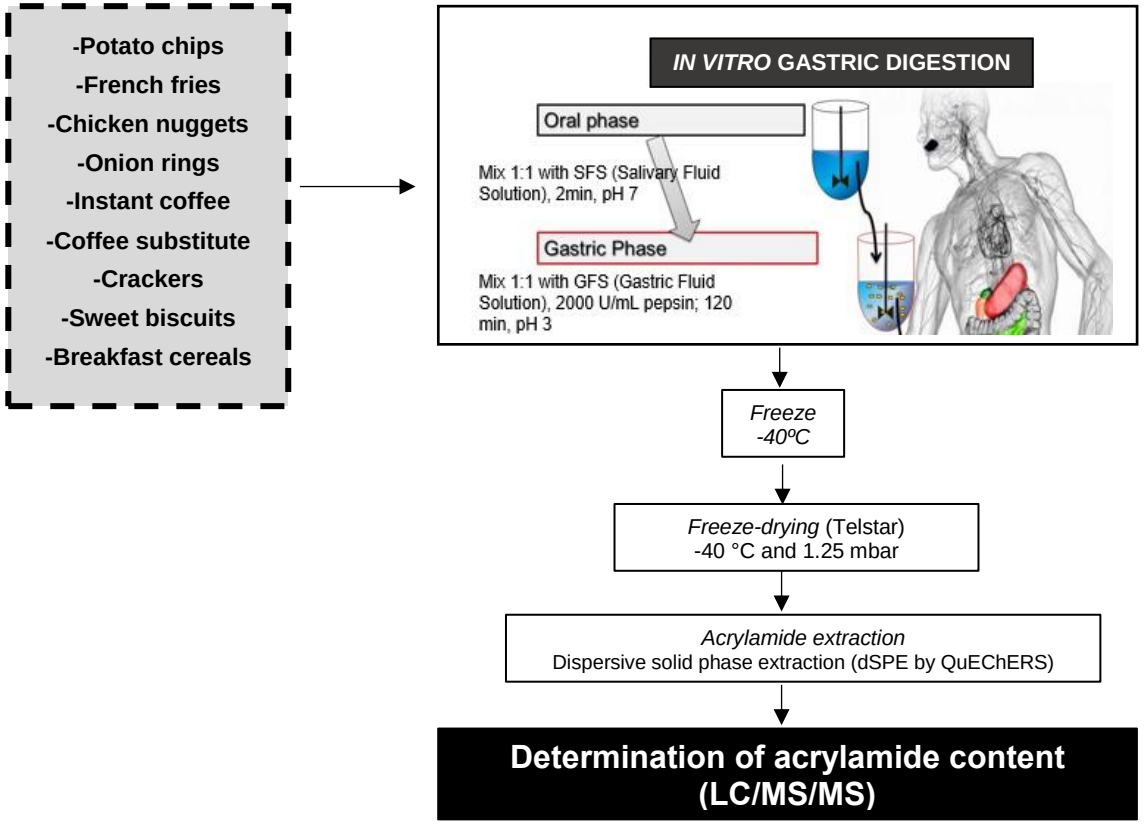
MICROSTRUCTURAL ANALYSIS

-Feret diameter
-Minimum distance between particles



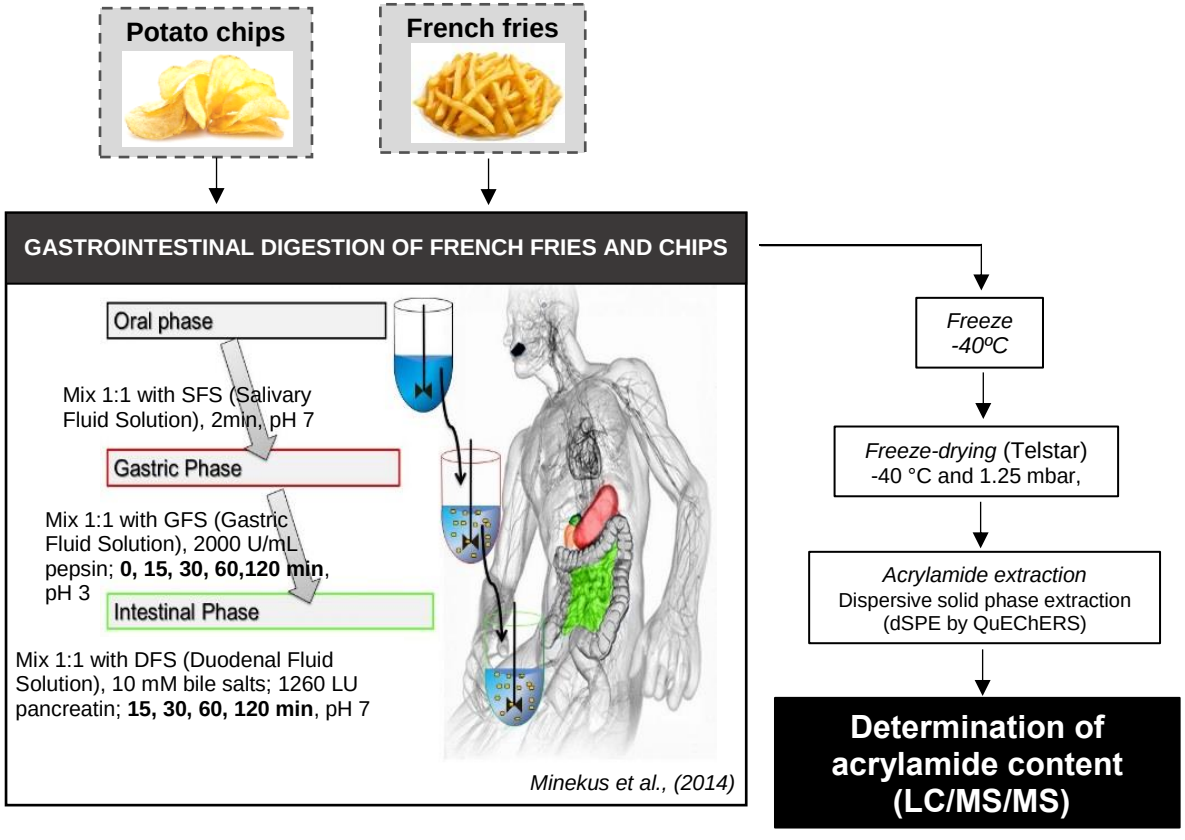
***iii. Evolution of acrylamide content during gastrointestinal digestion of different thermally processed foods*.**

Paper V (1st Part)



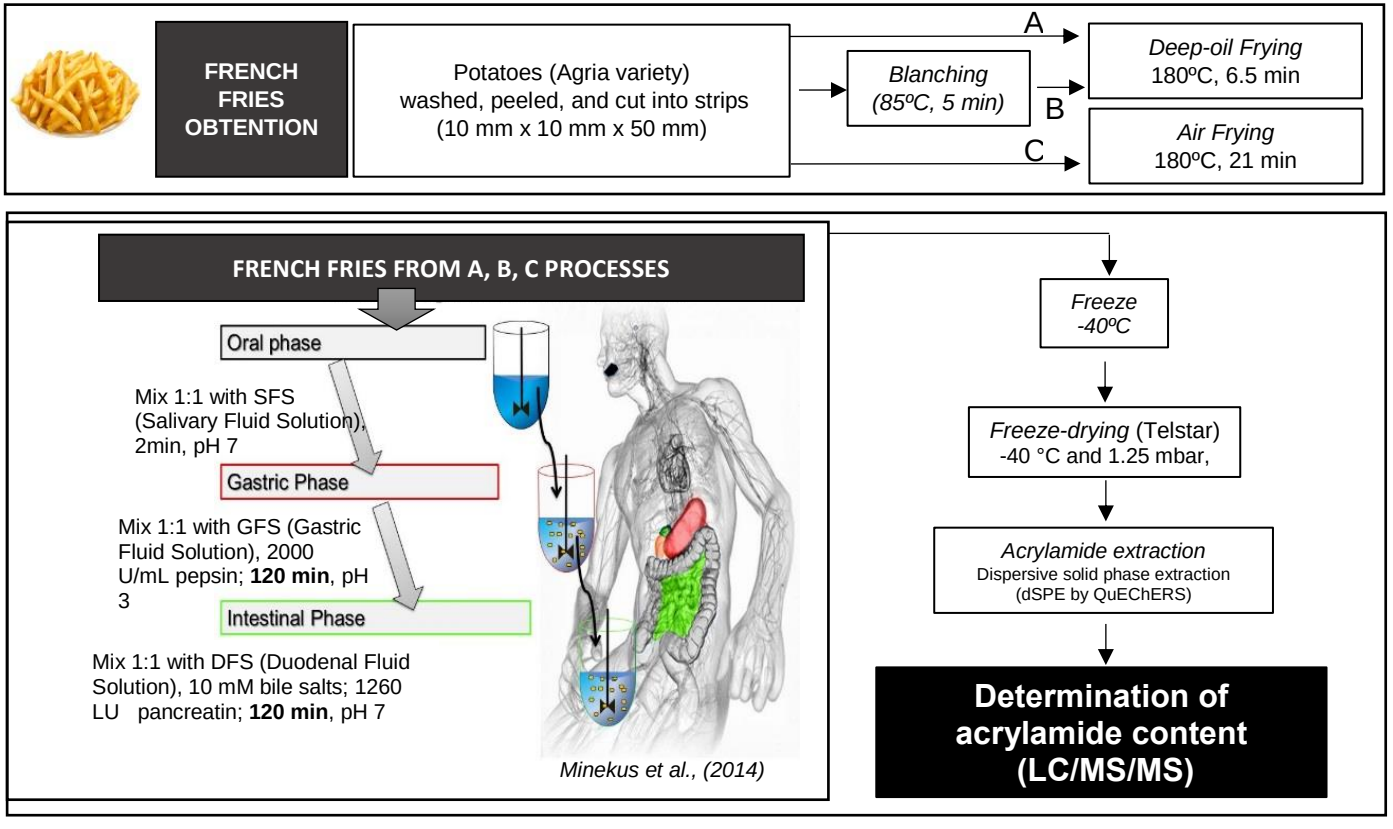
iii. Evolution of acrylamide content during gastrointestinal digestion of different thermally processed foods.

Paper V (2nd Part)



iii. Evolution of acrylamide content during gastrointestinal digestion of different thermally processed foods.

Paper V (3rdPart)



3. EXPERIMENTAL METHODOLOGY

3. EXPERIMENTAL METHODOLOGY

This section includes the materials and methods employed in the experimental plan carried out in this doctoral thesis. It is divided into four subsections: sample preparation, frying step (or pyrolysis step), *in vitro* simulated digestion and analytical determinations.

3.1. Sample preparation

i. Model systems

The studies developed in Papers II and III were carried out on model systems. Concretely, the main objectives were to evaluate the effect of the percentage of chitosan, glucose and/or fructose presence, pH, temperature and time of the reaction (Paper II), as well as deacetylation degree and molecular weight of chitosan (Paper III), on acrylamide generation.

Model reactions were carried out following the method proposed by Gökmen & Şenyuva, (2007) with some minor modifications. 25 mL threaded Pyrex tubes were used to execute the reactions. Concretely, 5 μmol of asparagine and 5 μmol of reducing sugars (fructose and/or glucose) were dissolved in 100 μl of 0.5% acid lactic solution (with or without chitosan). Tubes kept closed along experiments.

Chitosan is a biopolymer with many properties, and most of them depend on the deacetylation and polymerization degrees (Aranaz et al., 2009). For this reason, commercial chitosan was submitted to acetylation and deacetylation processes and acid hydrolysis to obtain chitosan with different deacetylation degrees and molecular weights as follows:

Acetylation and deacetylation of commercial chitosan

Chitosan (Poly (D-glucosamine), deacetylated chitin, high molecular weight) was used to obtain chitosan with different deacetylation degree (DD) by acetylation and deacetylation processes.

Acetylation process was performed according to Kiang, Wen, Lim, & Leong, (2004). Briefly, chitosan (15g) was dissolved in a solution of 2% acetic acid (300 mL), distilled water (400 mL) and methanol (800 mL), and stirred for 20 minutes. Then, 2 mL of acetic anhydride were added into the solution and the mixture was stirred for 12 hours. Subsequently, chitosan was precipitated with 1M NaOH, washed with water until neutral pH and dried under vacuum at 60ºC.

Deacetylation was done according to Zhou et al., (2008) with slight modifications: chitosan (10 g) was dissolved in 100 mL of NaOH solution (ratio of 1:2 (w/v)) for 30 minutes at 100ºC, washed repeatedly with distilled water and dried at 60ºC. This process was considered as a one cycle deacetylation process, and was done one or twice to obtain different DD. Once deacetylation process performed, chitosans were submitted to analyze in order to establish the DD of different chitosans as it is described in the analytical determinations section.

Depolymerization of commercial chitosan

Chitosan with different molecular weight (Mv) were obtained by acid hydrolysis according to the method described by Zhou et al., (2006) with minor modifications. Commercial chitosan (2g) was dissolved in 2% acetic acid (100mL), stirred and heated at 70ºC for different times (2, 4 and 8 hours). Then, the reaction mixture was neutralized with NaOH. Absolute ethanol was added (70 mL per liter of solution) in order to completely precipitate the chitosan. The samples were filtered, washed with distilled water, and dried at 60ºC.

The molecular weight (Mv) of the obtained chitosans was determined as described in analytical determinations.

ii. Batters systems

On the one hand, batter formulations with chitosan were prepared for evaluating the effect of chitosan on acrylamide generation when being an ingredient of a real food system. The impact of incorporating chitosan on the physical properties of raw batters, as well as on the texture, color, or oil content of fried ones was also studied (Papers II and III).

On the other hand, different batters of blends of rice and wheat flours were prepared with or without chitosan on thermal and rheological properties analyzed to stablish if capability of chitosan to modify/enhance thermal and rheological properties of rice and/or wheat flour-based batters (Paper IV).

Batter formulations consisted of a commercial wheat flour-formulation (Paper II and III) or blends of wheat and rice flours (Paper IV), with chitosan solution (at different concentrations) and 2.5% of salt in a water-to-dry-mix proportion of 1.2:1 (w:w). Formulations without chitosan were used as controls. Batter samples were kept for at least 30 min at room temperature before analysis or frying, to ensure an adequate hydration of the components.

iii. Pre-treated potatoes

Paper I aims to evaluate the effectiveness of different treatment prior to frying in reducing on acrylamide formation, as well as the impact of an emerging frying technique, hot-air frying, on this compound. Concretely, potato discs of *Frisia* variety (7 mm of thickness and 25 mm of diameter) were blanched (at 85 °C for 5 min) or dipped in solutions with different chemical agents (citric acid, glycine, calcium lactate, sodium chloride, or nicotinic acid) at 1% and 2% for 60 min at room temperature, and later fried by conventional deep-oil frying or hot-air frying. A control consisting of immersing the samples in distilled water for 60 min at room temperature was also performed. In all cases, the potato:solution ratio was 1:5 (w/w).

In Paper V, the changes undergone by acrylamide along gastrointestinal digestion were studied. Apart from some selected commercial food products with relevant content of dietary acrylamide (instant coffee, substitute coffee, salad and sweet biscuits, breakfast cereaks, onion rings, chicken nuggets, potato chips and French fries), *French* fries were prepared in the laboratory to study the effect of blanching and air-frying on the acrylamide content during the gastrointestinal digestion. For this purpose, potato strips of *Agria* variety (10mmx10mmx50mm) were air-fried and blanched at 85ºC for 5 min before deep-oil frying. Deep-oil fried samples were also obtained as control samples.

3.2. Pyrolysis procedure or frying step

Model systems (Papers II and III) were submitted to heat process in an oil bath as summarized in Table 3.1. During the heating processes, only the bottom of the tubes was covered with hot oil. After the reaction time, tubes were immediately placed on an ice bath for 5 min.

Table 3.1. Reaction times and tested conditions in each Paper where model systems were carried out.

	Paper II	Paper III
Reaction times	5, 10, 15, 20, 30 min	5, 10, 15 min
Tested conditions	Asn/Glu; Asn/Fru; Asn/Fru+Glu (1:1) pH 4 and 5 150 and 180 ºC 0, 0.5 and 1% of chitosan	Asn/Fru+Glu (1:1) pH 4 180 ºC 0 and 1% of chitosan

Raw batter samples (Papers II and III) were placed in an aluminum cylindrical instrument (11.5 ± 0.1 g sample weight) which was introduced in the fryer. The geometry of fried samples was 11 ± 1 mm (height) x 65 ± 2 mm (outer diameter) x 25 ± 1 mm (inner diameter). The aluminum tool used and the final shape is shown in Figure 3.1.

Figure 3.1. *Tool used for frying batters (left) and example of fried sample obtained (right).*

Batters and potatoes discs or strips were fried in a commercial deep-fat-fryer (model: FM 6720 Ideal 2000 Professional, Solac; 2000W) (Figure 3.2 (left)) at 180 ± 2 ºC. Different frying times were studied, depending on the food product. After taking the samples out of the fryer, the excess of oil was removed with paper for 20 s. Air-frying (Papers I and V) was carried out in a hot-air-frying equipment (model: AH-9000 Actifry, Tefal; 1400 W) at 180ºC (Figure 3.2 (right)). A total amount of 0.3 g of oil per 100 g of potatoes was added.

Figure 3.2. *Deep-oil-fryer (left) and air-frying equipment (right) used in the experimental trials.*

3.3. In vitro simulated gastrointestinal digestion

The effect of gastric digestion on acrylamide in nine food products (French fries, potato chips, instant coffee, coffee substitute, sweet biscuits, crackers, breakfast cereals, onion rings and chicken nuggets) and the kinetics of acrylamide content during gastric and intestinal digestion of French fries and chips were analyzed. In addition, blanched French fries and air-fried potatoes were subjected to gastric and intestinal digestion.

The standardized static in vitro method recently published by Minekus et al., (2014) was followed to simulate oral, gastric and intestinal digestions. The stock

solutions and corresponding fluids were also prepared according to the standardized protocol (Table 3.2).

***Table 3.2**. Electrolyte composition of salivary, gastric and intestinal fluids prepared from stock solutions (salivary, gastric and intestinal).*

	SSF	***SGF***	***SDF***
	mmol/ L	***mmol/ L***	***mmol/ L***
KCl	15.1	6.9	6.8
KH_2PO_4	3.7	0.9	0.8
$NaHCO_3$	13.6	25	85
NaCl	-	47.2	38.4
$MgCl_2(H_20)_6$	0.15	0.1	0.33
$(NH_4)_2CO_3$	0.06	0.5	-
$CaCl_2(H_20)_2$	1.5	0.15	0.6

Final volume was adjusted with distilled water after adjusting the pH.

Samples were mixed with Simulated Salivary Fluid (SSF) in a ratio 50:50 w/v during 2 min (hand blender, Ufesa 600W, Slovenia). 10 g of the mix were weighed in a Falcon tube, 10 mL of Simulated Gastric Fluid (SGF), containing 2000 U pepsin, were added (ratio 50:50 w/v) and the pH adjusted to 3 with HCl 1M (pH-meter Mettler Toledo, Schwerzenbach, Switzerland). Immediately, the mixture was placed in a thermostatically controlled chamber (Selecta, Spain) at 37 °C coupled to a shaker (Intelli-Mixer RM 2, Elmi Ldt., Baltics and Russia) in order to apply a constant stirring of 55 rpm in clockwise circular movements. The total duration of this stage was 120 min, and every 30 min the pH was measured and readjusted to 3 (if necessary). Subsequently, 20 mL of Simulated Intestinal Fluid (SDF) were added to the mix (ratio 50: 50 (v / v)), the pH adjusted to pH= 7 with NaOH 1M, and the tube introduced again in the camber for 120 min (intestinal phase). The final bile salt and pancreatin concentration were 10 mM and 1260 U, respectively, in each tube. The end of enzymatic reactions (after different times of gastric and intestinal simulated digestion) was conducted by immersing the tubes in ice and then, samples were frozen at -40 °C (model CVN-40/105, Matex,

Barcelona) for subsequent freeze-drying (-40 °C and 1.25 mbar, Telstar, Terrassa, Spain).

3.4. Analytical determinations

i. Acrylamide extraction and quantification

Acrylamide content was analyzed by LC/MS/MS, with an Agilent 1200 Series HPLC system coupled to an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies Inc.) The extraction and determination was followed as the method described by Mastovska & Lehotay (2006) with some modifications. The method was validated and results are included in Paper I.

Acrylamide extraction differed from food products (Papers I, II and V) or model systems (Papers II and III). In model systems, the extraction was done by adding 2 mL of Mili-Q water to the pyrolisates obtained after heating treatment, and tubes were agitated in a vortex for 1 min. The content was filtered (0.22 mm Nylon filters) and transferred to a vial for the LC/MS/MS analysis. Acrylamide extraction in food products (batters, fried potatoes and commercial foodstuffs) before and after digestion (Paper V) was performed as follows: 1 g (or 2g depending on the sample) was placed in a 50 mL Falcon tube and 5 mL of n-hexane added. The tube was shaken in a vortex for 30 s, after which 10 ml bidistilled water, 10 mL acetonitrile, 4 g MgSO4 and 0.5 g NaCl were added and stirred in the vortex for one minute. The suspension was then centrifuged at 2026 RCF (Centronic BL II (Selecta, Spain)) for 5 min. Hexane layer was discarded and 1 mL of the acetonitrile phase was transferred to a 2 mL polypropylene tube containing 50 mg PSA and 150 mg MgSO4, and stirred for 30 s. The homogenate was centrifuged at 2697 RCF (Labofuge 200 (Heraeus, Germany)) for 1 min and the supernatant was transferred to a vial for acrylamide analysis. In papers III and V, $^{13}C_3$-acrylamide was added as an internal standard. In contrast, the standard addition method was used in Papers I and II.

The chromatographic conditions are described below. The column used in this study was a Zorbax Eclipse XDB C-18 (2.1 x 50 mm^2, 1.8 µm). The mobile phase

used consisted of 2.5% methanol/97.5% of 0.1% formic acid (A) and methanol (B). The elution gradient was as follows: 0-3 min 100% of A; 3.1-3.5 min 70% A; 3.6 min 100% A, with 1 min post-time to equilibrate the column. The column oven temperature was set at 30ºC, the flow was maintained at 0.4 mL/min and the injection volume was 10 mL. The electrospray was operated in positive ion mode. The conditions used in the ionization source were: 350ºC at 12 L/min for the drying gas (N_2), a nebulizer pressure of 40 psi and a capillary voltage of 4000 V. Identification and quantification of acrylamide in the samples was performed using the multiple reaction monitoring mode (MRM), and the ions m/z 72 > 27 and m/z 72 > 55.2 were studied, respectively. When the internal standard ($^{13}C_3$-acrylamide) was added, the correspondent ion m/z 75 > 58 was also monitored.

ii. Others chemical determinations

Reducing sugars content

In Paper I, the content of reducing sugars after the implement of the pre-treatments was analyzed in order to stablish the possible relationship between the precursor content in the food matrix and the acrylamide content in the fried product.

The reducing sugars content was determined by spectrophotometry (model V-630; Jasco Inc., Tokyo, Japan) according to the method described by (Miller, 1959) This is a colorimetric method based on the reduction of acid 3.5-dinitrosalicylic (DNS) by the presence of reducing sugars in the sample. Ninety grams of L(+) potassium sodium tartrate tetrahydrate was added to 210 mL of DNS solution (2.3% of NaOH and 1.4% DNS (w/w) in distilled water) and made up to 300 mL. In order to carry out the determinations, 0.3 g of homogenized potato sample was mixed with 1 mL of distilled water and 2 mL of DNS solution. A blank consisting of 1 mL of distilled water and 2 mL of DNS solution was also prepared. After that, the mix was heated for 5 mL in boiling water and then cooled at room temperature for 10 min. Finally, 1 mL of the mix was diluted in 4 mL of distilled water and the absorbance determined at 546 nm. The content of reducing sugars (g/100 g of potatoes) was calculated using Eq. (*I*):

$$Red.sugars\ \left(\frac{g}{100\ g\ potatoes}\right) = (Abs - 0.00385)\ x\ 1.07893 \qquad (I)$$

Water content

Water content of the samples was determined by vacuum drying at 60ºC until constant weight was achieved (20.103, AOAC, 1980). This analysis was used in Papers III and V.

Oil content

Total oil content in fried samples was determined by solvent extraction with petroleum ether using the Soxhlet method (AACC, 1995). Oil content of products was analyzed in Paper III.

iii. Physical properties

Optical properties

Superficial color of fried samples (Paper I and III) were determined by using a spectrocolorimeter (MINOLTA, mod. CM-3600d). The color space coordinates CIEL*a*b* were obtained from the absorption spectrum between 380 and 770 nm by reflectance with the reference system: D65 illuminant and 10 ° observer, and specific lens depending on the product (potatoes or fried batters).

Coordinates L*, a* and b* where determined in Paper I to compare the influence of the different pre-treatments and air-frying on the superficial color of fried potatoes.

Chroma (C_{ab}*= (a*^{2}+b*^{2})1/2) and hue (h_{ab}=arctan(b*/a*)), as well as total color changes (ΔE=[(L*-L*control)^2+ (b*-b*control)^2+ (a*-a*control)^2]1/2) were calculated in Paper III.

Mechanical properties

Texture changes over frying time were evaluated (Paper III) by a puncture test using a Texture Analyser (mod. TA-XT PlusAname, Spain) equipped with a 50 kg load cell. Texture test was performed just after frying, at consuming temperature (55 ºC), and after cooling at room temperature (25 ºC). The plunger

used for the test was a cylinder with a flat base of 2 mm diameter and a holed platform was used to ensure a total sample perforation. The crosshead speed was 1 mm/s. The maximum shear force F_{max} (N) necessary to perforate the sample was recorded from the force-deformation curve.

Water Retention Capacity (WRC)

Water Retention Capacity (WRC) of raw batters was analyzed in Paper III. Chitosan is a hydrocolloid, and thus, favors the retention of water. Its WRC when use as an ingredient in the raw batter was analyzed as follows: 18 g of raw batter were weighed in a 30 mL centrifuge tube, tempered at different temperatures (10, 20, 30 and 40 ºC) and centrifuged at 17300 RCF for 10 min. The supernatant was removed and weighed to calculate the WRC (equation *II*).

$$\mathrm{WRC} = \frac{(W_s \cdot x_w) - W_w}{(W_s \cdot x_w)} \cdot 100 \qquad (II)$$

where W_s is the total sample mass (g); x_w is water mass fraction of batter (g water/ g batter) and W_w is the supernatant mass (g).

Rheological properties

Flow behavior of raw batters was analyzed in Paper III and IV. In both articles, the effect of the addition of chitosan to batters, in different percentages, on the rheological behavior of the batters was evaluated. Nevertheless, the formulation of the batters was different in both studies. A commercial mix was used (Yolanda®) in paper III and combinations of rice and wheat flours in Paper IV.

In Paper III, apparent viscosity of raw batter formulations was determined using a Haake Rheostress 1 rheometer (Thermo Electric Corporation, Germany) equipped with a plate-plate (60 mm of diameter) at 10, 20, 30 and 40 ºC. Apparent viscosity (Pa·s) was measured as a function of shear rate (γ) from 0 to 100 s-1 after 5 min of stabilization time. Rheological constants K (consistency index, $Pa \cdot s^n$) and n (flow behavior index) were adjusted to the Ostwald-De Waele model (equation *III*):

$$\tau = K \cdot \gamma^{n} \qquad (III)$$

In Paper IV, rheological parameters were analyzed using a strain/stress control rheometer MRC 102 (Physica/Anton Paar (GmbH In., Graz, Austria) equipped with a plate-plate (50 mm of diameter). The gap between plates was fixed to 1 mm, and the free surface of samples edges was covered with silicone oil to reduce dehydration during the measurements. Apparent viscosity (Pa·s) was measured at 20 ºC as a function of shear rate (γ) from 0 to 150 s-1 after 5 min of stabilization time. The obtained flow curves were evaluated and fitted according to Herschel-Buckley model. Rheological constants τ (shear stress), $\tau 0$ (yield stress), K (consistency index) and n (flow behavior index) were adjusted to the (equation *IV*):

$$\tau = \tau_0 + K \cdot \gamma^{n} \qquad (IV)$$

Thermal properties

Thermal properties of batter formulations from Paper V were analyzed with an Auto Q20 Differential Scanning Calorimeter (T.A. Instrument, Hüllhorst, Germany). Glass transition temperature, temperature and enthalpy of gelatinization and ice-melting were analyzed. 26 ± 1 mg of sample were placed in hermetic aluminum pans and an empty pan was used as the reference. After calibrated the ramps at 10 ºC/min with indium, the profile was performed as follows: from 15 ºC to 120 ºC at 10 ºC/minute (to obtain gelatinization temperature and enthalpy), after a cooling until -50 ºC. It included an isotherm step during 3 min and then a heating in order to thawing, until 40 ºC at 10 ºC/min to obtain, the glass transition temperature followed by melting temperature and melting enthalpy. Unfrozen water content (UFW, g water/g solids) was analyzed as Laaksonen & Roos, (2000), as follows (equation *V*):

$$UFW = \frac{w_{tot} - \frac{\Delta H_{mtot}}{\Delta H_{mw}}}{C_{tot}} \qquad (V)$$

where w_{tot} is total amount of water (g), ΔH_{mtot} is the total heat of melting of ice (J), ΔH_{mw} is latent heat of melting ice (334J/g) and C_{tot} is total amount of solids (g).

Microstructural observations

The microstructure of samples was observed by using a light microscope (Nikon, Shinjuku, Japan) at 10x of magnification, taking ten micrographs for each sample. One drop of dispersion (previous dilute with hexane) was placed on a glass side and covered with a cover slip carefully placed over the sample, parallel to the plane of the slide and centered to ensure sample thickness was uniform. Micrographs were captured using a digital camera (Model 2.1 Rev 1; Polaroid Corporation, NY, USA). The Image Pro-plus 6.0 software (Media Cybernetics Inc Bethesda, USA) was used to analyze the images.

Particles size were determined according with (Glicerina, Balestra, Dalla Rosa, & Romani, 2013), by evaluating the Feret diameter, defined as the distance between two tangent lines to the two opposite sides of the particles (Allen, 1997). An Euclidean Distance Map (EDM) was further generate in order to evaluate the distance between particles. The map indicates, for each pixel in the image (black points) the shortest distance between them (Bayod, 2008; Danielsson, 1980; Glicerina, Balestra, Dalla Rosa, & Romani, 2016). The distance between black points (particles) was expressed as grey values. On the other hand, the white points represented the empty space. For this reason, applying an EDM to the original image it is possible to obtain information about the minimum distance between particles and about the amount and distribution of void spaces (Krislock & Wolkowicz, 2012).

iv. Characterization of chitosan

Deacetilation degree

The titration method described by Wang et al. (2006) was used to determine the deacetylation degree (DD) of the chitosan obtained from the above described process. 0.2 g of chitosan was dissolved in 20 mL of HCl 0.1 M under stirring for 4 h. Measurements were performed with a solution of NaOH 0.1 M by using a Metrohm's high-end titrator. The DD of chitosan was calculated as follows (equation *VI*):

$$DD = \frac{\Delta V \cdot C_{NaOH} x\ 10^{-3} \cdot 16}{M \cdot 0.0994} \qquad (VI)$$

where ΔV is NaOH volume of between two inflexion points, C_{NaOH} is concentration of NaOH solution, M is the mass of the sample, and 16 and 0.0994 are the molecular weight and theoretical amount of amino groups, respectively.

Molecular weight (M_v)

Molecular weight of obtained chitosans by acid hydrolysis was analyzed by viscosimetry as Bof, Bordagaray, Locaso, & García, (2015). The measurements were performed using an Ubbelohde capillary viscometer No. 2121R, (Ø= 0.4 mm) equipped with a thermostat bath at 25.0 °C ± 0.01 ºC. Chitosan was dissolved in 0.1M acetic acid/0.2M NaCl, into different concentrations: $5.0 \cdot 10^{-4}$, $6.5 \cdot 10^{-4}$, $8.5 \cdot 10^{-4}$ y 10^{-3} g/mL, being filtered (0.45µm) before viscosity determinations. Draining times of a fixed volume of chitosan solutions (t) and pure solvent (t_0) were measured. From these, relative viscosity (η_r) and specific viscosity (η_{sp}) of were calculated using the following equations *VII* and *VIII*:

$$\eta_r = \frac{\eta}{\eta_0} = \frac{t}{t_0} \qquad (VII)$$

$$\eta_{sp} = \eta_r - 1 \qquad (VIII)$$

where η is chitosan solution viscosity and η_0 is viscosity of the pure solvent, and their corresponding draining times (t and t_0).

The reduced viscosity (η_{red}) was calculated from the specific viscosity (η_{sp}) and the concentration of chitosan solution (*equation IX*):

$$\eta_{red} = \eta_{sp} / C \qquad (IX)$$

where *C* is concentration of chitosan solution (g/mL).

The intrinsic viscosity *[η]* was determined graphically, extrapolating values of reduced viscosity (η_{sp}/C) to zero concentration. The intrinsic viscosity was used to determine the viscosity average molecular weight *(M_v)* from Mark-Houwink-Sakurada-Staudinger equation (*equation X)*:

$$[\eta] = K_m \cdot (M_v)^a \qquad (X)$$

where *Km* and *a* are two constants dependent on the particular polymer-solvent system ($1.81 \cdot 10^{-3}$ and 0.93, respectively) (Roberts & Domszy, 1982).

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4. RESULTS

4.1. EFFECT OF PRETREATMENTS AND AIR-FRYING, A NOVEL TECHNOLOGY, ON ACRYLAMIDE GENERATION IN FRIED POTATOES

Mariola Sansano, Marisol Juan-Borrás, Isabel Escriche, Ana Andrés, Ana Heredia

Journal of Food Science, 80 (2015), 120-128

http://dx.doi.org/10.1111/1750-3841.12843

Instituto de Ingeniería de Alimentos para el Desarrollo
Universitat Politècnica de València, Valencia, Spain

EFFECT OF PRETREATMENTS AND AIR-FRYING, A NOVEL TECHNOLOGY, ON ACRYLAMIDE GENERATION IN FRIED POTATOES

Mariola Sansano, Marisol Juan-Borrás, Isabel Escriche, Ana Andrés & Ana Heredia

A worrying concern of part of the population to follow a healthy and balanced diet has encouraged food industry to improve the nutritional and safety profile of the products. In this context, air-frying is an emerging frying technique that allows obtaining low-fat fried products with similar sensorial attributes than under conventional deep-oil frying. Air-frying equipment mainly consisted of a chamber in which food is in continuous motion, and completely surrounded by an emulsion of fine oil drops homogeneously distributed in the dispersing phase (hot air). The high temperature of the oil-in-air emulsion favors the cooking of the product, the formation of a crust due to the impact of the drops of hot oil on the surface of the product, as well as the characteristic golden color generation. Mass transfer mechanisms (water and oil) together with the superficial textural and color changes taking place during air frying of potatoes have been reported in some studies. However, the impact of this technique on acrylamide generation has not been analyzed yet.

In this context, this present work focuses on the study of the effect of air frying technique on acrylamide generation in potato chips. In addition, some widely studied pre-treatments (blanching, dipping in 1 or 2 % solutions of NaCl, nicotinic acid, glycine, citric acid or calcium lactate) that aimed at acrylamide reduction have been applied and their effectiveness studied when combined with air-frying. Apart from acrylamide determination, the effect of pre-treatments on reducing sugars lixiviation from raw potatoes and the superficial color of fried potatoes have also analyzed because of the relationship between acrylamide and each one of these parameters. All experiments have been performed by conventional deep-oil frying (as a control) as well. Reducing sugars content were analyzed by a spectrophotometric method and the colorimetric parameters with a Minolta

colorimeter. In order to determine acrylamide content in fried potatoes, the analytical method was validated, using the Mastovska & Lehotay, (2006) method as a basis for acrylamide extraction and quantification.

Air-frying technique produced potato chips with 90% less acrylamide than those fried by conventional deep-oil frying. The application of any of these studied pre-treatments resulted unnecessary because no additional reductions were obtained. When potato chips were frying by deep-oil frying, nevertheless, the immersion of raw potatoes in solutions of nicotinic acid, citric acid, 1% glycine or 2% NaCl resulted in deep-fried potatoes with much lower levels of acrylamide (reductions up to 90%) compared to non-pretreated ones. A lack of correlation was found between reducing sugars content of raw material before frying and acrylamide content in fried potatoes. Finally, kinetics of acrylamide generation and surface's color development were lower under air-frying than deep-oil frying conditions.

From the obtained results, it can be concluded that air-frying technique, whose domestic application is on the rise because it processes fried foods with lower fat content and therefore in calories, reduces the content of acrylamide in fried potatoes, resulting notably healthier one.

EFFECT OF PRETREATMENTS AND AIR-FRYING, A NOVEL TECHNOLOGY, ON ACRYLAMIDE GENERATION IN FRIED POTATOES

Mariola Sansano, Marisol Juan-Borrás, Isabel Escriche, Ana Andrés, Ana Heredia

Abstract

This paper investigated the effect of air-frying technology, in combination with a pre-treatment based of soaking the samples in different chemical agent solutions (citric acid, glycine, calcium lactate, sodium chloride or nicotinic acid (vitamin B3)), on the generation of acrylamide in fried potatoes. The influence of reducing sugars on the development of surface's color was also analyzed. The experiments were conducted at 180 ºC by means of air-frying and deep oil-frying, as a reference technology. Based on the evolution of color crust with frying time, it could be concluded that the rate of Maillard reaction decreased as the initial reducing sugars content increased in the raw material, and was also lower for deep-oil frying than for air-frying regardless of pre-treatments applied. Air-frying reduced acrylamide content by about 90% compared with conventional deep-oil frying without being necessary the application of a pre-treatment. However, deep-oil fried potatoes pre-treated with solutions of nicotinic acid, citric acid, glycine at 1% and NaCl at 2% presented much lower acrylamide levels (up to 80% to 90% reduction) than non-pretreated samples.

Keywords: Acrylamide, Air-frying, Additives, Color, Reducing sugars

1. Introduction

In June 2010, the European Union published recommendations for the control of acrylamide content in food (2010/307/UE). This publication was the result of the adventitious discovery of the excessive incidence of acrylamide in different foods, mainly submitted to frying and baking, by researchers of the Stockholm University and the publication of analytical data of various foods on the web site of the Swedish National Food Agency in 2002 (Rosén and Hellenäs 2002). According to epidemiological studies, the toxicity of acrylamide, a probable

carcinogenic compound for humans classified in Group 2A (IARC), it is not only because of its consumption, but also because of its role as a precursor in the development of other compounds during hepatic metabolism for example, glycidamide (Blank 2005).

Acrylamide is an early product in Maillard reaction which is produced by the reaction between the amino acid asparagine and reducing sugars (glucose, fructose and so on) when food is subjected to temperatures above 120 ° C in processes such as frying or baking (Yaylayan and others 2003). Acrylamide generation is especially relevant in processed potato products because of the high presence of the precursors (asparagine and reducing sugars) in the matrix. The presence of the precursors can vary depending on the variety, soil conditions and postharvest storage conditions (Kumar and others 2004).

The influence of the main frying variables (oil temperature, frying time, potato:oil ratio, surface:volume ratio, and so on) on acrylamide generation has been extensively studied over the past 10 y (Bråthen, and Knutsen 2005; Gökmen, and Palazoglu 2009), as well as its relationship with potato properties such as pH, water activity, capillarity and porosity (Low and others 2006). It has been reported that the higher the frying temperature and the lower the moisture content in the potato, the higher the acrylamide content in the fried product (Pedreschi and others 2005). Some alternatives such as low pressure-frying (Troncoso and Pedreschi 2009; Dueik and others 2012), microwave assisted frying or microwave prethawing (Ngadi and others 2009; Tuta and others 2010), and predrying (Pedreschi and Moyano, 2005) have been studied in order to obtain healthier fried products in terms of fat and/or acrylamide content. Air-frying is a technology that permits the reduction of fat content in fried products by 90% (Andrés and others 2012). The technology consists of direct contact between the product and a dispersion of oil droplets in hot air in a frying chamber. The product is constantly in motion to promote homogeneous contact between both phases. In this way, the product is dehydrated and the typical crust of fried products gradually appears (Andrés and others 2012). To the authors' knowledge, no

publication provides scientific information about the impact of this technology on acrylamide generation.

The application of a treatment prior to frying for acrylamide reduction has raised interest in recent years. Some pre-treatments based on the immersion of the product in solutions with chemical agents in order to mitigate acrylamide generation during frying could be grouped according to the following objectives: (i) to reduce the content of asparagine and reducing sugars in the raw material, for example, by blanching (Pedreschi and others 2005); (ii) to enzymatically hydrolyze asparagine, that is, asparaginase (Zyzak and others 2003, Ciesarova and others 2006, Pedreschi and others 2011); (iii) to add amino acids (for example, glycine (Low and others 2006)) or cations (for example,. Ca^{2+} or Na^{+}: (Gökmen and Senyuva 2007) able to compete with asparagine in Maillard reaction and (iv) to kinetically control the rate of acrylamide formation by pH reduction (Stadler and Scholz 2004) or by lactic acid fermentation (Anese and others 2009; Bartkiene and others 2012). The criteria taken into account to select the substances employed in this study to perform pre-treatments were mainly two: (i) the number of studies in which the substance was used and its effectiveness. This is the case of blanching, citric acid or sodium chloride; (ii) the functionality of the substance. In this sense, the incorporation of a bioactive compound such as glycine, nicotinic acid (vitamin B3) or calcium during pre-treatment could contribute to the functionality of the final fried product.

Moreover, it is important to point out that the majority of published studies concerning the effect of pre-treatments by immersion in different additives to reduce acrylamide have been carried out in model systems, but not in real foods in which the structure could play an important role. In this study, the advantages of the application of these different pre-treatments could accurately be established because of all of them are applied at the same frying conditions and raw material.

The main objective of this study was to evaluate the effect of air-frying, in combination with different chemical pre-treatments, on acrylamide reduction in a real food matrix such as fried potato.

2. Materials and Methods

2.1. Reagents

Citric acid, nicotinic acid and acrylamide standard (≥ 99%) were obtained from Merck (Darmstadt, Germany), calcium lactate and sodium chloride from Scharlab (Barcelona, Spain). Glycine, L (+) potassium sodium tartrate tetrahydrate, formic acid (99% to 100% purity) and magnesium sulfate were purchased from VWR (Fontenay-sous-Bois, France). 3.5-dinitrosalicylic acid (DNS) and hexane were from Panreac (Barcelona, Spain). Primary secondary amine (PSA) was obtained from Supelco (Bellefonte, Pa, U.S.A.). Double distilled water was prepared for chromatographic use (Milli-Q, Millipore Corp., Bedford Mass., U.S.A.). All chemicals used were analytical grade, and those used for chromatographic analysis were HPLC grade. The acrylamide standard was purchased from Merck, the stock solution (1mg/mL) was prepared by dissolving 100mg of the acrylamide in 100mL of acetonitrile, and was kept at 4ºC until use. All working solutions were prepared daily by serial dilution in acetonitrile.

2.2. Raw material

The potatoes (Solanum tuberosum L., Frisia variety) were provided by a local supplier. This variety was chosen because of its availability during all the year in Spain. This variety is characterized by yellow skin, light yellow-white flesh, and oval shape. The potatoes were sorted, washed, peeled, cut into 7 mm thick slices and cored with a stainless steel core borer (25 mm in diameter) to produce discs. The experiments were performed in two different periods of time: March-April and May-June, being necessary to analyze the total reducing sugars content of potatoes used in each period because of the relevance of these compounds in acrylamide generation as well as color changes along frying.

2.3. Experimental methodology

2.3.1. Pre-treatments

The potato discs were subjected to various treatments prior to frying. Specifically, samples were blanched at 85 °C for 5 min or immersed in solutions of different chemical agents: citric acid, glycine, calcium lactate, sodium chloride, or nicotinic acid at 1 and 2% for 60 min at room temperature. Likewise, a control consisting of dipping the samples in distilled water at room temperature for 60 min was also performed. In all cases, the potato:solution ratio was 1:5 (w/w).

The potato discs were removed from the solution and the excess liquid was soaked up with absorbent paper for 2 min prior to frying.

2.3.2. Frying step

The experiments were carried at a fixed frying temperature of 180 °C in a commercial deep oil fryer (model: FM 6720 Ideal 2000 Professional, Solac with a nominal power of 2000 W) and hot-air-frying equipment (model: AH-9000 Actifry, Tefal with a nominal power of 1400 W). For deep oil experiments, a potato-to-oil ratio of 1:20 (w/v) was used. Therefore, a total amount of 2 L of oil was employed to deep-oil fry 100 g of potatoes. Commercial sunflower oilseed was used in all frying experiments. This ratio was large enough to avoid important changes in terms of product-to-oil ratio and therefore in the oil composition and temperature. The oil was renewed every 2 experiments. For hot-air-frying experiments, 0.003 kg of oil per kilogram of potatoes was added to the air chamber according to the specifications of the equipment, that is, a total amount of 0.3 g of oil per 100 g of potatoes. A constant frying temperature was confirmed by means of two PT-100 temperature sensors (model: TF101K) located at the top and the bottom of each fryer. Samples were immersed in the fryers when the initial frying temperature of 180 °C was achieved. Each experiment was conducted in triplicate.

The concentration of reducing sugars was determined in raw potatoes in order to evaluate its influence on color development. Color development was based on the CIEL*a*b* colorimetric characteristics of fried potatoes. A reference frying

time was complementary established based on a visual evaluation of color on the surface and the external (crispy outer crust) and internal cooking level of the samples. For this purpose, samples were removed from the fryers for color determination at different time intervals depending on the frying technology and initial reducing sugars content. Concurrently, samples with low reducing sugar content were removed every 2 min until 16 min and at 26 min for deep-oil-frying conditions, and every 2 min until 24 min and at 34 min for air-frying. Samples with high reducing sugar content were removed every minute until 7 min for deep-oil-frying, and every 2 min until 20 min for air-frying. The sampling time was chosen according to a previous study on mass and heat transfer phenomena for both frying technologies (Andrés and others 2012). Before the analytical determinations, the excess oil was removed from the samples with absorbent paper.

Determination of the acrylamide contents was only carried out at the reference frying time.

2.4. Analytical determinations

All determinations were carried out in triplicate.

2.4.1. Optical properties

The determination of the optical properties of the potato disks in each experiment and frying time was carried out on the surface of the samples by means of a spectrocolorimeter (MINOLTA, mod. CM-3600d). The color space coordinates CIEL*a*b* were obtained from the absorption spectrum between 380 and 770 nm by reflectance with the reference system: D65 illuminant and 10° observer, and a 7 mm lens. Previously, samples were measured with both black and white calibration tiles in order to study the possible translucency of the samples. Since the same spectrum was obtained with the black and white tiles, the opacity of the samples was confirmed, and only data corresponding to the black tile were analyzed.

2.4.2. Reducing sugars content

The reducing sugars content was determined by spectrophotometry (model V-630; Jasco Inc., Tokyo, Japan) according to the protocol described by Miller (1959). This colorimetric method is based on the reduction of acid 3.5-dinitrosalicylic by reducing sugars. Ninety grams of Rochelle salt (L (+) potassium sodium tartrate tetrahydrate) was added to 210 mL of DNS solution (2.3 % of NaOH and 1.4 % DNS (w/w) in distilled water) and made up to 300 mL. In order to carry out the determinations, 0.3 g of homogenized potato sample was mixed with 1 mL of distilled water and 2 mL of DNS solution.

A blank consisting of 1mL of distilled water and 2 mL of DNS solution was also prepared. After that, the mix was heated for 5 min in boiling water and then cooled at room temperature for 10 minutes. Finally, 1 mL of the mix was diluted in 4 mL of distilled water and the absorbance determined at 546 nm. The quantification of reducing sugars content (g/100 g of potatoes) was calculated using Eq. (*I*):

$$\text{Reducing sugars (g/100 g of potatoes)} = (\text{Absorbance} - 0.00385) \times 1.07893 \qquad (I)$$

2.4.3. Analysis of acrylamide

The acrylamide content determinations were carried out using the method of dispersive solid phase extraction called QuEChERS, originally designed for pesticide analysis in food and modified by Mastovska and Lehotay (2006) for the extraction of acrylamide, following the method described by Al-Tasher (2011) with some modifications. Three potato disks were ground in a blender and a sub-sample (2 g) of potato was placed in a 50mL Falcon tube and 5 mL of n-hexane was added. The tube was shaken in a vortex for 30 s and after that 10 ml bidistilled water, 10 mL acetonitrile, 4 g MgSO4 and 0.5 g NaCl were added and stirred in the vortex for 1 min. The suspension was then centrifuged at 2026 RCF (Centronic BL II; Selecta, Barcelona, Spain) for 5 min, the hexane layer (upper phase) was discarded and 1mL of the acetonitrile phase, containing the acrylamide, was transferred to a 2 mL polypropylene tube containing 50 mg PSA

and 150 mg MgSO4, and stirred for 30 s. The homogenate was centrifuged at 2697 RCF (Labofuge 200; Heraeus, Hanau, Germany)) for 1 min and the supernatant was transferred to a vial for analysis by LC/MS/MS. The vials were amber type with a 1.5 mL of capacity.

The chromatographic analysis was performed with an Agilent 1200 Series HPLC system coupled to an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies Inc.) with an electrospray type ionization source. The column used in this study was a Zorbax Eclipse XDB C-18 (2.1 x 50mm2, 1.8µm). The mobile phase used consisted of 2.5% methanol and 97.5% aqueous formic acid (0.1%) (A) and methanol (B). The elution gradient was as follows: 0 to 4 min 100% of A; 4.1 to 6 min 70% A; 6.1 min 100% A, with 2 min post-time to equilibrate the column and the total run time was 8 min. The column oven temperature was set at 30 °C, the flow was maintained at 0.2 mL/min, and the injection volume was 10 µL; after each injection a needle wash was performed.

The electrospray operated in positive ion mode. The conditions used in the ionization source were: 350 ºC at 12 L/ min for the drying gas (N_2), a nebulizer pressure of 40 psi and a capillary voltage of 4000 V. Identification and quantification of acrylamide in the samples was performed using the multiple reaction monitoring (MRM) mode, and the two most abundant ions, *m*/*z* 72 > 27 and *m*/*z* 72 > 55.2, were studied respectively.

Before analysis, the chromatography method was validated according to the directive 2002/657/CE. The validation included the determination of linearity, recovery, precision (repeatability and reproducibility), LOD (limit of detection) and LOQ (limit of quantification). The linearity and matrix effect of the analytical procedure were studied using calibration standards prepared in neat solvent and in fortified samples submitted to QuEChERS extraction. The five-point-calibration curves (10, 20, 50, 100, and 200 *µ*g/kg) in solvent and in fortified raw and fried potatoes were constructed and compared. The slope ratios (slope matrix/slope solvent) were < 1 in all cases. This indicated that a matrix effect existed with a suppression of ionization (Cuadros-Rodriguez and others 2003). Therefore,

acrylamide content (*μ*g/kg) in fried potatoes was quantified from the “fried matrix curve”; whereas acrylamide content (*μ*g/kg) in pretreated samples was quantified from the “raw matrix curve”. The presence of Maillard reaction products in fried potato matrices leads to a different MS/MS response than in raw potatoes samples in which no Maillard reaction products exist (Zhang and others 2011).

The accuracy of both the fried and raw matrix was evaluated through recovery experiments by fortifying an appropriate sample at three different levels (10, 50 and 100 *μ*g/kg), with six replicates at each level (n=6).

Repeatability was calculated from the analysis of six blank samples fortified at each one of the three specified levels of fortification (10, 50 and 100 *μ*g/kg), and performed by the same operator on the same day. To evaluate reproducibility the analyses were repeated on three consecutive days by two different operators.

The LOD and LOQ of the method were obtained by fortifying samples with acrylamide at different concentrations. The values were determined as the amount of analyte for which signal-to-noise ratios (S/N) were higher than 3 and 10, respectively.

2.4.4. Statistical analysis

Statistical ANOVA was performed by Statgraphics Centurion to estimate the effect of process variables (reducing sugars content in raw material, type of pre-treatment and frying technology) on the obtained results. Evaluations were based on a 95% significance level.

3. Results and discussion

3.1. Influence of frying technology, pre-treatment and initial reducing sugars content on color changes during frying

It is well known that browning reactions induce changes in the crust of fried products. The kinetics of Maillard reaction depends on several factors such as precursor concentrations in raw material, pre-treatments, frying technology and frying variables (De Wilde and others 2005). Additionally, a correlation between

acrylamide development and reducing sugars content in raw material has been reported in previous studies (Williams 2005; De Wilde and others 2005).

Statistical analysis showed (Table 4.1) that frying technology, initial reducing sugars in raw potatoes, and their interaction, had a significant influence on the evolution of CIEL*a*b* parameters with frying time.

Table 4.1. *Values of F ratio and level of significance from ANOVA multifactor of the influence of the main effects (pre-treatment, frying technique, and initial reducing sugars) on the CIEL*a*b* along frying.*

	F ratio		
Main effects	***L****	***a****	***b****
A: Pre-treatment	1.77 NS	1.60NS	1.76NS
B: Frying Technique	19.61**	50.09**	192.47**
C: Initial Reducing sugars	4.29*	7.17**	81.36**
Interactions			
AB	1.42NS	1.64NS	3.99**
BC	3.80NS	10.45**	35.32**

**Statistical significance ≥95% (p-value ≤ 0.05); **Statistical significance ≥99% (p-value ≤ 0.01); NS (not statistical significance, p-value > 0.05)*

Moreover, frying technology was the factor which most influenced the evolution of color parameters during frying (higher value of *F*ratio), and especially on *a**. Pre-treatment was not found to have a significant effect which means that it is possible to apply pre-treatments without affecting the color of fried potatoes. Figure 4.1, 4.2 and 4.3 show the evolution of lightness (L*), *a** and *b** with regard to air and deep-oil frying time, of untreated, control and blanched samples as well as after dipping them in 1 and 2% of citric acid. The authors do not consider it necessary to show the results corresponding to all pre-treatments in Figures 4.1 to 4.3. On one hand, the results showed that the higher the initial reducing sugars content, the faster the rate of browning reactions. Color changes in potato products are the direct consequence of non-enzymatic browning reactions which reducing sugars (that is, glucose and fructose) participate in (Manzoco and others 2001). On the other hand, frying technology clearly affected the kinetic of color

changes. In fact, Maillard reaction was much faster for deep-frying than air-frying. This is because of the differences between both technologies in terms of mass and heat transport kinetics, even if the temperature of the external medium (air or oil) was 180 ºC in both cases. The thermal properties of oil favor heat transport by convection from the external medium to the potato surface, and lead to a faster frying process (Andrés and others 2012).

For oil-frying, lightness (L*) increased initially but tended to decrease with time resulting in a darkening of the fried potatoes; whereas it almost remained constant in samples submitted to air-frying (Figure 4.1). Browning was directly reflected in a gradual increase of the *a** color parameter. As it can be observed in Figure 4.2, *a** values gradually increased from negative values to positive ones. Finally, the CIE *b** parameter (related to yellow tint when positive) experimented a notable increase during frying, regardless of frying technology (Figure 4.3). Samples submitted to oil-frying reached higher *b** values than air-fried potatoes and the rate of changes of *b** and *a** was faster in fried potatoes with high initial reducing sugars content. The frying time required to achieve positive values of *a** in fried potatoes with low initial reducing sugars content was 26 and 34 min in oil and air-frying conditions, whereas the time at which samples with high initial reducing sugars content was 7 and 20 min in oil and air-frying, respectively. The reference frying times, those at which *a** values were lower than 0, were 16 and 24 min in samples with low reducing sugars content for oil and air-frying, respectively, whereas 5 and 16 min were required in samples with high reducing sugars content for oil and air-frying, respectively. It is important to point out that the reference time varied depending on each technology and the initial reducing sugars content, but not on the type of additives used for pre-treatment.

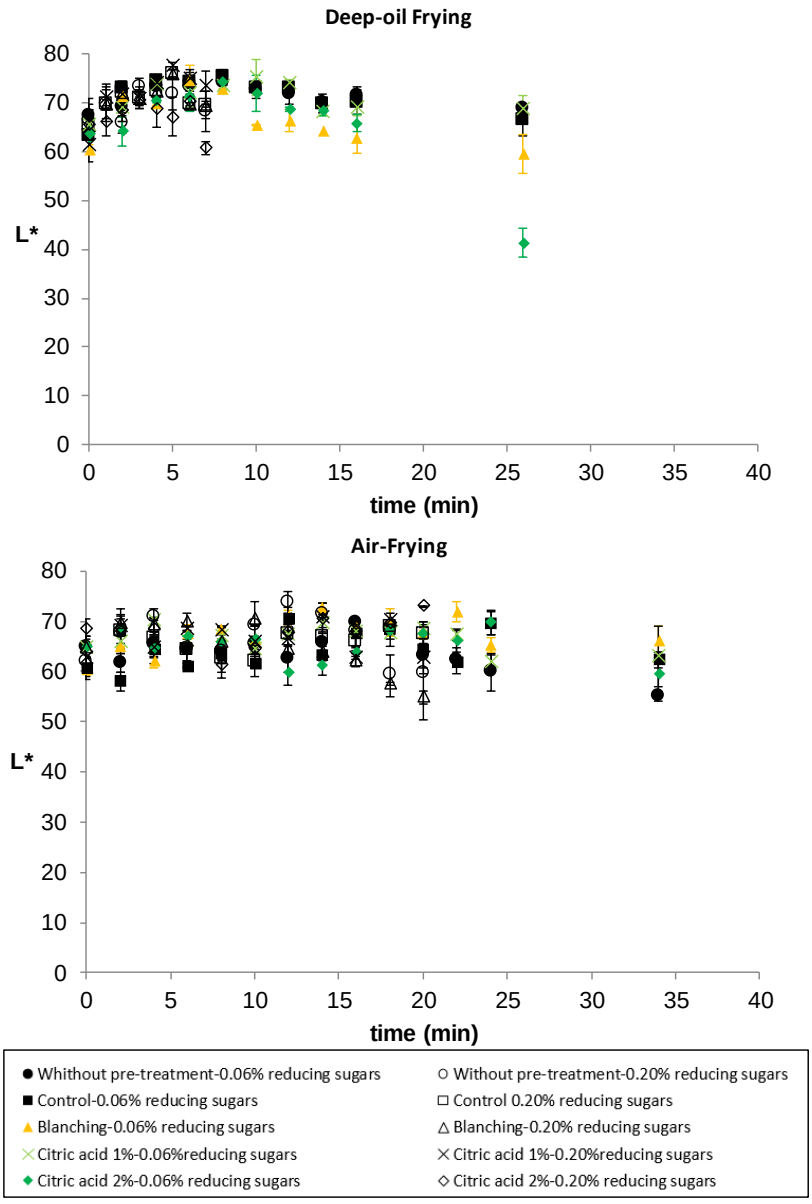

Figure 4.1. *Evolution of lightness (L*) along deep-oil and air-frying of unpretreated (without any pre-treatment), control (60 min at room temperature in distilled water), blanched and dipped in citric acid at 1% and 2% samples with low and high-medium initial reducing sugars content in raw potatoes.*

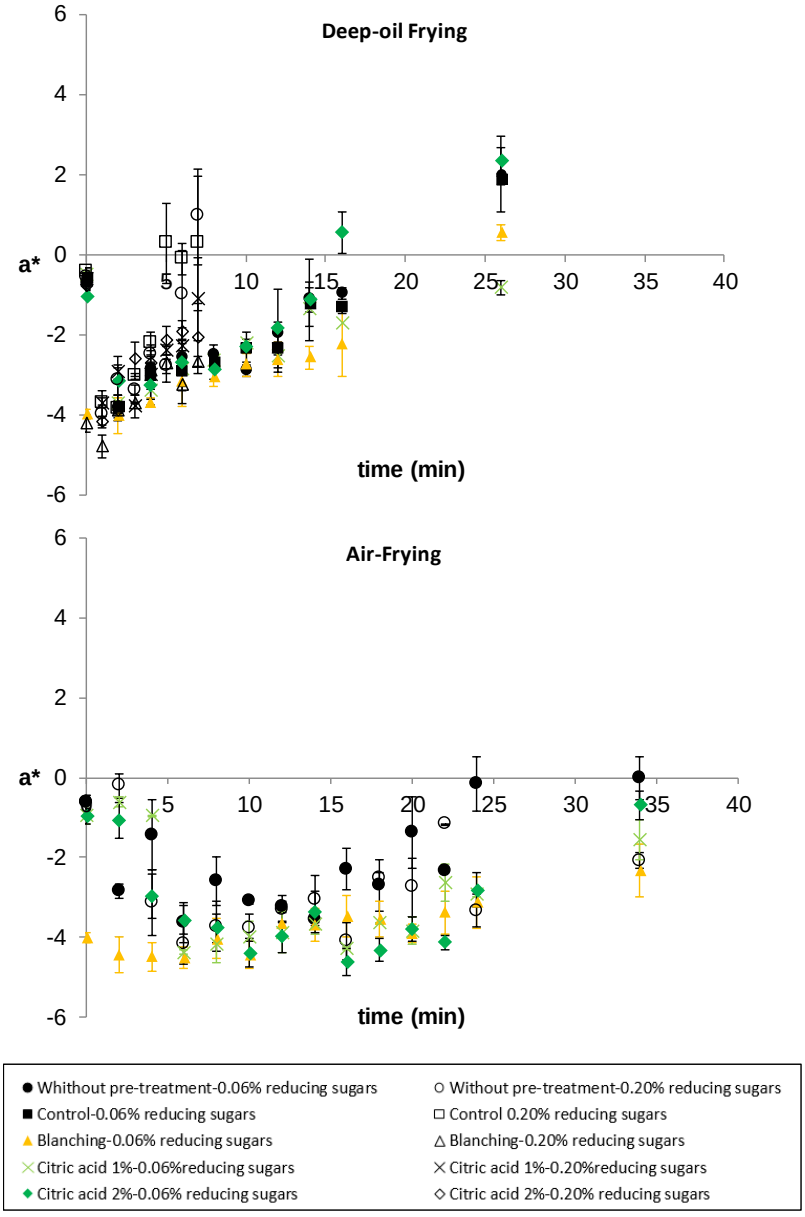

Figure 4.2. *Evolution of a* colorimetric parameter along deep-oil and air-frying of unpretreated (without any pre-treatment), control (60 min at room temperature in distilled water), blanched and dipped in citric acid at 1% and 2% samples with low and medium-high initial reducing sugars content in raw potatoes.*

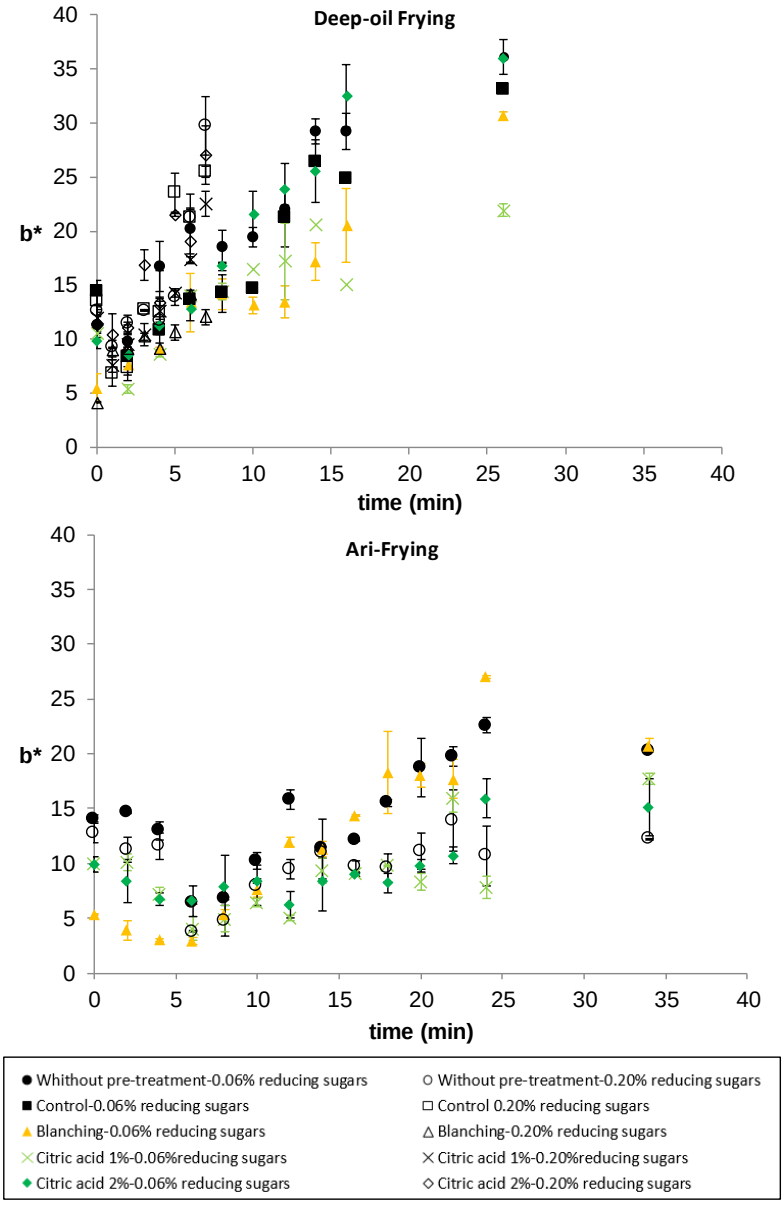

Figure 4.3. *Evolution of b* colorimetric parameter along deep-oil and air-frying of unpretreated (without any pre-treatment), control (60 min at room temperature in distilled water), blanched and dipped in citric acid at 1% and 2% samples with low and medium-high initial reducing sugars content in raw potatoes.*

3.2. Validation of the analytical method for acrylamide determination

As there is no official methodology described for the analysis of acrylamide, validation of the analytical procedure was carried out as first step in order to ensure the quality of the obtained results. The calibration curves for raw and fried samples were linear in the range 10 to 200 μg/kg, with a correlation coefficient (R^2) of 0.9926 and 0.9986, respectively. The recoveries, performed by adding known quantities of acrylamide were in the range 97% to 108% for raw matrix and 98% to 116% for the fried one. The relative standard deviation for recovery data ranged from 4.4% to 15.5% and 4.8% to 16.0%, respectively.

Repeatability and reproducibility, expressed as the relative standard deviation, were 0.5% to 3.5% and 0.5% to 12.8% for the raw matrix, whereas for the fried matrix they were 2.6% to 15.6% and 4.1% to 14.6%, respectively. All the relative standard deviations are in agreement with the 2002/657EC Directive, which permits up to 20%. The limit of quantification of the method assayed, in both cases, was 10 μg/kg.

The results of validation demonstrate that the method used is accurate and precise, and therefore it can be concluded that this analytical procedure guarantees the quantification of acrylamide in the samples.

3.3. Influence of frying technology and pre-treatment on reducing sugars content and acrylamide generation

The effectiveness of pre-treatment in initial reducing sugars lixiviation as well as the influence of chemical agents involved in the pre-treatments and the frying technology on acrylamide reduction at reference frying time were also studied. This analysis was only conducted in samples with high initial reducing sugars (0.203±0.005%), as this condition is the most disadvantageous for acrylamide formation.

Table 4.2 shows the reducing sugars content of the samples after pre-treatment. The results have been expressed as g of reducing sugars per 100 g of fresh potatoes in order to compare the effect of pre-treatment. For this purpose,

the net mass variation occurring under the different experimental conditions (data not shown) was taken into account. The relative variation of initial reducing sugars content (%) taken place during pre-treatment was also calculated.

Table 4.2. *Reducing sugar content (g/100g fresh potato) of the samples after pre-treatments and variation of initial reducing sugars (%) as a consequence of pre-treatments*

Pre-treatment	***Reducing sugars content (g/100g fresh potato)***	***Variation of initial reducing sugars (%)***
Raw potatoes	0.203 (0.005)d	
Control	0.20 (0.03)d	-6.54(1.03)
Blanching	0.15 (0.03)bc	-26(5)
Nicotinic acid 1%	0.146 (0.016)bc	-31(6)
Nicotinic acid 2%	0.156 (0.009)bc	-23(5)
Citric acid 1%	0.133 (0.013)abc	-35(6)
Citric acid 2%	0.1237 (0.0112)abc	-39(6)
Glicine 1%	0.13 (0.02)bc	-39(5)
Glicine 2%	0.118(0.013)ab	-42(6)
NaCl 1%	0.21(0.02)d	1.3(1.4)
NaCl 2%	0.097(0.015)a	-55(5)
Calcium lactate 1%	0.158(0.006)c	-22(3)
Calcium lactate 2%	0.130(0.005)abc	-36(2)

[abcd] *Different letters indicate differences between homogenous groups at a 95% of significance level (P value ≤ 0.05). Mean (standard deviation).*

On one hand, pre-treatment by immersion in different chemical agents, as well as blanching, resulted in a statistically significant loss of the initial reducing sugars of the raw potatoes.

It is important to point out that placing the samples in water alone for 60 min (control) resulted only in a 6% reduction; therefore it could be considered that no reduction took place under control conditions, whereas the presence of a solute in the external immersion medium produced reductions of over 22%, excepting in 1% NaCl. In fact, the presence of a solute in the immersion medium generates a mild chemical potential gradient acting as a driving force for mass fluxes,

leading to an exchange of soluble solutes between the potato tissue and the surrounding solution (Pointing 1973). Concretely, there is an intake of the chemical agent, from the external solution to the potato tissue, at the same time as a partial removal of native hydrosoluble solutes, such as reducing sugars and asparagine, from the liquid phase of the potato to the external solution (Wicklund and others 2006). However, an increase of solute concentration in the external solution did not significantly affect the variation of reducing sugars (Table 4.2), whereas some chemical agents such citric acid, glycine or calcium lactate and sodium chloride at 2% improved the reducing sugars lixiviation. The results also demonstrated the advantage of applying short high-temperature treatments (blanching) instead of long low-temperature ones (control). In this way, blanching (5 min at 85 ºC) resulted in a 26% loss of reducing sugars instead of 6% for control conditions (60 min at room temperature). Pedreschi and others (2004) reported similar levels of reduction after blanching potatoes of *Tivoli* var.; whereas Mestdagh and others (2008a) reported lower values of reduction. During blanching, the alteration of amylaceous tissue takes place resulting in a higher migration of acrylamide precursors (Pedreschi and others 2005).

Previous studies reported that lixiviation of reducing sugars in raw materials and acrylamide generation on frying are linked, since reducing sugars content is one of the main limiting acrylamide precursors in potato products (Amrein and others 2003; De Wilde and others, 2005). In this study, the roles of different chemical agents and frying technologies in inhibiting acrylamide formation were also studied. The acrylamide content (μg/kg) of pretreated deep-oil fried and air-fried potatoes at their respective reference frying times (5 min and 16 min in deep-oil frying and air-frying, respectively) is shown in Figure 4.4.

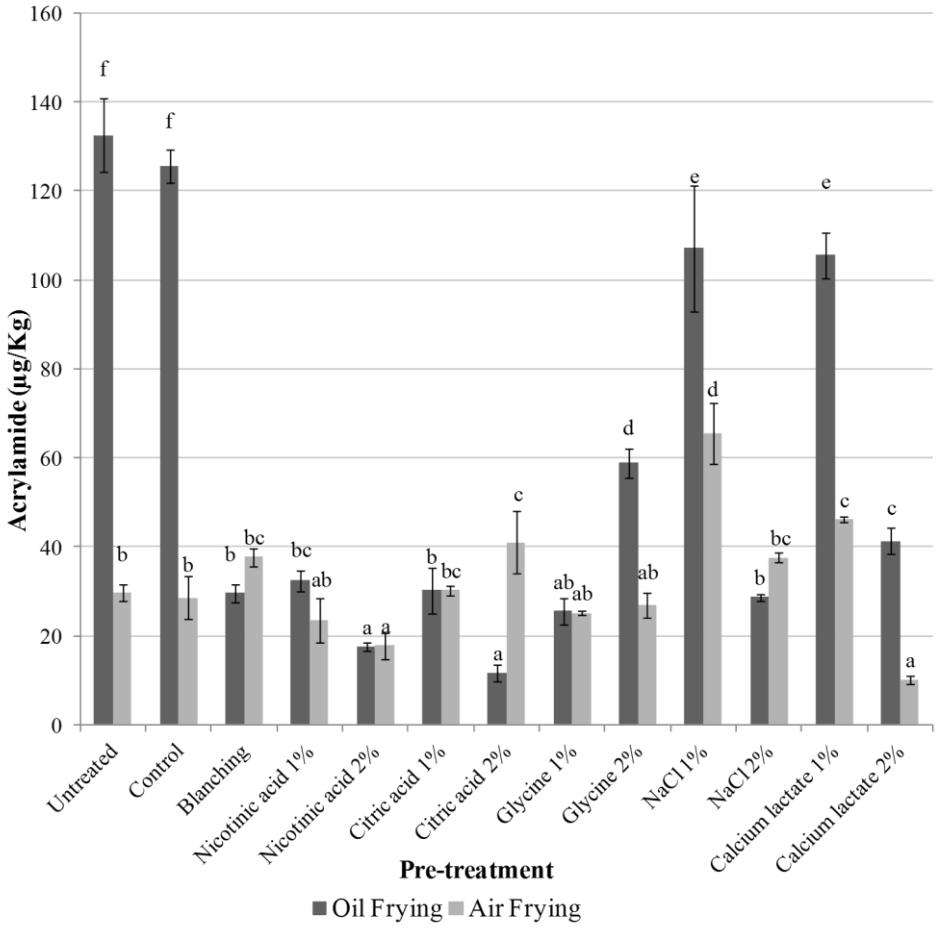

***Figure 4.4**. Acrylamide content (µg/kg) of fried potatoes submitted to the different studied pre-treatments and at the reference frying time of deep-oil-frying and air-frying. These values correspond to fried potatoes with medium-high initial reducing sugars content in raw potatoes*.

Air-frying resulted in a 77% reduction in acrylamide formation for unpretreated samples in comparison to deep-oil fried ones. Therefore, the application of a pre-treatment with the aim of reducing this toxic compound is not necessary for air-frying. The effectiveness of air-frying in limiting acrylamide formation could be related to the higher relative humidity of the product/external medium interphase

for air-frying compared with deep-oil frying. Moreover, Gökmen and Palazoglu (2009) reported that a certain amount of acrylamide formed in potatoes during frying is lost through evaporation. In fact, the higher fluxes of water from the core of the product to the surface and finally to the air, linked to the long frying time during air-frying (Andrés and others 2012), prolonged the constant-rate frying step characterized by the saturation of the product surface with water. On the other hand, deep-oil frying mainly occurs at a falling-rate. It is well known that the presence of water on the potato surface prevents acrylamide formation in fried products.

Surprisingly, samples pre-treated with 1% of NaCl or Calcium lactate presented higher acrylamide content than the control and the unpretreated air-fried samples. The presence of any of these mono o divalent cations, Ca^{2+} or Na^{+}, at the product surface could diminish the water activity of the surface and favor acrylamide formation in the fried product. This result revealed a strong interaction between the frying technology and type of pre-treatment as these cations reduced acrylamide formation for deep-oil frying. In addition, pre-treatments with blanching, nicotinic acid, citric acid, glycine at 1% and NaCl at 2% limited acrylamide generation in deep-oil fried potatoes. Zeng and others (2009) previously reported that nicotinic acid was the most effective water-soluble vitamin in the inhibition of acrylamide with up to 70% mitigation. Moreover, since no undesirable flavor was found in fried potatoes after treatment, nicotinic acid could be a promising inhibitor of acrylamide formation in food processing. The effect of citric acid (77% and 91% reduction at 1% and 2% of solution concentration, respectively) could be attributed to both an important pH drop and a spatial hindrance that hinder the reaction between acrylamide precursors (Mestdagh and others 2008b). The ability of glycine to reduce the acrylamide content in fried potatoes compared with other amino acids was previously reported in model systems (Bråthen and Knutsen, 2005; Low and others, 2006) and blanched potato crisps (Kim and others 2005). Unexpectedly, a reduction of 80% and 55% was found in this study at 1% and 2% of glycine, respectively. Alternatively, concentrations above 1% of glycine in the soaking medium, that is,

in the pretreated potato, did not result in a higher acrylamide reduction. The effectiveness of glycine, and other free amino acids or proteins, was attributed to the promotion of competitive reactions with asparagine to react with reducing sugars and/or by covalently binding the formed acrylamide through Michael type addition reactions (Mestdagh and others 2008a, 2008b). The effect of mono and divalent cations Na^+ and Ca^{2+} at 2% gave as a result significant acrylamide mitigation. A loss of 78 and 68% took place at this concentration with NaCl and calcium lactate, respectively; whereas a reduction of ≈20% occurred at 1% with both cations. Mestdagh and others (2008) reported a maximum reduction of 10% and 49% by adding 50 µmol/ g mixture of NaCl and 100 µmol/ g mixture of calcium lactate to a model system. However, Gökmen and Senyuva (2007) found a complete prevention of acrylamide formation by Ca^{2+}, whereas monovalent cations, such as Na^+, almost halved the acrylamide formed in the model system. Calcium lactate and NaCl, as food additives, are widely used as firming and preservative agents in commercial foods and they could be also used by the food industry to control the formation of acrylamide.

Finally, a correlation between reducing sugars content at the beginning of frying (Table 4.2) and acrylamide content (Figure 4.4) at the reference frying time was separately performed for both frying technologies (data not shown). Results showed that reducing sugars content at initial frying time had lack of correlation with acrylamide content (R^2=0.648 for deep-oil frying and 0.252 for air-frying). This fact again reflected that acrylamide inhibition is a complex mechanism in which not only the amount of the precursors (reducing sugars and asparagine) has a major role but also the interference of additives in Maillard reaction and the kinetics and conditions of frying.

4. Conclusions

From the results obtained in this study, it could be concluded that the lower the initial reducing sugars content the higher the time required for reaching the typical tonality of fried potatoes. The rate of Maillard reaction during air-frying technology was much lower than for deep oil-frying conditions leading to a

drastically acrylamide reduction (about ≈ 90%) with compared with conventional frying even in unpretreated samples. This fact permits to affirm that air-frying technology is a promising technology for obtaining healthy fried products.

The application of a pre-treatment became an important step for acrylamide mitigation for deep-oil frying. In this sense, both the extraction efficiency of reducing sugars and the penetration of additives during pre-treatment played a combining role for acrylamide mitigation. Concretely, dipping the potatoes into a solution of nicotinic acid, citric acid, glycine at 1% or NaCl at 2% might be a viable approach for the minimization of acrylamide content. Nevertheless, the sensory repercussion of any strategy to reduce acrylamide generation should be evaluated before application.

Acknowledgments

Authors would like to thank Generalitat Valenciana (GV/2012/072) for the financial support given to this investigation.

Author Contributions

M. Sansano collected experimental data and drafted the manuscript. M. Juan-Borrás and I. Escriche performed method validation for LC/MS/MS acrylamide analysis. A. Andrés planned the study and interpreted the results. A. Heredia planned the study, interpreted the results, and drafted the manuscript

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4.2. PROTECTIVE EFFECT OF CHITOSAN ON ACRYLAMIDE FORMATION IN MODEL AND BATTER SYSTEMS

Mariola Sansano, Mª Luisa Castelló, Ana Heredia, Ana Andrés

Food Hydrocolloids, 60 (2016), 1-6

https://doi.org/10.1016/j.foodhyd.2016.03.017

Instituto de Ingeniería de Alimentos para el Desarrollo
Universitat Politècnica de València, Valencia, Spain

PROTECTIVE EFFECT OF CHITOSAN ON ACRYLAMIDE FORMATION IN MODEL AND BATTER SYSTEMS

Mariola Sansano, Mª Luisa Castelló, Ana Heredia & Ana Andrés

Chitosan is a polymer obtained from chitin that can be obtained from the waste resource of shrimp shells, thus contribute to the reduction of the environment impact. The main properties of chitosan, biocompatibility, biodegradability, non-toxicity, and non- adsorption favors its application in a wide range of industries from water treatment, biomedicine & pharmaceutical, industrial, food and beverages, cosmetics, agrochemical and others. Its structure, rich in free amino groups, aroused our interest about its application as an ingredient with the purpose of reducing acrylamide content. We hypothesized that those free amino groups of chitosan would compete with amino groups of asparagine to bind to carbonyl group of reducing sugars and thus, would reduce acrylamide formation. Although chitosan is a compound whose properties have been widely studied, scientific publications related to this proposal were not found in the literature.

The main objective of this work was to study the addition of chitosan in model and batter systems as a way to mitigate acrylamide. Model systems allow a better control of the experimental work and a better understanding of the influence of different factors. In this sense, the effect of chitosan concentration, pH, reducing sugars and the temperature on acrylamide formation in model systems were also evaluated.

Model reactions were conducted using a reference method, published by Vural Gökmen, a leading expert on acrylamide field, who has more than 25 publications related with acrylamide, in scientific journals and chapters in books. An equimolar mix of asparagine and reducing sugars (fructose and/or glucose), prepared with lactic acid solution (at pH 4 and 5) and chitosan content of 0, 0.5 and 1%. was heated at 150 and 180ºC for 0, 5, 10, 15, 20 and 30 minutes. After the chemical reactions, acrylamide was extracted and analyzed by HPLC/MS/MS.

The statistical analysis showed the potential chitosan effect on acrylamide content, as well as, the great influence of time and temperature and the molecular specie of reducing sugar. However, between pH 4 and 5 no statistical differences in acrylamide formation were observed. As expected, at 180ºC the content of acrylamide was higher than at 150ºC. At 150ºC, the acrylamide content grew progressively with treatment time, and at 180ºC, the amount of acrylamide reached the maximum values (at 10-15 min) and started to decrease with time. Final acrylamide content was significantly higher in fructose-model systems than in glucose-model systems, even when chitosan was present. 0.5% of chitosan content mitigated acrylamide formation (compared to the control) by 52% and 65% at 180 and 150ºC respectively; using 1% of chitosan, the mitigation was by 75% and 76% at 180 and 150ºC respectively.

The evidence of chitosan mitigation effect in model systems required the study in real systems. Thus, batter systems were chosen for this purpose, as it could be easily added as an ingredient of a batter formulation. Formulations included a mix of commercial flours (wheat and rice), salt, and chitosan (0, 0.27 and 0.54%). Batters were fried at 180ºC for 2, 4 and 7 minutes in order to test the effect on samples before the optimum frying time, at the optimum time and on over fried samples. Samples fried until the optimal time showed acrylamide reductions of 59 ± 6% when 0.27% of chitosan was added; the addition of higher amount of chitosan (0.54%) did not reduce significantly the final acrylamide content.

Acrylamide mitigation has become a new property of chitosan that could help developing healthier products. The addition of other hydrocolloids to fried batters has also shown acrylamide reductions, although 5% of pectin addition was needed to get similar reductions as those we obtained with 0.27% of chitosan. It is important to highlight that the main purpose of hydrocolloid addition in batter systems is focused on getting healthier food products, with less oil content. Results from this work showed that to develop fried batters with less acrylamide, and thus, healthier food, can also be achieved by using chitosan, which evidences a new property of this hydrocolloid.

PROTECTIVE EFFECT OF CHITOSAN ON ACRYLAMIDE FORMATION IN MODEL AND BATTER SYSTEMS

Mariola Sansano, Mª Luisa Castelló, Ana Heredia, Ana Andrés

Abstract

In recent years high contents of acrylamide, a potentially carcinogenic substance, have been found in a wide range of fried and baked foods. For this reason, the health authorities together with the food industry have carried out research to find ways to minimize the presence of acrylamide during food processing. The addition of chitosan may be an excellent alternative for achieving this goal because due to their richness in amino groups, they would interfere with the Maillard reaction that unleashes the formation of acrylamide. The main aims of this study were to analyze the addition of different concentrations of chitosan in model systems as a new way of mitigating generation of acrylamide during frying processes, while evaluating the influence of pH, reducing sugars (glucose and fructose) present in the system and frying temperature, and to determine the functionality of adding chitosan in fried batter systems. The results showed that chitosan is capable of inhibiting the formation of acrylamide in model systems and in fried batters. In model systems, a reduction in acrylamide ranging from 49 to 85 % was achieved for 1% of chitosan, the maximum inhibition taking place in asparagine-fructose model systems and the lowest in asparagine-glucose model systems. In fried batter, acrylamide was mitigated by 59 ± 6 % with a chitosan concentration of 0.27% in batter formulations. Double concentrations of chitosan (0.54 %) did not considerably improve the inhibition capacity.

Keywords: Acrylamide, Chitosan, Model systems, Batter systems

1. Introduction

It is well-known that food processing can improve nutrition, quality and safety. However, toxic substances such as acrylamide can sometimes be formed through the interaction of food compounds, from natural and added ingredient.

According to some epidemiological studies, acrylamide is potentially carcinogenic compound for humans (IARC, 1994), not only due to its consumption, but also to its role as a precursor in the development of other compounds during hepatic metabolism such as glycidamide (Blank, 2005). Acrylamide is mainly used in industrial processes used to make paper, dyes, plastics and treating drinking water. However, it can also be present in small amounts in food packaging, some adhesives and cigarette smoke (Rudel, Ackerman, Attfield, & Brody, 2014). Acrylamide was also found to be formed in some starchy foods, especially potato products, during high-temperature cooking and under low moisture conditions, such as frying, baking and roasting, formation being lower in protein-rich foods (Tareke et al., 2002). Acrylamide is formed during Maillard reactions, and mainly between the reaction of asparagine and reducing sugars at high temperatures (Becalski, Lau, Lewis, & Seaman, 2003; Mottram, Wedzicha, & Dodson, 2002; Stadler et al., 2002). Several studies have proven the importance of temperature, time, levels of precursors, pH, nature of the matrix, etc. on acrylamide formation in food. Consequently, a wide range of strategies have been developed in the last decade to reduce the final content of acrylamide in model systems and foods processed at high temperatures. Some strategies based on controlling processing conditions such as time and temperature (Tareke et al., 2002), as well as frying in low pressure conditions or novel frying techniques, such as, microwave or air frying have achieved a significant inhibition of acrylamide formation (Barutcu, Sahin, & Sumnu, 2009; Sansano, Juan-Borrás, Escriche, Andrés, & Heredia, 2015; Troncoso & Pedreschi, 2009). It is also advantageous to apply treatments before frying, such as blanching, or soaking the food products in acids, vitamins, cations or amino acids in order to reduce acrylamide precursors, and to interfere with and modify Maillard reactions triggering acrylamide formation (Gökmen & Şenyuva, 2007; Jung, Choi, & Ju, 2003; Pedreschi, Kaack, & Granby, 2004; Rydberg et al., 2003; Zeng et al., 2009).

Hydrocolloids are hydrophilic polymers that modify the functional properties of food systems, such as thickening, gelling and emulsifying properties (Saha & Bhattacharya, 2010). Some studies have tested the use of hydrocolloids to

control moisture diffusion and consequently, oil absorption during frying. Lower contents of fat were obtained when including hydrocolloids such as soy protein isolate, whey protein isolate, methylcellulose and hydroxypropyl methylcellulose as an edible film coating before frying (Albert & Mittal, 2002; Balasubramaniam, Chinnan, Mallikarjunan, & Phillips, 1997) or, what seems to be most effective, introducing them as an ingredient in batter formulation (Holownia, Chinnan, Erickson, & Mallikarjunan, 2000; Sanz, Salvador, & Fiszman, 2004). Zeng et al. (2010) tested some hydrocolloids (agar, alginic acid, carrageenan, carob gum, gelatin, hydroxyprpyl distarch phosphate, pectin and xanthan gum) in acrylamide formation in model and real systems. They found positive results mainly for pectin and alginic acid, but these hydrocolloids did not significantly change the water content of the fried potatoes strips. Therefore, they are unlikely to modulate the formation of acrylamide due to their property of water retention. These authors suggested that the formation of surface coatings might also modulate heat transfer from the surrounding oil to the product.

Among the different hydrocolloids, chitosan, a polycationic polymer and waste product from the sea food processing industry, is an abundant natural resource that has, as yet, not been fully utilized. The advantages of this polymer include availability, low cost, high biocompatibility, biodegradability and ease of chemical modification. Chitosan has many applications in several sectors because of its multiple properties: it is not digestible by humans, so it is considered to be a dietary fiber; which binds lipids and helps in reducing cholesterol (Muzzarelli, 1996), and it is protective, fungistatic and antibacterial (El Ghaouth, Arul, Ponnapalam, & Boulet, 1991; Tsai & Su, 1999). Moreover, chitosan is a molecule which is rich in amino groups, this being the main characteristic leading to our hypothesis: amino groups of chitosan would compete with amino groups of asparagine to bind to carbonyl group of reducing sugars and thus, would modulate acrylamide generation (Lindsay & Jang, 2005). If this hypothesis is confirmed chitosan would be proven to have another function: protecting against acrylamide formation. The main purpose of this study was to analyze the addition of chitosan as a way to mitigate the generation of acrylamide during frying

processes in model systems and fried batter systems. The effect of pH of the reaction, the type of reducing sugars (glucose and/or fructose) present in the model system and the temperature were also evaluated.

2. Materials and methods

2.1. Chemicals and consumables

Asparagine, glucose and fructose were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Chitosan (Poly (D-glucosamine)*Deacetylated chitin) was also purchased from Sigma- Aldrich (St. Louis, MO, USA). Chitosan was used in coarse ground flakes and powder, presented a deacetylation degree superior to 75% with a high molecular weight (lot: MKBH5816V). Formic acid, acetonitrile and magnesium sulfate were purchased from VWR-Prolabo (Fontenay-sous-Bois, France), methanol and hexane were obtained from Panreac (Barcelona, Spain). Acrylamide standard (> 99%) was purchased from Merk (Darmstadt, Germany), sodium chloride was obtained from Scharlab (Barcelona, Spain) and Primary secondary amine (PSA) was purchased from Supelco (Bellefonte, USA). Double distilled water was prepared for chromatographic use (Milli-Q, Millipore Corp., Bedford, MA). All chemicals used were analytical grade, and those used for chromatographic analysis were HPLC grade. To test the effect of chitosan in a real system, a commercial formulation was used (Yolanda, Murcia, Spain). This formulation consists of wheat and rise flours, an acidity regulator (E-334), bulking agent (E-500ii) and coloring (E-160b). Moisture and ash contents (11.5% and 1.8%, respectively) were measured using AACC methods (1995), protein and fat contents (10.0% and 1.4%, respectively) were supplied by manufacturers, and particle size (78.0 μm) was analyzed with the Mastersizer 2000 (Malvern Instruments, Germany) coupled with the Scirocco 2000 module for dry measurement.

2.2. Preparation of reaction mixtures for pyrolysis

In order to confirm our hypothesis, we carried out chemical model reactions following the method proposed by Gökmen and Şenyuva (2007) with some minor

modifications. The reaction was carried out using a 25 mL threaded Pyrex tube which contained 5 µmol of asparagine and 5 µmol of reducing sugars, and 100 µl of acid lactic solution on which chitosan was previously dissolved at 0, 0.5 or 1%. Eighteen different model systems were formulated depending on the type of sugar used: glucose, fructose or an equimolecular mixture of both; the pH (4 and 5) and the concentration of chitosan (0, 0.5 and 1%).

The samples were placed in an oil bath previously preheated at the two temperatures tested (150 and 180 ºC) and the total heating time for the samples was 30 min. After the reaction time, the tubes were immediately cooled in an ice-water bath for 5 min.

2.3. Preparation of batters systems for frying

Batter formulations consisted of the commercial formulation with chitosan solutions (at 0, 0.5 and 1%) at pH=4 with 2.5% of salt in a water-to-dry-mix proportion of 1.2/1. The final chitosan contents in the formulations were 0, 0.27 and 0.54% respectively. Batter samples were kept for at least 30 min at room temperature before frying. The frying step was carried out in a commercial deep-fat fryer with a capacity of 2 L (model: FM 6720 Ideal 2000 Professional, Solac) at 180 ± 2 ºC. Samples (11.5 ± 0.1 g) were placed in an aluminum cylindrical instrument and then introduced in the fryer in order to obtain homogenous ring shaped fried samples (height: 11±1mm; outer diameter= 65mm ± 2 and inner diameter= 25 ± 1mm). Triplicate samples (n = 3) were fried for 2, 4 and 7 min for the three formulations tested. The excess oil was removed with paper on both sides for 20 s after taking the samples out of the fryer.

2.4. Analysis of acrylamide

2.4.1. *Extraction of acrylamide from pyrolysates (model systems)*

Two mL of Mili-Q water were added to the pyrolisates obtained and tubes were agitated in a vortex for 1 min. The tube content was filtered (0.22 µm Nylon filters) and transferred to a vial for the following acrylamide content determination, studied in triplicate (n=3).

2.4.2. Extraction of acrylamide from the fried batter systems

The acrylamide content was determined by means of dispersive solid phase extraction (QuEChERS) according to Mastovska and Lehotay (2006) with some modifications. The standard addition was used rather than the traditional calibration curve in order to remove the matrix effect, fortifying at five different levels (10, 20, 50, 100 and 300 $\mu g/kg^{-1}$), with six replicates for each level (n=6). Fried batter systems were subjected to a previous acrylamide extraction as follows: three samples were ground in a blender and a sub-sample (1 g) was placed in a 50mL Falcon tube, following which 5 mL of n-hexane were added. The tube was shaken in a vortex for 30 s, after which10 mL bidistilled water, 10 mL acetonitrile, 4 g MgSO4 and 0.5 g NaCl were added and stirred in the vortex for one minute. The suspension was then centrifuged at 2026 RCF (Centronic BL II (Selecta, Spain)) for 5 min, following which the hexane layer (upper phase) was discarded. 1 mL of the acetonitrile phase, containing the acrylamide, was then transferred to a 2 mL polypropylene tube containing 50 mg PSA and 150 mg MgSO4, and stirred for 30 s. The homogenate was centrifuged at 2697 RCF (Labofuge 200 (Heraeus, Germany)) for 1 min and the supernatant was transferred to a vial for acrylamide analysis.

2.4.3. LC/MS/MS analysis

The acrylamide analysis was performed with an Agilent 1200 Series HPLC system coupled to an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies Inc., CA, USA) with an electrospray type ionization source. The column used in this study was a Zorbax Eclipse XDB C-18 (2.1mmx50mm, 1.8µm). The mobile phase used consisted of 2.5% methanol/ 97.5% of 0.1% formic acid (A) and methanol (B). The elution gradient was as follows: 0-3 min 100% of A; 3.1-3.5 min 70% A; 3.6 min 100% A, with 1 min post-time to equilibrate the column. The column oven temperature was set at 30°C, the flow was maintained at 0.4 mL/min and the injection volume was 10µL. The electrospray was operated in positive ion mode. The conditions used in the ionization source were: 350 ºC at 12 L/min for the drying gas (N_2), a nebulizer pressure of 40 psi

and a capillary voltage of 4000 V. Identification and quantification of acrylamide in the samples was performed using the multiple reaction monitoring mode (MRM), and the ion m/z 72 > 27 and m/z 72 > 55.2 were studied respectively.

2.5. Water content determination

Water content was analyzed by vacuum drying at 60 ºC until constant weight was achieved (20.103, AOAC, 1980).

2.6. Statistical analysis

Statistical analysis of variance (ANOVA) was performed by Statgraphics Centurion to estimate the effect of process variables (pH, reaction temperature and time, reducing sugars and chitosan content) on the obtained results. Evaluations were based on 95 and 99% significance levels.

3. Results and discussion

3.1. Effect of chitosan on acrylamide formation in model systems

The acrylamide content generated in model systems formulated with glucose, fructose and the equimolecular mixture of both sugars are shown in Figure 4.5. In all cases the higher the percentage of chitosan, the lesser the amount of acrylamide produced. Temperature was an important factor, considering that at 150 ºC the acrylamide content grows gradually with heating time, but at 180 ºC, after 10-15 min the amount of acrylamide generally reaches the maximum value and starts to decrease lightly with treatment time, likely due to polymerization (Stadler et al., 2004). The statistical analysis revealed that except pH, all the factors considered in this study (type of reducing sugars present in the system, the reaction time, the % of chitosan and the temperature) have a significant influence on acrylamide formation (Table 4.3). As the pH was not a significant factor, Fig 4.5 only includes results at pH 4.

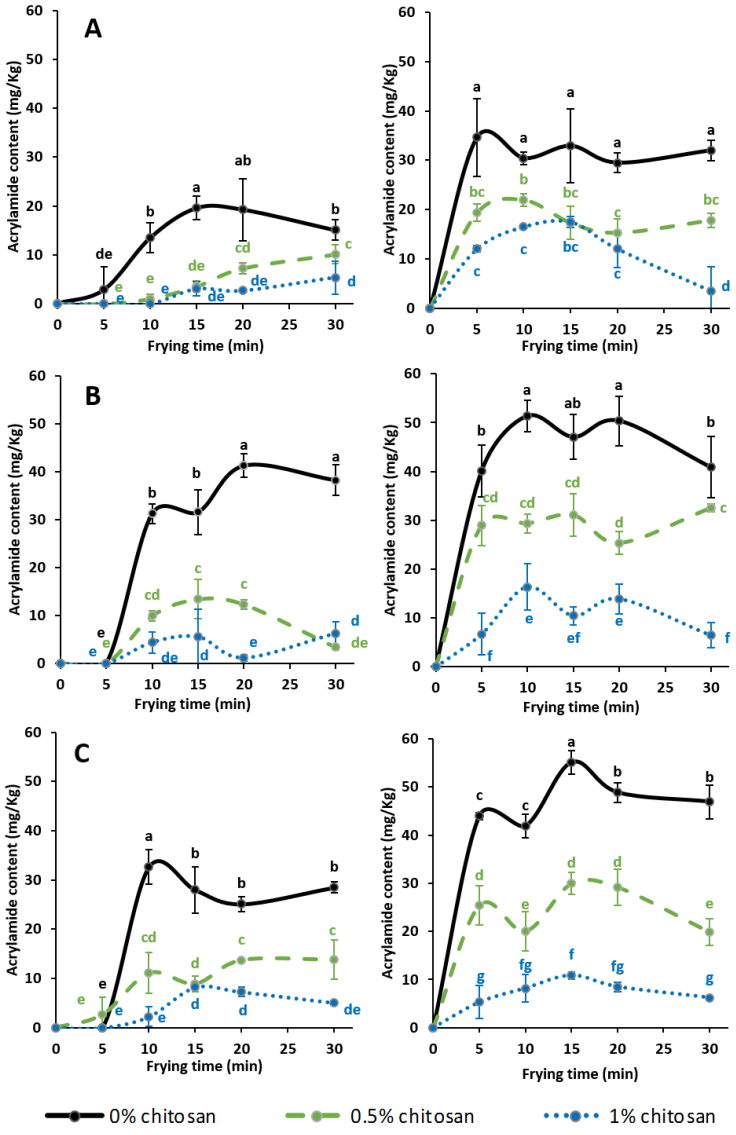

Figure 4.5. *Average acrylamide content (mg/kg) generated in model systems with 0, 0.5 and 1% of chitosan, pH 4 at 150 ºC (left) and 180 ºC (right) after 5, 10, 15, 20 and 30 min of frying. (A) asparagine-glucose; (B) asparagine-fructose; (C) asparagine-glucose-fructose.*

Error bars represent standard deviations (n=3).

Homogeneous groups are represented by the same letter.

Table 4.3. *Multifactor ANOVA for Acrylamide content (mg/kg) of main effects and their interactions in model systems.*

MAIN EFFECTS	*Df*	*F-Ratio*
pH	1	0.13 (NS)
Reducing Sugar	2	54.86**
Time (min)	5	175.00**
Chitosan (%)	2	618.45**
Temperature (ºC)	1	624.55**
INTERACTIONS	*Df*	*F-Ratio*
pH- Reducing sugar	2	6.01**
pH- Time	5	4.18**
pH-Chitosan	2	4.43*
pH- Temperature	1	1.39 (NS)
Reducing sugar-Time	10	5.26**
Reducing sugar-Chitosan	4	17.61**
Reducing sugar-Temperature	2	2.56 (NS)
Time- Chitosan	10	29.79**
Time -Temperature	5	41.27**
Chitosan-temperature	2	77.67**

***Statistical significance ≥99% (p-value ≤ 0.01);*Statistical significance ≥95% (p-value ≤ 0.05); NS (not statistical significance, p-value > 0.05).Df: degrees of freedom*

According to the F-Ratio values, the temperature and the percentage of chitosan are the most significant factors, as well as the interaction between them. Time is also a significant element, as has been proven in many studies, as well as the temperature and their interaction (Gertz & Klostermann, 2002).The potential of generating acrylamide from suitable precursors has mainly been attributed to the concentration of asparagine, which directly provides the backbone of the acrylamide molecule. However, there is some indication in literature that the type of sugar, or in general the carbonyl compound, may significantly affect the final amount of acrylamide generated through the Maillard reaction. Some authors have speculated on the role of physical properties of

precursors and suggested that the melting point of sugars is a possible parameter to consider (Stadler et al., 2004).

In Figure 4.6, differences in acrylamide content depending on the type of reducing sugar in the system can be appreciated. Higher amounts of acrylamide were produced with fructose than with glucose. Other authors have stated that mixtures with fructose generate acrylamide earlier, meaning at a lower temperature, than those containing glucose (at about 125 and 140 ºC, respectively), which means the final content of acrylamide was higher with fructose than with glucose (Robert et al., 2004).

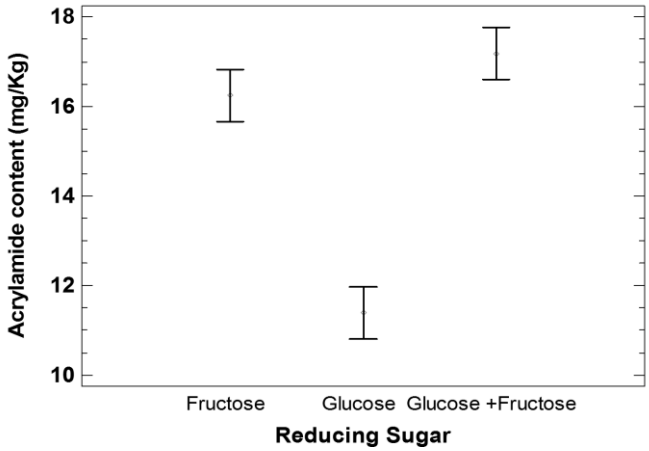

Figure 4.6. *Influence of the type of reducing sugars tested on acrylamide formation (mg/kg) in model systems.*
Error bars represent 95% LSD (Least significance difference).

Figure 4.7 shows the inhibitory effect of chitosan on acrylamide formation. In fact, adding 0.5% of chitosan led to an inhibition of acrylamide formation (according to the control) of 52% and 65% at 180 and 150 ºC respectively, and 1% of chitosan, 75% and 76% at 180 and 150 ºC respectively. The influence of the temperature on acrylamide formation is well known but when the interaction between the temperature and the concentration of chitosan was analyzed, it can be observed that chitosan drastically reduces the influence of temperature on the acrylamide formation. This seems to indicate that there is likely to be a reaction

of chitosan with reducing sugars at temperatures below acrylamide formation temperatures. In spite of the small concentration of chitosan, the resulting inhibitory effect was significant, ranging between 40% and 84% (results not shown) depending on the reducing sugar and the treatment temperature. The protective effect of chitosan is very high as compared to the results reported by Zeng et al., (2010), who used other hydrocolloids in model systems and in which at the concentration of 1% none of the hydrocolloids showed a significant inhibition of the formation of acrylamide, although around 60% was achieved when 2% of alginic acid and pectin were tested.

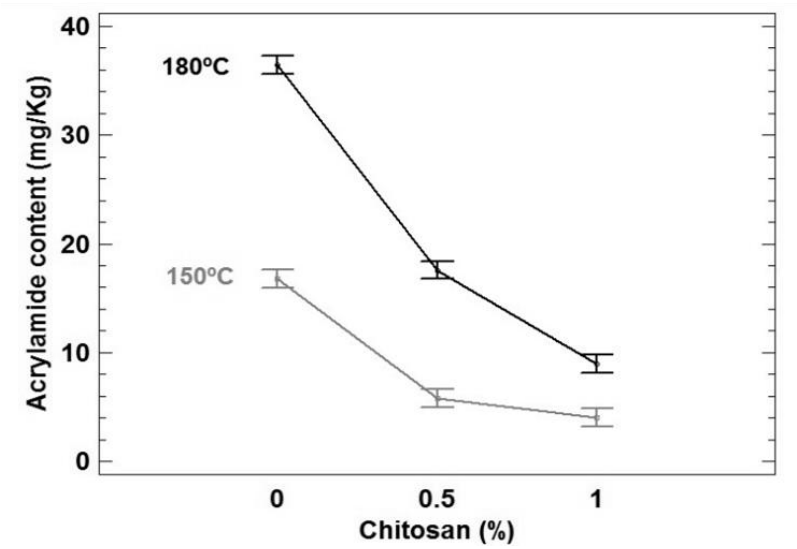

Figure 4.7. *Influence of process temperature (150 and 180ºC) and chitosan content (0, 0.5 and 1%) on acrylamide formation (mg/kg) in model systems. Error bars represent 95% LSD (Least significance difference).*

3.2. Effect of chitosan on acrylamide formation in fried batter systems

As evidenced in the model systems, the effect of chitosan on acrylamide inhibition was observed in real systems. The results showed that the concentration of chitosan was again the most significant factor followed by the frying time (F-ratio: 44.38 and 33.43, respectively) and their interaction (chitosan %-frying time). The ability of chitosan to compete with asparagine to bind to

reducing sugars was quite significant even at low concentration and no significant differences are found between using 0.27 or 0.54% of chitosan at 2 and 4 min (Figure 4.8). At 7 min, which is above the optimum frying time, the reduction in acrylamide formation was dependent on the percentage of chitosan, being more effective at 0.54 than 0.27%.

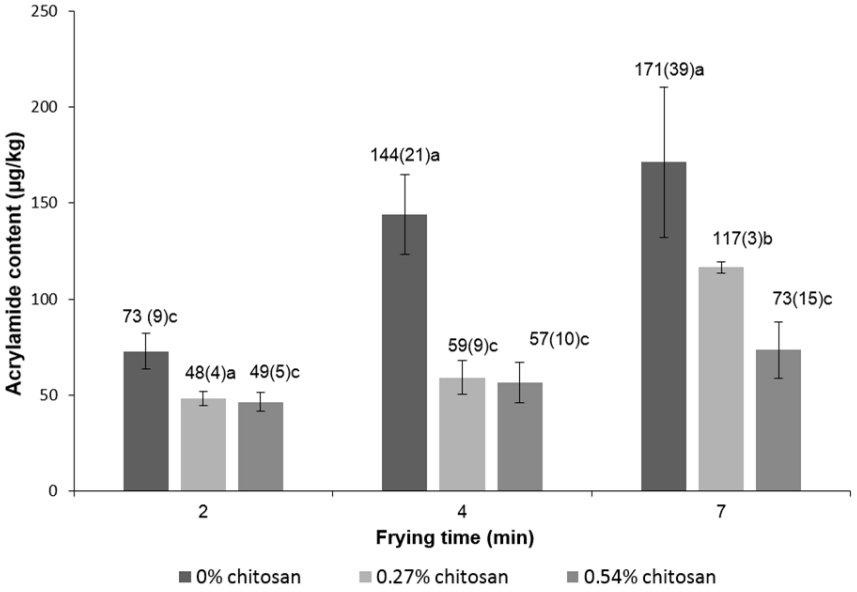

Figure 4.8. *Acrylamide content (mean and standard deviation) in fried batter systems with 0, 0.27 and 0.54% of chitosan at 2, 4 and 7 min.*
Homogeneous groups are represented by the same letter.

It is generally known that water content is a key factor that has to be considerate in fried products in terms of acrylamide formation. Chitosan, as a hydrocolloid, joins water, but water content of fried samples was not a significant variable in acrylamide formation in this study. Chitosan did not significantly modified water content of fried samples (p-value= 0.9725), but, as was expected, frying time was a significant factor (p-value=0.0000, F-ratio=60.99). Values of percentage of moisture content at 2, 4 and 7 min were 20 ± 2^{a}, 17 ± 2^{b}, 5.8 ± 1.9^{c} for control samples; 21 ± 3^{a}, 16.3 ± 0.6^{b}, 4.5 ± 1.8^{c} when 0.27% of chitosan was added, and 22 ± 2^{a}, 16 ± 2^{b}, 4.4 ± 0.2^{c} for 0.54% of chitosan.

Figure 4.9 shows that at similar heating times, 85±9% of reduction was the highest inhibitory rate found in model systems when 1% of chitosan was present in the medium. In fried batter systems, the inhibition rate reached by adding chitosan to batter formulation was about 60 ± 7% regardless of the percentage of chitosan tested (0.27 and 0.54%).

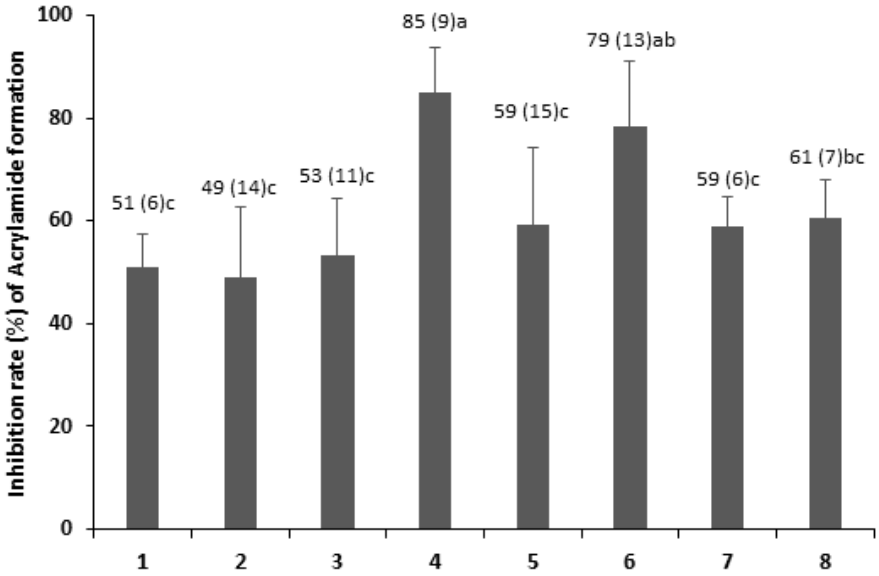

Figure 4.9. *Inhibitory effect of chitosan (%) (mean and standard deviation (n=3)) on acrylamide formation in model systems (after 5 min of reaction time) and fried batter systems (after 4 min of frying) at 180 ºC. X-axis legend: (1) (2) asparagine-glucose and 0.5 or 1% of chitosan; (3) (4) asparagine-fructose and 0.5 or 1% of chitosan; (5) (6) asparagine-glucose-fructose and 0.5 or 1% of chitosan; (7) (8) fried batters with 0.27 or 0.54% of chitosan.*
Homogeneous groups are represented by the same letter.

These are excellent results as compared to other hydrocolloids tested in real foods, i.e., the maximum inhibitory rate reported by Zeng et al. (2010) that tested the addition of different hydrocolloids to the formulation of a cracker, was 43% when 5% of pectin was incorporated to the formula.

4. Conclusions

Adding small amounts of chitosan in model and fried batter systems has been proven to be a new way to mitigate the generation of acrylamide. In fried batters, 0.27% of chitosan was capable of reducing the content of acrylamide in the final product by 59% and in model systems, the reduction depended largely on the reducing sugar tested, but to an even greater extent, on the percentage of chitosan, especially when fructose was present in the medium (up to 85%). The proposed mechanism of acrylamide reduction is based on the richness of amino groups of chitosan, which compete with asparagine amino groups to bind carbonyls (e.g. reducing sugars), the first stage of acrylamide formation. For this reason, chitosan has a high potential to provide consumers with healthy food products (lower acrylamide content) if it is incorporated into batters on a commercial scale.

Acknowledgments

The authors would like to thank the Generalitat Valenciana (GV/2012/072) for the financial support given to this investigation.

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4.3. ACRYLAMIDE FORMATION AND QUALITY PROPERTIES OF CHITOSAN BASED BATTER FORMULATIONS

Mariola Sansano, Mª Luisa Castelló, Ana Heredia; Ana Andrés

Food hydrocolloids, 66 (2017), 1-7

http://dx.doi.org/10.1016/j.foodhyd.2016.10.019

Instituto de Ingeniería de Alimentos para el Desarrollo
Universitat Politècnica de València, Valencia, Spain

ACRYLAMIDE FORMATION AND QUALITY PROPERTIES OF CHITOSAN BASED BATTER FORMULATIONS

Mariola Sansano, Mª Luisa Castelló, Ana Heredia & Ana Andrés

The ability of chitosan to reduce acrylamide formation in model systems has been previously shown. But, chitosan functional properties are strongly dependent on its particular structure. In this sense, properties such as the molecular weight and the deacetylation degree are the main characteristics that differentiate chitosans. Because of this, the first objective of this work was to study how these properties could modulate acrylamide inhibition in order to make a selection of the most adequate degree of deacetylation (DD) and molecular weight (Mw) of chitosan based on acrylamide mitigation criteria.

In addition, the effectiveness of chitosan as an additive to mitigate acrylamide formation in fried batters was also tested and evidenced; subsequently, it arose the necessity to study if the final fried product with chitosan would maintain the quality attributes. This is the reason why the oil uptake, the texture and the color of fried batters with and without chitosan were evaluated. In addition, the flow properties and water retention capacity of the raw batter with chitosan were also analyzed. So, the second objective of this work was to evaluate the influence of including chitosan in batter formulations on some important technological parameters of raw batters (flow behavior and water retention capacity) and on some quality properties of fried batters (oil uptake, color and texture).

Acetylation and deacetylation processes were tested on commercial chitosan to obtain four final deacetylation degrees. In addition, commercial chitosan was subjected to acid hydrolysis in order to obtain lower molecular weights (finally, 4 different Mv). Resulted chitosans were dried at 60ºC and after grinding, their powder was obtained. Titration and viscosimetry methods were used to analyze deacetylation degree and molecular weight of the different chitosans obtained. Model systems were prepared as in the previous chapter, adding 1% of the obtained chitosans and comparing with control samples (without chitosan).

Batters were composed of a commercial dry mix (rice and wheat flours basis), salt and a 1% of commercial chitosan solution, in a water-to-dry proportion (1:1.2), and samples without chitosan were used as control. Rheological parameters and water retention capacity of the raw batter formulation were evaluated, and after frying (2, 4 and 7 min, 180ºC), water loss and oil uptake as well as color (cieLab coordinates) and texture at 55ºC and 25ºC were also analyzed.

Results corresponding to the first objective showed that deacetylation degree and molecular weight of chitosan affect the acrylamide formation in model systems. High deacetylation degree, what means greater amount of free amino groups in chitosan structure, resulted in greater acrylamide reduction. Concretely, between 44% and 81% of acrylamide reduction was obtained when higher deacetylation degree chitosans (86.5 and 92.8%) were added to the model system, as compared to the control. The higher the molecular weight of chitosan the less acrylamide content was obtained. The acid hydrolysis of chitosan was the chosen process for obtaining smaller molecular weights. But, this process seemed to modify notably other chitosan structural properties, what could be the reason to explain the worse inhibitory performance on acrylamide formation than chitosan without acid hydrolysis treatment.

The addition of commercial chitosan increased the consistency, the apparent viscosity and the water retention capacity of raw batters, depending on batter temperature. Although color and water loss were not affected by chitosan addition, fried batters absorbed less oil content and hardening during the cooling period was reduced in samples with chitosan. Those results arise the importance of molecular specifications of chitosan in case manufacturers would like to use it on industrial scale with for acrylamide mitigation purposes. In the same way, the higher viscosity of the resulting batters with chitosan, have to be considered for the optimization of the process, since mixing, pumping and coating could be affected. Results related to acrylamide and oil uptake revealed the potential of chitosan-based fried batters to obtain healthier food products.

ACRYLAMIDE FORMATION AND QUALITY PROPERTIES OF CHITOSAN BASED BATTER FORMULATIONS

Mariola Sansano, Mª Luisa Castelló, Ana Heredia; Ana Andrés

Abstract

The potential of chitosan to mitigate acrylamide formation has been already demonstrated. The two main objectives of this study were: 1) to select the most adequate degree of deacetylation (DD) and molecular weight (Mw) of chitosan based on acrylamide mitigation criteria and 2) to evaluate the influence of including chitosan in batter formulations on some important technological parameters of raw batters (flow behavior and water retention capacity) and on some quality properties of fried batters (oil uptake, color and texture). Results in model systems showed that chitosans with higher deacetylation degree (86.5 and 92.8%) achieved a decrease of acrylamide between 44 and 81%, depending on reaction time, compared to the control (without chitosan). Furthermore, acid hydrolysis process of chitosan was found to negatively affect its inhibitory effect on acrylamide formation independently of the molecular weight. Raw chitosan based batter formulations presented higher consistency and water retention capacity than the control; chitosan addition to batters reduced the hardening of the fried samples during the post-frying cooling period. No significant differences in water loss were observed between batters with or without added chitosan; however, chitosan-batter formulations showed lower oil uptake during frying as compared to control samples.

Keywords: Acrylamide, chitosan, deacetylation degree, molecular weight, batters

1. Introduction

Consumers are becoming more health-conscious and demand high quality food products, binding the food industry to take measures to provide these needs. Healthier fried products, with low-fat content and/or free of acrylamide, could be examples of this growing demand. Acrylamide is a probable carcinogenic for

humans (Group 2A) according to the IARC classification that could be formed in foods, especially in starchy foods such as potatoes or cereals, submitted to processes taken place at temperatures above 120 ºC, being an intermediate-product of Maillard reactions (Mottram, Wedzicha, & Dodson, 2002; Stadler et al., 2002; Becalski et al., 2003). On 4 June 2015, European Food Security Administration (EFSA) published its first full risk assessment of acrylamide in food reconfirming that acrylamide in food potentially increases the risk of developing cancer for consumers in all age groups. FoodDrinkEurope consortium annually updates to the Acrylamide Toolbox rapport which recompiles different strategies from scientific literature addressed to Food Industry, policy-makers and consumers to inhibit the acrylamide generation. Most of these are addressed to reduce the acrylamide precursors (asparagine or reducing sugars) in the food matrix and/or stablish mechanisms to avoid its generation interfering in Maillard reactions (Medeiros Vinci et al., 2012; Morales et al., 2008). In the last years, the influence of hydrocolloids in acrylamide formation has been tested in model and fried systems. Certain hydrocolloids (pectin and alginic acid) have demonstrated good potential to mitigate up to 50% of acrylamide content in foods when they are incorporated above of 5% in crackers (Zeng et al., 2010). Recently, the capacity of chitosan to limit acrylamide generation has been proved in model and fried batter systems (Sansano, Castelló, Heredia, & Andrés, 2016; Chang, Sung, & Chen, 2016). Concretely, the addition of small amounts of chitosan (~ 0.5%) to model systems and batters reduces acrylamide generation in 58±23 and 61±7 %, respectively. The action mechanism is based on the free amino groups present in chitosan which are able to compete with asparagine in binding to the reducing sugars, which implies a reduction in acrylamide formation. Chitosan is a polysaccharide obtained by deacetylation of chitin, an abundant polysaccharide in nature. Chitosan has been found to be biocompatible, biodegradable, biofunctional, and has strong antimicrobial and antifungal activities (Aider, 2010). Some of the current applications of chitosan are based on its antioxidant character (Darmadji & Izumimoto, 1994), antitumor (Tsukada et al., 1990), anti-cholesterolemic, anti-ulcer and its antiuricemic properties, which are related to

the capacity of chitosan to bind specifically fatty acids, bile acids, phospholipids and uric acid (Muzzarelli, 1996). It should be highlighted that those biological properties of chitosan (antimicrobial, antioxidant and anti-cholesterolemic) mainly depend on its deacetylation and polymerization degrees (Aranaz et al., 2009). Therefore, these properties might affect its potential to reduce acrylamide formation as well. For this reason, these properties should also be considered to select the specific chitosan to be added as ingredient with this specific purpose. The influence of deacetylation degree (DD) and molecular weight (Mw) of chitosan on acrylamide reduction needs to be correlated. Chang et al., (2016) reported the effect of chitosan with low molecular weight on acrylamide generation in model systems, indicating a statistically significant capacity to reduce acrylamide formation of 50-190 KDa-chitosan compared to the control, but there are no scientific papers published of the effect of deacetylation degree of chitosan on acrylamide mitigation.

Batters are used as coatings for fried products such as onion rings, tempura products (vegetables, prawns...), battered squid rings, chicken or fish nuggets, and they are composed basically of flour, water, salt and leavenings. It is well known that the selection of batter ingredients determines the visual appearance, color, flavor, crispiness, adhesion and therefore consumer acceptance (Hsia, Smith, & Steffe, 1992). In this sense, the use of chitosan as ingredient in batter formulations with the main goal of inhibits acrylamide could have an impact on different properties of raw and fried batters as well.

For these reasons, the main objective of this study was to evaluate the influence of deacetylation degree and molecular weight of chitosan on the acrylamide generation in model systems in order to select the most appropriate chitosan to be included in batter formulations. The influence of chitosan addition on the rheological parameters and water retention capacity of raw batter, as well as the influence on water loss and oil uptake during frying were also evaluated. Finally, fried samples were also compared in terms of color and texture.

2. Materials and methods

2.1. Reagents

Reducing sugars (glucose and fructose), asparagine, chitosan (Poly (D-glucosamine) *Deacelyled chitin, high molecular weight) and acetic anhydride were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Acetic acid, sodium hydroxide, lactic acid, methanol were from Panreac (Barcelona, Spain). Formic acid was purchased from VWR-Prolabo (Fontenay-sous-Bois, France). The standard of acrylamide (≥ 99%) was from Merck (Darmstadt, Germany) and the internal standard, $^{13}C_3$-labelled acrylamide (99%) from Cambridge Isotope Laboratories (Andover, MA). All chemicals were analytical grade, except those used for chromatographic analysis (HPLC grade). The bidistilled water was obtained by a purification process of water (Milli-Q, Millipore Corp., Bedford, MA).

2.2. Acetylation and deacetylation of commercial chitosan

Commercial chitosan was used to obtain chitosan with different deacetylation degree (DD) by acetylation and deacetylation processes. Acetylation process was performed according to Kiang, Wen, Lim, & Leong, (2004). Briefly, 15 g chitosan were dissolved in a solution of 2% acetic acid (300 mL), distilled water (400 mL) and methanol (800 mL), and stirred for 20 minutes. Then, 2 mL of acetic anhydride were added into the solution and the mixture was stirred for 12 hours. To end the reaction, 1M NaOH was added to the solution in order to precipitate the chitosan, which was washed several times until neutral pH with distilled water and dried under vacuum at 60°C. Deacetylation was done according to Zhou et al., (2008) with slight modifications: chitosan (10 g) was dissolved in 100 mL of NaOH solution (ratio of 1:2 (w/v)) for 30 minutes at 100ºC, washed repeatedly with distilled water and dried at 60ºC. This process was considered as a one cycle deacetylation process, and was applied one or twice to obtain different DD.

The titration method described by Wang et al. (2006) with minor modifications was used to determine the deacetylation degree (DD) of the chitosan obtained from the above described process. 0.2 g of chitosan was dissolved in 20 mL of

HCl 0.1 M under stirring for 4 h. Measurements were performed with a solution of NaOH 0.1 M by using a Metrohm's high-end titrator. The DD of chitosan was calculated as follows (equation 1):

$$DD = \frac{\Delta V \cdot C_{NaOH} x\, 10^{-3} \cdot 16}{M \cdot 0.0994} \qquad (I)$$

where ΔV is NaOH volume of between two inflexion points, C_{NaOH} is concentration of NaOH solution, M is the mass of the sample, and 16 and 0.0994 are the molecular weight and theoretical amount of amino groups, respectively.

2.3. Depolymerization of commercial chitosan by acid hydrolysis

Chitosan with different molecular weight (Mw) were obtained by acid hydrolysis according to the method described by Zhou et al., (2006) with minor modifications. Commercial chitosan (2g) was dissolved in 2% acetic acid (100mL), stirred and heated at 70ºC for different times (2, 4 and 8 hours) in order to obtain chitosan with different molecular weights. After that, the reaction mixture was neutralized with NaOH. Absolute ethanol was added (70 mL per liter of solution) in order to completely precipitate the chitosan. The samples were filtered, washed with distilled water, and dried at 60ºC.

The molecular weight of the chitosan (M_v) was determined by viscosimetry (Bof et al., 2015). The measurements were performed using an Ubbelohde capillary viscometer No. 2121R, (Ø= 0.4 mm) equipped with a thermostat bath at 25.0° C ± 0.01ºC. Chitosan was dissolved in 0.1M acetic acid/0.2M NaCl, into different concentrations: $5.0 \cdot 10^{-4}$, $6.5 \cdot 10^{-4}$, $8.5 \cdot 10^{-4}$ y 10^{-3} g/mL, being filtered (0.45µm) before viscosity determinations. Draining times of a fixed volume of chitosan solutions (t) and pure solvent (t_0) were measured. From these, relative viscosity (η_r) and specific viscosity (η_{sp}) of were calculated using the following equations *II* and *III*:

$$\eta_r = \frac{\eta}{\eta_0} = \frac{t}{t_0} \qquad (II)$$

$$\eta_{sp} = \eta_r - 1 \qquad (III)$$

where η is chitosan solution viscosity and η_0 is viscosity of the pure solvent, and their corresponding draining times (t and t_0).

The reduced viscosity (η_{red}) was calculated from the specific viscosity (η_{sp}) and the concentration of chitosan solution (*equation IV*):

$$\eta_{red} = \eta_{sp} / C \qquad (IV)$$

where C is concentration of chitosan solution (g/mL).

The intrinsic viscosity $[\eta]$ was determined graphically, extrapolating values of reduced viscosity (η_{sp}/C) to zero concentration. The intrinsic viscosity was used to determine the viscosity average molecular weight (M_v) from Mark-Houwink-Sakurada-Staudinger equation (*equation V*):

$$[\eta] = K_m \cdot (M_v)^a \qquad (V)$$

where Km and a are two constants dependent on the particular polymer-solvent system ($1.81 \cdot 10^{-3}$ and 0.93, respectively) (Roberts & Domszy, 1982).

2.4. Acrylamide generation in model systems with chitosan

Chemical model reactions were carried out following the method described by Sansano et al., (2016). The reaction was carried out at pH = 4 using a 25 mL threaded Pyrex tube which contained 5 µmol of asparagine and 5 µmol of a mixture of glucose-fructose 1:1 (w/w), and 100 µl of a 0.5% acid lactic solution containing 0 (control samples) and 1% (w/w) of each one of the chitosan with different DD and molecular weight. Tubes kept closed along experiments. Samples were subjected to 180ºC in an oil bath (model: FM 6720 Ideal 2000 Professional, Solac with a nominal power of 2,000 W) during 5, 10 and 15 minutes, in triplicate. During heating processes only the bottom of the tubes was covered with oil. After time of reaction, the tubes were immediately placed on ice for 5 min. Two mL of Milli-Q water were added and tubes were vortexed for 1 min. The mixture was filtered with Nylon filter (0.45 µm) and transferred to a vial for chromatographic analysis of acrylamide. 100 ng of $^{13}C_3$-acrylamide were added to each sample as internal standard.

2.5. Acrylamide determination by chromatography

Acrylamide was analyzed by triplicate according to Sansano et al. (2015) with minor modifications using an Agilent 1200 Series HPLC system coupled to an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies Inc., CA, USA), in positive electrospray ionization mode. A Zorbax XDB C-18 column (2.1mmx50mm, 1.8µm) was used. Six different levels (20, 50, 100, 200, 300 and 500 µg/L), with six replicates for each level (n=6) were studied, being 20 µg/L the limit of quantification. The column temperature was set at 30ºC, the sample injection volume was 5µL and flow rate was maintained at 0.4mL/minute. The mobile phase consisted of 2.5% methanol/ 97.5% of 0.1% formic acid (A) and methanol (B), and the elution gradient was as follows: 0-3 min 100% of A; 3.1-3.5 min 70% A; 3.6 min 100% A, with 1 min post-time. The ionization source was nitrogen at 350ºC at 12 L/min and 40 psi of nebulizer pressure and 4000V of capillary voltage. Multiple reactions monitoring mode (MRM) was used to identify and quantify acrylamide. The MRM transition 72>55.2 was used to quantify and 72>27 was also monitored for acrylamide and 75>58 for $^{13}C_3$-acrylamide (internal standard).

2.6. Batter formulations

The batter formulations were prepared from a commercial dry mix for battering products (Yolanda®, Murcia, Spain), consisting on wheat and rice flours, acidity regulator (E-334), bulking agent (E-500ii) and coloring (E-160b). Batters were prepared according to Sansano et al., (2016). Flour basis was added in a water-to-dry-mix proportion of 1.2:1 (w/w), with addition of 2.5% of table salt. Chitosan batters were prepared in the same way but replacing the water by a chitosan solution at 1% dissolved in 0.5% lactic acid at pH=4. The final chitosan content in the batters formulation was 0.54 % (final pH= 5.7) and batters without chitosan were used as control. Protein and fat contents of the commercial dry mix were 10.0 and 1.4%, respectively (data supplied by manufacturers); moisture (11.5%) and ash contents (1.8%) were measured following AACC methods (1995). Average particle size (78.0 µm) was analyzed with a Mastersizer 2000 coupled

with Scirocco 2000 module for dry measurement (Malvern Instruments, Germany). Batters were kept for 30 minutes at room temperature before frying.

2.7. Flow properties and water retention capacity of raw batters

Apparent viscosity of raw batter formulations was determined using a Haake Rheostress 1 rheometer (Thermo Electric Corporation, Germany) equipped with a plate-plate (60 mm of diameter) at 10, 20, 30 and 40 ºC. Apparent viscosity (Pa·s) was measured as a function of shear rate (γ) from 0 to 100 s^{-1} after 5 min of stabilization time. Rheological constants *K* (consistency index, $Pa \cdot s^n$) and *n* (flow behavior index) were adjusted to the Ostwald-De Waele model (*equation VI*):

$$\tau = K \cdot \gamma^n \quad (VI)$$

Water Retention Capacity of raw batters was analyzed as follows: 18 g of each batter formulation were weighed in a 30 mL centrifuge tube, tempered at different temperatures (10, 20, 30 and 40 ºC) and centrifuged at 17300 RCF for 10 minutes. The supernatant was removed and weighed to calculate the WRC (*equation VII*).

$$WRC = \frac{(W_S \cdot x_W) - W_W}{(W_S \cdot x_W)} \cdot 100 \qquad (VII)$$

where W_s is the total sample mass (g); x_w is water mass fraction of batter (g w/ g batter) and W_w is the supernatant mass (g).

Both determinations were done by triplicate.

2.8. Frying process

Frying of batters was carried out in a commercial deep-fat fryer of 2 L of capacity (model: FM 6720 Ideal 2000 Professional, Solac) at 180±2ºC. Samples (11.5 ± 0.1g) were placed in an aluminum cylindrical cake mold and introduced in the fryer in order to obtain homogenous ring shaped fried samples (height: 11±1mm; outer diameter= 65mm ±2 and inner diameter= 25 ±1mm). Three samples (n = 3) were fried for each frying time (2, 4 and 7 minutes). The excess

of oil was removed with paper on both sides for 20 seconds after taking out the samples from the fryer.

After frying, the following determinations were carried out by triplicate (n=3) at each time of frying, except for mass fluxes, color and texture (n=5 for each frying time).

Water content was analyzed by vacuum drying at 60ºC until constant weight was achieved (20.103, AOAC, 1980). Total oil content was determined by solvent extraction using the Soxhlet method (AACC, 1995), performing the extraction procedure with petroleum ether.

Net changes of components (ΔM^i_t) (concretely, oil uptake (ΔM_t^{oil}) and water loss (ΔM_t^w) during frying were obtained according to *equation VIII*

$$\Delta M_t^i = \frac{\left(M_t \times x_t^i\right) - \left(M_0 \times x_o^i\right)}{M_0} \qquad (VIII)$$

where M_0 is the total mass of the sample at initial time (g), M_t is the total mass at time t (g), x_0^i is the mass fraction of component (water or oil) at initial time (g/total g) and x_t^i is the mass fraction of component (water or oil) at time t (g/total g). Superscript i is "*oil*" or "*w*" for oil and water component, respectively.

2.9. Texture properties of deep-oil fried batters

Texture changes over frying time were evaluated by a puncture test using a Texture Analyser (mod. TA-XT PlusAname, Spain) equipped with a 50 kg load cell. Texture test was performed twice, just after frying but tempering the samples at a consuming temperature (55ºC) and after cooling the samples at room temperature (25ºC). The plunger used for the test was a cylinder with a flat base of 2 mm diameter. Samples were placed on a holed platform to ensure a total sample perforation. The crosshead speed was 1 mm/s. The maximum shear force F_{max} (N) necessary to perforate each sample was recorded from the force-deformation curve.

2.10. Optical properties of deep-oil fried batters

Optical properties of the fried samples were determined by using a spectrocolorimeter (MINOLTA, mod. CM-3600d). The color space coordinates CIEL*a*b* were obtained from the absorption spectrum between 380 and 770 nm by reflectance with the reference system: D_{65} illuminant and 10 ° observer, and a 12 mm lens. Chroma ($C_{ab}^* = (a^{*2}+b^{*2})^{1/2}$) and hue ($h_{ab}=\arctan(b^*/a^*)$), as well as total color changes ($\Delta E=[(L^*-L^*_{control})^2+ (b^*-b^*_{control})^2+ (a^*-a^*_{control})^2]^{1/2}$) of fried batters were calculated.

2.11. Statistical analysis

The influence of degree of deacetylation and the molecular weight of chitosan on acrylamide generation in model systems, as well as the effect of chitosan on the analyzed physicochemical properties of raw and fried batters were analyzed using Statgraphics Centurion XVI. Analysis of variance was carried out with a multifactorial ANOVA, obtaining a significance level of 95%.

3. Results and discussion

3.1. Influence of deacetylation degree and molecular weight on acrylamide formation in model systems

The estimated deacetylation degree was 64.8±0.8 % for commercial chitosan, 49.4±0.3 % for the chitosan obtained from the acetylation process, and 86.5±0.6 % and 92.80±0.12 % for the chitosan obtained from 1 or 2 deacetylation cycles, respectively. Briefly, it can be observed that the application of two cycles of deacetylation did not substantially increase the degree of deacetylation of chitosan compared to the DD achieved after 1 cycle.

The inhibitory capacity of chitosan on acrylamide generation in model systems was confirmed independently of the deacetylation degree (DD) (reductions between 44-74% with respect to the control) (Fig.4.10).

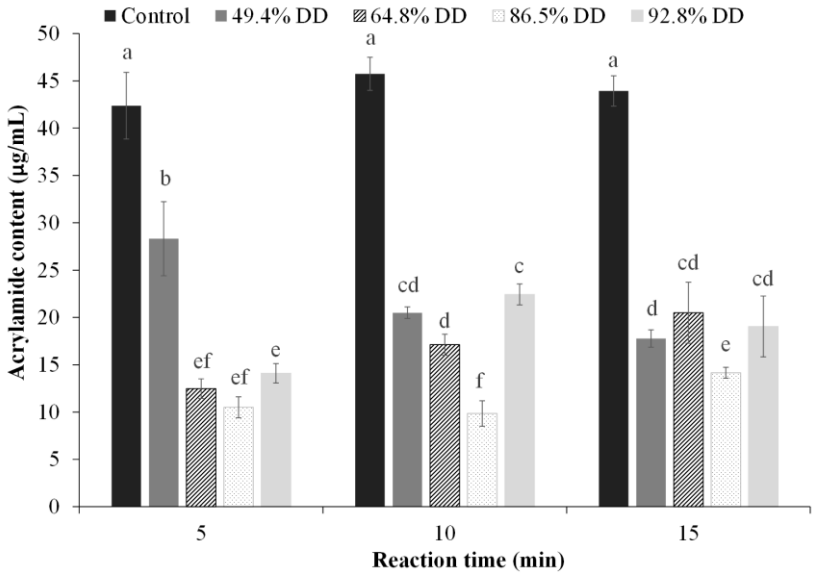

Figure 4.10. *Acrylamide content (µg/mL) versus reaction time generated in different model systems consisting of 5µmol of asparagine, 5µmol glucose:fructose (1:1) and 0 (control) and 1% of chitosan with different deacetylation degree (DD).*
Homogeneous groups are represented by the same letter.

Nevertheless, results did not show a clear relationship between chitosan-DD and the degree of the acylamide mitigation. At short-time of reaction (5 min), the lowest DD (49.4%) seemed to be the least effective against acrylamide generation, while no difference on acrylamide reduction was observed between those with higher deacetylation degree. At longer reaction times (10 and 15 min) similar acrylamide generation was found in model systems with acetylated chitosan (DD 49%) and commercial chitosan (DD 64.8%).

A lightly higher reduction of acrylamide formation was observed when chitosan obtained from one cycle deacetylation process (DD 86.5 %) was added to the model system. However, the results obtained with 92.8%-DD chitosan does not allow to conclude that the higher the DD the higher the inhibitory capacity of chitosan. The method used to obtain this DD, a 2-cycle deacetylation process,

could be co-responsible of these results, but it should be confirmed. Apparently, two consecutive deacetylation cycles could not only modify the deacetylation degree, but also affect other properties. Tsai, Su, Chen, & Pan, (2002) reported that chitosan with a high level of deacetylation tends to form aggregates in aqueous solutions which contribute to form intermolecular interactions that might reduce available sites on the chitosan molecule to inhibit acrylamide formation.

In addition to the DD of chitosan, the influence of molecular weight of chitosan on acrylamide formation was explored using the same model systems (Fig.4.11). Table 4.4 shows the viscosity average molecular weight (M_v) measured on the commercial chitosan (0 h) and on those obtained by hydrolysis process of different duration (2, 4 and 8 hours).

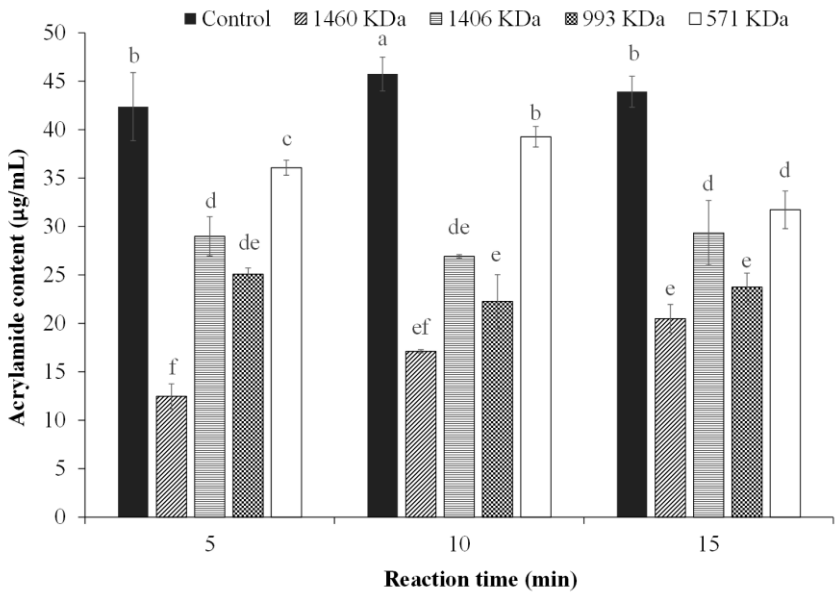

Figure 4.11. *Acrylamide content (µg/mL) versus reaction time generated in different model systems consisting of 5 µmol of asparagine, 5 µmol glucose:fructose (1:1) and 0 (control) and 1% of chitosan with different molecular weight (Mv).*

Homogeneous groups are represented by the same letter.

Table 4.4. *Intrinsic viscosity and viscosity average molecular weight (M_v) corresponding to hydrolyzed chitosan samples. 0 h corresponds to commercial Chitosan.*

Hydrolysis time (h)	[η] intrinsic viscosity (mL/g)	M_v (KDa)
0	902 (3)d	1460 (5)d
2	868 (5)c	1406 (3)c
4	613 (7)b	993 (11)b
8	353 (13)a	571 (21)a

[a, b, c, d]*Homogeneous groups are represented by the same letter.*

Chang et al. (2016) recently reported the effect of chitosan with low molecular weight on acrylamide generation in model systems, indicating a statistically significant acrylamide reduction capacity of 90-150 KDa-chitosan compared to the control, but no statistical effect of including chitosan with 190-310 KDa or 310-375 KDa in model systems on acrylamide generation. The range of molecular weight evaluated in the present studied was above the interval studied by Chang et al. (2016), being the lowest M_v achieved in this study 571 ± 21 KDa with the longest hydrolysis time (8 h). The longer the duration of the hydrolysis process the greater the reduction on molecular weight of chitosan, but in any case it is possible to reduce the formation of acrylamide at levels lower than those achieved with the commercial chitosan (Fig.4.11). These results can be explained if they are considered as the overall result of two opposite effects: the first one related to structural changes induced by the acid hydrolysis with a negative influence on inhibiting acrylamide formation and a positive second one related to the decrease of molecular weight. In fact, Kumar (2000) recommended the enzymatic hydrolysis against the acid one because of the better control of the process and the absence of chemical modifications of the structure. Furthermore, the hydrolysis can promote changes in the chain conformation of chitosan and new intermolecular bonds (Rege & Block, 1999). The negative effect of hydrolysis is observed independently of the duration of the process because of commercial

chitosan (1460 KDa) was more effective inhibiting acrylamide formation than chitosan with similar Mv but submitted to a 2 hours acid hydrolysis process (1406 KDa) (Fig.4.11). This effect is slightly countered by the decrease of Mv in chitosan of 993 KDa while it seems to be the predominant effect in model systems with 571 KDa chitosan.

3.2. Quality properties of chitosan based batter formulations

The above results together with those published by the same authors in a previous study (Sansano et al., 2016) were the base to select the commercial chitosan (64.8% DD and 1460 KDa) as ingredient in batter formulations and study the main quality properties of the new formulation. In this study, commercial chitosan was used to formulate batters according to Sansano et al., (2016) in order to evaluate the effect of this hydrocolloid on the rheological behavior and water retention capacity of raw batters as well as water loss and oil uptake during frying, and color and textural changes of fried samples.

Table 4.5 shows the rheological parameters, consistency (K) and flow behavior index (n) of batters with and without chitosan, their apparent viscosity at 20 s^{-1} (reference shear stress), as well as their water retention capacity (WRC). For the entire range of studied temperatures (from 10 to 40 ºC), the addition of chitosan resulted in a significant increase of both the consistency (K) and the apparent viscosity at 20 s^{-1} of the studied batters. The increase of consistency of batters when chitosan is added will need to be considered for future applications because of its influence on batter pick-up, yield and crispiness (Sanz et al., 2004).

As refers to flow behavior index, it was not affected by chitosan addition at 10 and 20ºC and only a slight decrease was observed at 30 and 40ºC. Flow behavior index (n) was highly dependent on temperature regardless of chitosan addition. This fact indicates that the control of temperature is a key-variable during the line production of batters formulation at industrial scale (Baixauli, Sanz, Salvador, & Fiszman, 2003). Similar results have been reported by other authors for other hydrocolloids such as methylcellulose, guar gum or xanthan gum (Hsia et al., 1992; Sanz et al., 2004).

Table 4.5. *Rheological parameters: consistency (K) and flow index behavior (n), apparent viscosity at 20 s^{-1}, and water retention capacity (WRC (%)) of batter formulations with and without chitosan at 10, 20, 30 and 40°C.*

	T (ºC)	***K (Pa s^n)***	***n***	***Apparent viscosity, shear rate = 20s^{-1} (Pa·s)***	***WRC (%)***
Without chitosan	10	2.63 (0.07)d	0.73 (0.00)a	1.10 (0.04)c	34.4 (0.1)d
	20	2.44 (0.08)e	0.71 (0.01)b	0.96 (0.05)d	34.9 (0.2)d
	30	2.01 (0.01)f	0.70(0.00)c	0.784 (0.009)e	34.6 (0.5)d
	40	1.92 (0.02)f	0.69 (0.01)d	0.708 (0.004)e	36.1 (0.6)c
With chitosan	10	4.16 (0.03)a	0.73 (0.00)a	1.73 (0.02)a	44.3 (0.2)a
	20	3.15 (0.12)bc	0.71 (0.00)b	1.23 (0.05)b	43.9 (0.3)a
	30	3.36 (0.01)b	0.69 (0.01)d	1.21 (0.03)b	39.6 (0.3)b
	40	3.01 (0.16)c	0.67 (0.00)e	1.06 (0.07)c	40.3 (0.1)b

Homogeneous groups are represented by the same letter.

In a similar way than in other hydrocolloids, such as methylcellulose (Sanz et al., 2004), the WRC was higher in the raw batters containing chitosan than in the control (0% of chitosan), thus showing the ability of chitosan polymeric chain to retain water.

Do Amaral et al., (2015) and Sayas-Barberá et al., (2011) reported that using chitosan as an ingredient in formulations of sausages and hamburgers contributed to increase water retention after oven cooking at 150ºC (internal temperature in the product 72ºC), compared to the control indicating that chitosan encouraged water retention after thermal processing. However, chitosan based batters presented lower WRC at 30 and 40ºC than at 10 or 20ºC, probably due to very different interface conditions as compared with sausages.

Additionally to the evaluation of the above-parameters in raw batter formulations, other relevant properties were analyzed during and after the frying process, since the impact of chitosan addition (~ 0.5%), on acrylamide mitigation in fried batters has been recently published by the same authors (Sansano et al., 2016) (average reductions of 32, 60 and 59% after 2, 4 and 7 min of frying, respectively). Table 4.6 shows the results of water loss and oil uptake along frying and the texture after tempering the fried samples at 55 and 25ºC.

No statistically significant differences were found on water loss during frying between batters with and without chitosan. It suggests that water retention capacity of chitosan in raw batters gets lost during frying. In this sense, Sayas-Barberá et al., (2011) did not found significant differences of moisture contents of fried burgers with chitosan (up to 1%) and the control. However, Do Amaral et al., (2015), who incorporated a higher chitosan percentage (1-2%) than in this study, reported a higher retention of moisture in sausages compared to the control. Likewise, Ansarifar et al., (2015) obtained samples with higher water and fat contents in fried cheese nuggets when chitosan was incorporated at 0.5 and 1.5%. All these results point out that the effect of chitosan on the final moisture content of the fried product, and then on water loss, is quiet dependent on the type of matrix and the cooking method. On the other hand, some hydrocolloids,

such as wheat and soy protein isolates, methylcellulose or hydroxypropylmethylcellulose, have been proved to reduce oil uptake and water loss during frying (Albert & Mittal, 2002; (García, Ferrero, Bértola, Martino, & Zaritzky, 2002). In this study, chitosan seems to slightly limit the oil uptake in batters after 2 and 4 min of frying as compared to the control; while Usawakesmanee, Wuttijumnong, Chinnan, Jangchud, & Raksakulthai, (2005) did not find significant differences between using chitosan as an ingredient of fried breaded potato and the control in terms of final fat content of fried products. Once again, the influence of chitosan in the fat retention will be different depending on the food matrix composition. Some studies showed that including dextrin or dried egg combined or not with chitosan reduced breaking force compared to the control (Baixauli et al., 2003) and some others showed that low molecular chitosan increased the hardness of fried batters (Ansarifar et al., 2012; 2015).

Concerning to the chitosan influence on the color and texture of fried batters, results from texture test showed an increase of maximum force with frying time, mainly due to the decrease of water content at the surface, meaning an increase of crispness because of the crust formation. The addition of chitosan did not imply significant differences in maximum force values, i.e. in hardness of the external crust, of fried batters at 55ºC (serving temperature). However, after cooling the samples at 25ºC, the maximum force registered in samples without chitosan was higher than in samples with chitosan, except for over-fried samples (7 min). This result reveals that the presence of chitosan protects tightening during cooling, maybe because its capability of binding water. Finally, Table 4.7 includes the optical parameters of fried batters with or without chitosan at each frying time. As can be observed, lightness (L*) and hue (h_{ab}) of batters were not affected by chitosan addition. However, both parameters decreased as expected with frying time due to brownish. According to ANOVA results, chitosan addition provided lower C_{ab}* values at 2 minutes compared to control samples, but it increased the color saturation (C_{ab}*) of fried samples at 4 and 7 minutes.

Table 4.6. *Average values and standard deviations of water loss and oil uptake (n=3) and F_{max} at 55 and 25 ºC (n=5) of fried batters with or without chitosan at different frying times. Homogeneous groups are represented by the same letter.*

	Frying time (min)	***Water loss (ΔM_w)***	***Oil uptake (ΔM_{oil})***	***F_{max} (N) at 55ºC***	***F_{max} (N) at 25ºC***
Without chitosan	2	-0.37 (0.04)a	0.24 (0.02)a	2.06 (0.03)b	1.1 (0.4)d
	4	-0.44 (0.09)b	0.28 (0.08)a	5 (3)b	5 (3)c
	7	-0.51 (0.04)c	0.30 (0.05)a	14.5 (1.1)a	23 (4)a
With chitosan	2	-0.35 (0.05)a	0.21 (0.04)b	2 (2)b	1.2 (0.8)d
	4	-0.424 (0.014)b	0.203 (0.012)b	3.6 (0.8)a	3.5 (1.4)cd
	7	-0.525 (0.017)c	0.24 (0.04)a	12 (0.9)a	17 (3)b

Table 4.7. *Average values and standard deviations (n=5) of chromatic parameters L*, C*, h* and ΔE of fried batters with or without chitosan at different frying time. Homogeneous groups are represented by the same letter*.

	Frying time (min)	***L****	***C_{ab}****	***h_{ab}***	***ΔE***
Without chitosan	2	61.5 (1.4)a	34.4 (1.5)b	89.1 (0.8)a	-
	4	53 (2)b	34.0 (1.3)b	79.9 (0.7)b	-
	7	47 (3)c	30.4 (1.1)c	69.9 (1.8)c	-
With chitosan	2	60.9 (1.5)a	31.4 (1.7)c	92.3 (1.4)a	5.2 (1.6)b
	4	50 (3)b	37.0 (0.9)a	79.7 (1.7)b	6.3 (1.7)ab
	7	44 (3)c	37.2 (0.9)a	67.9 (1.4)c	6.7 (1.2)a

Other hydrocolloids, such as guar gum or xanthan gum, contributed to a lower color development, while gum Arabic included in batters for chicken nuggets increased darkness, probably due to the reduction of protein content, at replacing flour by a hydrocolloid (Sahin, Sumnu, & Altunakar, 2005). The increase of darkness when chitosan is present in the food system may be due to Maillard reactions progress as a consequence of the reaction between the amino groups of this polysaccharide and carbonyl groups of glucose at high temperature (Rao et al., 2011; Phisut & Jiraporn, 2013). Color differences (ΔE values) were similar for all frying times and low, which implies that probably, consumers will not perceive color changes promoted by chitosan addition.

4. Conclusions

The results showed that the higher the deacetylation degree of chitosan, the higher the reduction in acrylamide compared to the control samples (without chitosan). However, the two cycles deacetylation process provokes additional changes that results in less efficiency in acrylamide mitigation. Additionally, the acid hydrolysis was found to be a process that not only decrease the molecular weight, which would contribute to a high inhibition of acrylamide formation, but also promote changes with negative effect on chitosan capacity to reduce acrylamide. Therefore, commercial chitosan with high molecular weight and DD was found to be the most appropriate to evaluate quality properties of a new batter formula.

Chitosan as ingredient in battering formulations in a concentration of ~ 0.5%, which implies an acrylamide reduction between 32 and 69% depending on frying time, increased both the consistency and the water retention capacity of raw batters without significant modifications of the final color or texture of fried batters. Therefore, batter formulations with chitosan (~ 0.5%) can be used to largely mitigate acrylamide without prejudice to the main quality properties valued by the consumers.

Acknowledgments

The authors thank the Universitat Politècnica de València for the PhD scholarship given to Mariola Sansano Tomás.

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4.4. INFLUENCE OF CHITOSAN ON THERMAL, MICROSTRUCTURAL AND RHEOLOGICAL PROPERTIES OF RICE AND WHEAT FLOURS- BASED BATTERS

Mariola Sansano[a], Ana Heredia[a]; Virginia Glicerina[b], Federica Balestra[b], Santina Romani[bc], Ana Andrés[a]

Under review in LWT-Food Science & Technology (May 2017)

[a]Instituto de Ingeniería de Alimentos para el Desarrollo. Universitat Politècnica de València, Valencia, Spain

[b]Interdepartmental Centre for Agri-Food Industrial Research, Alma Mater Studiorum, University of Bologna, Via Quinto Bucci, 336, Cesena FC, Italy

[c]Department of Agricultural and Food Sciences, University of Bologna, Piazza Goidanich 60, 47521 Cesena (FC), Italy.

INFLUENCE OF CHITOSAN ON THERMAL, MICROSTRUCTURAL AND RHEOLOGICAL PROPERTIES OF RICE AND WHEAT FLOURS- BASED BATTERS

Mariola Sansano, Ana Heredia, Virginia Glicerina, Federica Balestra, Santina Romani, Ana Andrés

Total wheat flour substitution by rice flour is an option to produce gluten-free batters, suitable for celiac population. Rice flour, is also used in batters because of its low capability to retain oil during frying. However, rice flour needs hydrocolloids to develop the network of the mix, in order to offset gluten function. As seen in paper II, chitosan addition to batters reduced acrylamide formation and oil absorption during frying, evidencing the potential use in healthier foods. However, there are not previous studies focused on the influence of chitosan and the specific interactions with rice flour batters, as well as wheat flour and their combination, on their, rheological and structural properties. Therefore, the aim of this work was to analyze the influence of chitosan addition on rheological and thermal properties of raw rice and wheat flours-based batters. In addition, microstructural analysis was carried out in wheat and rice flour combinations with 0 and 1% of chitosan.

Batter formulations were made with different combinations of wheat and rice flours (100:0, 70:30, 30/70, 0:100), salt and sodium bicarbonate in a water-to-dry mix proportion of 1.2:1 (w/w). The studied chitosan concentrations were: 0% (as the control), 0.25, 0.5 and 1%.

The influence of chitosan (0, 0.25, 0.5 and 1%) in the flow behavior of the different wheat and rice combinations was evaluated. Concretely, apparent viscosity at 20ºC ($Pa{\cdot}s^n$) and rheological parameters from Herschel-Buckley model were analyzed. Microstructure observations were performed by a light microscope and particle size determined by Feret diameter evaluation, and an Euclidean Distance Map done to evaluate the distance between particles. Thermal properties (starch gelatinization, ice melting and glass transition) were analyzed by using a DSC (Differential Scanning Calorimeter). From the total

water content and ice melting enthalpy it was also analyzed the unfrozen water content.

Wheat flour replacement (partially and totally) by rice flour in batters decreased the consistency (*K*) and the yield stress (τ_0) and increased the flow behavior index (*n*) because of the absence of gluten or its lower content. This phenomenon was observed at microscopic scale where a reduction of the structure aggregation of the batters. Additionally, chitosan addition favored the presence of high agglomeration areas, especially in batters with rice flour as the main flour in the formulation. 0.25% of chitosan addition in 100% rice flour batter formulation, triggered similar levels of viscosity and consistency than 100% wheat flour.

In terms of thermal properties, in general, chitosan incorporation to blends did not significantly modify them, as flour combination did. However, chitosan addition to 100% of rice-flour batters, reduced melting enthalpy (ΔH_m) and melting temperature (T_m), and increased the bonding water content. Additionally, batters formulated with rice flour presented higher glass transition temperature (Tg') than those made with of wheat flour, probably because the higher bonding water content. Gelatinization enthalpy and gelatinization peak temperature were lower in wheat-flours based batters, compared to rice-flour based systems. However, chitosan uniformed the onset temperature in wheat flour based batters (100% wheat flour; and 70% wheat flour /30% rice flour), probably because chitosan controls water transference to the gluten net and manages the starch hydration process.

This work evidences again the potential use of chitosan as ingredient in batter formulations. Concretely, the possibility to produce gluten-free batters with similar physical properties than wheat flour-based batters, offers new possibilities to population with gluten intolerance. In addition, a positive impact of chitosan addition was observed in rheological and structural properties of the batters with different combinations of rice and wheat flour-based batters.

INFLUENCE OF CHITOSAN ON THERMAL, MICROSTRUCTURAL AND RHEOLOGICAL PROPERTIES OF RICE AND WHEAT FLOURS- BASED BATTERS

Mariola Sansano, Ana Heredia, Virginia Glicerina, Federica Balestra, Santina Romani, Ana Andrés

Abstract

Wheat flour replacement by rice flour is one of the key strategies in gluten-free batter formulations for food industry. Rice flour, without gluten, needs hydrocolloids to offset the development of the network of the mix. In this context, the aim of this work was to analyze the influence of chitosan (0, 0.25, 0.5 and 1% (w/w)) addition on the microstructural, rheological and thermal properties of rice and wheat: rice flours batters (100:0; 70:30, 30:70 and 0:100 (w/w)). Obtained results showed that the increasing replacement of wheat flour by rice one in batters decreased the consistency (*K*) and the yield stress (τ_0) and increased the flow behavior index (*n*) because of the absence or lower gluten content. However, the incorporation of only 0.25% chitosan to rice-flour formulation enhanced viscosity of the batters and enhanced structural agglomeration and therefore the stability and the interaction among ingredients, especially in batters with high content of rice flour (30:70 and 0:100). Lastly, chitosan incorporation to blends did not significantly modify thermal properties, excepting in 100% of rice-flour batters, reducing ΔHm, Tm, and increasing the bonding water content.

Keywords: chitosan; wheat-rice flours; rheology; thermal properties; microstructure

1. Introduction

Batters are complex liquid systems composed mainly of flour and water, in which the food product is dipped before frying. Commonly, other ingredients such as starch, hydrocolloids, salt and seasoning are incorporated to improve their functionality and sensory properties. During frying, the uniform layer covering the product generates a crispy crust as a result of a rapid loss of water. This crust

entails a barrier effect to further water loss and oil gain. Minimizing the oil uptake is one of the key proposals to obtain healthier fried-products. In spite of wheat flour is the main solid ingredient in batter formulations, rice flour has been lately incorporated because its addition enhances some properties of frying batters. Proteins and starch from rice flour have the particularity to be gluten-free and to retain less oil, resulting in a final product with less calories as well as with lower acrylamide content compared with wheat flour-based batter (Shih & Daigle, 1999; Shih, Boué, Daigle, & Shih, 2004). However, rice flour batters form thin slurries which require additives to develop adequate viscosity and other desirable batter properties (Shih & Daigle, 1999). For this reason some authors considered advantageous the use of hydrocolloids in batters (Albert & Mittal, 2002; García et al., 2002; Garmakhany, Mirzaei, Nejad, & Maghsudlo, 2008; Sahin et al., 2005). Hydrocolloids are substances characterized by the capability to link water increasing the viscosity of a solution. This property causes changes in the coating pick-up and yield; moreover after cooking, hydrocolloids affect freeze-thaw stability and improve mechanical resistance of the crust, and thus, the final texture (Varela & Fiszman, 2011). Additional benefits have been described related to the use of hydrocolloids. Zeng et al., (2010) reported a decrease of acrylamide generation in model systems, crackers and fried potatoes when pectin or alginic acid were used. Acrylamide is a potentially carcinogenic compound which is generated during frying or baking as a consequence of Maillard reactions (Mottram, Wedzicha, & Dodson, 2002; Stadler et al., 2002; Romani, Bacchiocca, Rocculi, & Dalla Rosa, 2009). Recently, Sansano, Castelló, Heredia, & Andrés (2016) reported a reduction of 61 ± 7% in acrylamide formation when 0.5% of chitosan was added as an ingredient in fried batters. The mechanism of acrylamide reduction that authors proposed is based on the richness of amino groups of chitosan, which compete with asparagine amino groups to bind carbonyls (e.g. reducing sugars).

However, there are no previous studies focused on the influence of chitosan addition to rice flour batters on their microstructural, rheological and thermal properties. Ansarifar, Shahidi, Mohebbi, Razavi, & Ansarifar, (2015) studied the

flow behavior of wheat flour batter formulations for chicken nuggets, and reported higher viscosities of the batters related with chitosan content, due to its high water binding capacity. Moreover, changes in the rheological properties of a material reveal changes in its molecular structure. Consequently, the rheological properties of a batter influence its flow characteristics and are themselves influenced by structural changes generated by the process or formulation (Xue & Ngadi, 2007a).

Since the type of flour used provides different rheological characteristics, it is necessary to study how they affect these and other properties. Interactions between components of batter formulations and their influence during heating treatment determine physical and chemical changes that can be analyzed by studying thermal properties. Chitosan incorporation in batters and specifically its interactions with the other components have not been analyzed in terms of thermal properties.

In this context, the aim of this work was to analyze the influence of chitosan addition on the microstructural, rheological and thermal properties of raw rice and wheat flours-based batters.

2. Materials and methods

2.1. Batter formulations

Battering formulations consisted in different combinations of wheat and rice flours, 2.5% of salt and 3.1% of sodium bicarbonate (dry weight basis) in a water-to-dry mix proportion of 1.2:1 (w/w). The rice and wheat flours were combined in the following ratios of wheat:rice flours: 100:0; 70:30, 30:70 and 0:100 (w/w). The batter systems were formulated with 0, 0.25, 0.5 and 1% chitosan, adding chitosan as a solution (2%, w/w), made as follows: 2g of chitosan were dissolved in 198 g of 1% acid lactic solution and stirred at 40ºC during 24 hours. Water and lactic acid were added to complete their final content (0.545 % and 54.54% wet basis, respectively). Batters were manually mixed during 60 seconds to

guarantee uniformity and were kept at room temperature for 30 minutes before analyzing.

Flours were bought in the local market, and their composition were: 77.4% of carbohydrates, 0.5% of fat and 7.1% of proteins for the rice flour and 75% of carbohydrates, 1.2% of fat and 9% of proteins, for the wheat flour. Chitosan (Poly (D-glucosamine)*Deacelyled chitin, high molecular weight, was purchased from Sigma-Aldrich (St. Louis, MO, USA), and lactic acid was from Panreac (Barcelona, Spain).

2.2. Rheological measurements

Rheological properties were studied using a strain/stress control rheometer MRC 102 (Physica/Anton Paar,GmbH, Inc., Graz; Austria) equipped with a plate-plate (50 mm of diameter). The gap between plates was fixed to 1mm. The free surface of samples edges was covered with a thin film of silicone oil in order to reduce sample dehydration during the measurements.

Apparent viscosity (Pa·s) was measured in triplicate, at 20ºC as a function of increasing shear rate (γ) from 0 to 150 s^{-1} after 5 min of stabilization time, in order to attemperate the product. The obtained flow curves were evaluated and fitted according to the Herschel-Buckley model, that is represented by the following equation *(I)*:

$$\tau = \tau_0 + K \cdot \gamma^n \qquad (I)$$

where τ is the shear stress (Pa), τ_0 the yield stress (Pa), *K* the consistency index (Pa s^n) and *n* the flow behavior index, that is the dimensionless flow .

2.3. Microstructural analysis

The microstructure of samples was observed by using a light microscope (Nikon, Shinjuku, Japan) at 10x of magnification, taking ten micrographs for each sample. The magnification was chosen after preliminary trials in order to obtain a wider field of view to see the whole structure and the interaction between particles. One drop of dispersion (previous dilute with hexane) was placed on a glass side and covered with a cover slip carefully placed over the sample, parallel

to the plane of the slide and centered to ensure sample thickness was uniform. Micrographs were captured using a digital camera (Model 2.1 Rev 1; Polaroid Corporation, NY, USA). The acquired images were subsequently elaborated using the software Image Pro-plus 6.0 (Media Cybernetics Inc Bethesda, USA). Particles size were determined according with Glicerina, Balestra, Dalla Rosa, & Romani, (2013), by evaluating the Feret diameter, that can be defined as the distance between two tangent lines to the two opposite sides of the particles (Allen, 1997). An Euclidean Distance Map (EDM) was further generated in order to evaluate the distance between particles. The map indicates, for each pixel in the image (black points) the shortest distance between them (Bayod, 2008; Danielsson, 1980; Glicerina et al., 2016). The distance between black points (particles) was expressed as grey values. On the other hand, the white points represented the empty space. For this reason, applying an EDM to the original image is possible to obtain information about the minimum distance between particles and about the amount and distribution of void spaces (Krislock & Wolkowicz, 2012).

2.4. Thermal properties

Thermal properties of batter formulations were analyzed with an Auto Q20 Differential Scanning Calorimeter (T.A. Instrument, Hüllhorst, Germany). Glass transition temperature, temperature and enthalpy of gelatinization and ice-melting were analyzed. 26±1mg of sample were placed in hermetic aluminum pans and an empty pan was used as the reference.

The ramps were calibrated 10ºC/min with indium, and then, the thermal profile was performed as follows: from 15ºC to 120ºC at 10ºC/minute (to obtain gelatinization temperature and enthalpy), and cooling until -50ºC. It included an isotherm step during 3 minutes and then a heating in order to thawing, until 40ºC at 10ºC/min to obtain, the glass transition temperature followed by melting temperature and melting enthalpy. Unfrozen water content (*UFW*, g water/g solids) was analyzed following the equation *II* (Laaksonen & Roos, 2000):

$$UFW = \frac{w_{tot} - \frac{\Delta H_{mtot}}{\Delta H_{mw}}}{C_{tot}} \qquad (II)$$

where w_{tot} is total amount of water (g), ΔH_{mtot} is the total heat of melting of ice (J), ΔH_{mw} is latent heat of melting ice (334J/g) and C_{tot} is total amount of solids (g).

2.5. Statistical analysis

The influence of rice-wheat flours ratio and chitosan content on thermal and rheological properties of batter formulations was analyzed using Statgraphics Centurion XVI. Variance was evaluated by a one-way analysis for rheological properties and a multifactorial analysis was carried out for thermal properties, with a significance level of 95%.

3. Results and discussion

3.1. Effect of wheat-rice flours combinations and chitosan on flow behavior of batters.

Rheological parameters corresponding to all batter formulations, with the exception of the formulations obtained with 100% rice flour with 0.5 and 1% of chitosan, were obtained by fitting the experimental flow curves to Herschel-Bulkley model and are reported in table 4.8. Samples made up from 100% rice flour with respectively 0.5 and 1% of chitosan could not be analyzed because of their excessive consistency and hardness. The incorporation of chitosan above 0.25 % together with the particle size of rice flour negatively limited the flow behavior of batters. Formulations without chitosan exhibited significant differences in rheological parameters depending on the type of flour. The presence of rice flour in batters (100%RF, 70%WF/30%RF and 30%WF/70%RF) decreased consistency (K) and the yield stress (τ_0) and increased the flow behavior index (n). This effect might be related to a decrease of gluten that helps water retention. In fact, replacing wheat by rice flour in batters the gluten concentration decreases (Dogan, Sahin, & Sumnu, 2005; Mukprasirt, Herald, & Flores, 2000; Xue & Ngadi, 2006). It is noteworthy that the formulation of 100% of rice flour without chitosan showed a visible syneresis because of its inability to retain water, due to its lack of gluten.

***Table 4.8**. Rheological parameters (τ_0, K, n) obtained from Herschel–Bulkley model depending on batter samples formulated with different type of flour and chitosan percentages.*

Formulation	Chitosan content (%)	τ_0 (Pa)	K (Pas s^n)	n	R^2	Apparent viscosity 20s^{-1} (mPa· s)
100% WF	0	1.56 (0.13)Da	1.840 (0.101)Da	0.796 (0.001)Ac	0.9994	1050 (53)Da
70%WF/30%RF	0	1.154 (0.009)Ca	1.380 (0.003)Ca	0.783(0.004)Ab	0.9996	776 (9)Ca
30%WF/70%RF	0	0.78 (0.02)Ba	1.0539 (0.0004)Ba	0.790 (0.005)Ac	0.9995	596 (9)Ba
100% RF	0	0.030 (0.007)Aa	0.45 (0.05)Aa	0.933 (0.015)Bb	0.9998	371 (20)Aa
100% WF	0.25	2.37 (0.12)Aa	2.47 (0.05)Bb	0.792 (0.002)Ac	0.9988	1476 (40)Cb
70%WF/30%RF	0.25	1.93 (0.03)Aa	2.205 (0.004)ABa	0.776 (0.011)Ab	0.9993	1296 (46)Ba
30%WF/70%RF	0.25	2.5 (1.0)Aa	2.0 (0.4)Aa	0.75 (0.04)Abc	0.9997	1081 (131)Ab
100% RF	0.25	2.0 (0.4)Ab	1.9 (0.2)Ab	0.75 (0.02)Aa	0.9994	1006 (61)Ab
100% WF	0.5	6.01 (1.05)Ab	4.99 (0.19)Ac	0.746 (0.014)Ab	0.9995	2704 (55)Ac
70%WF/30%RF	0.5	8 (4)Ab	5.1 (1.3)Ab	0.73 (0.04)Aa	0.9985	2727 (303)Ab
30%WF/70%RF	0.5	9 (2)Ab	5.1 (0.5)Ab	0.702 (0.016)Aab	0.9987	2649 (186)Ac
100% WF	1	14.6 (1.0)Ac	9.57 (0.04)Ad	0.725 (0.008)Ba	0.9988	5133 (47)Ad
70%WF/30%RF	1	13.0 (1.9)Ac	8.9 (0.7)Ac	0.727 (0.06)Ba	0.9990	4740 (215)Ab
30%WF/70%RF	1	35 (5)Bc	12.9 (1.6)Bc	0.690 (0.03)Aa	0.9850	7513 (90)Bd

Mean values and standard deviation. Different letters indicate differences between homogenous groups, in capital letters for the type of flour combination, and small letters to chitosan content.

The incorporation of chitosan increased τ_0 values, *K* and apparent viscosity and decreased *n*. This effect has been previously reported for other hydrocolloids, whose presence favors an increase of viscosity and consistency. Concretely, the addition of 0.2% of xantham gum and 1% of methylcellulose significantly increased the consistency index of rice batter formulations, due to a higher amount of free water available to encourage the hydration of the hydrocolloid compared to wheat-flour based formulations (Xue & Ngadi, 2007a).

Other ingredients such as phosphorylated starch or gelatinized rice flour have been used to increase poor thickening properties of rice flour and reduce oil uptake as well (Shih & Daigle, 1999). Baixauli, Sanz, Salvador, & Fiszman, (2003) reported that 1.5 % dried egg addition also increased consistency and reduced flow index of wheat flour-based batters at different temperatures, while dextrin was not effective.

However, 100% RF-0.25% chitosan formulation showed similar rheological behavior to wheat flour–based formulations, in particular apparent viscosity values at 20 s^{-1} were 1050 and 1006 for samples without and with chitosan respectively. The addition of 0.25% chitosan greatly affected the flow behavior of the different formulations tested. As shown in Figure 4.12, 100% wheat (WF) and 100% rice flours (RF) had a very different flour behavior. The addition of 0.25% of chitosan increased shear stress in both formulations; while 100% rice flour (with 0.25% of chitosan) showed values close to 100% wheat flour without chitosan.

With regard to the rheological properties, it could be concluded that an absence of gluten seemed to be offset by the addition of chitosan in 0.25% in rice flour batters. A similar viscosity would mean a similar pick up and stickiness of a batter or tempura formulation. These results can be used as a new strategy to produce gluten-free batters based on rice flour.

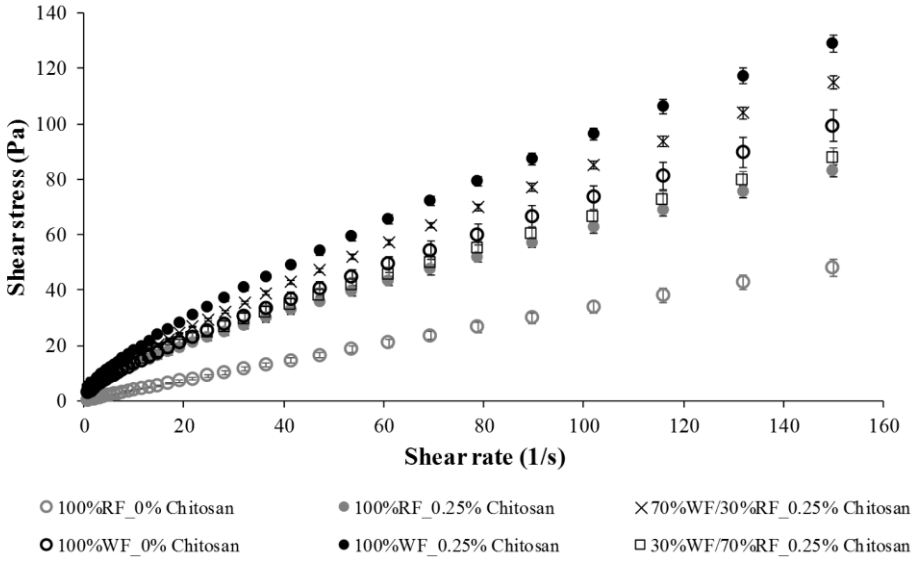

Figure 4.12. *Flow behavior properties of the following batter samples: 100% wheat flour (WF) and 100% rice flour (RF) without chitosan (0%); and formulations 100% WF, 70% wheat flour + 30% rice flour; 30% wheat flour + 70% rice flour and 100% RF with 0.25% of chitosan.*

3.2. Microstructural analysis of batters made with different wheat-rice flours combinations and chitosan.

In order to better explain rheological results, microstructural analysis was also performed. In particular, the formulations related to the extremes of the experimental plan were analyzed in order to obtain the most representative information on the interactions between different percentage of rice and wheat flour with (1%) or without chitosan. Moreover, as previous mentioned in the rheological section, because of the high consistency and hardness, it was no possible to perform fundamental analysis on sample realized with 100% of rice and 1% of chitosan in formulation, that was however characterized from a structural point of view. In Figure 4.13 (A, B, C, D) the micrographs of the different batter samples acquired at 10x of magnification are shown. As shown, in formulation without chitosan, a reduction in the structure aggregation was

observed as wheat flour was replaced by rice one. The decrease in wheat amount parallel to the increase in rice flour involves a reduction in the contact point between particles and an increase of void spaces between particles and aggregates. This effect might be attributed, as previous reported in the rheological section, to a decrease of gluten presence that reduce the batter's water holding capacity, and thus the network formation (Lai, 2002). As known by literature in fact gluten proteins absorb water twice its own weight and tend to hold it through complex chemical bonds, that give arise to a more aggregate structure (Sozer, 2009). Even though rice flour has low capacity of absorbing water, it is one of the most suitable cereal flour used in gluten-free products, thanks to the fact that it is natural, hypoallergenic, colorless and with bland taste (Ronda, Villanueva, & Collar, 2014).

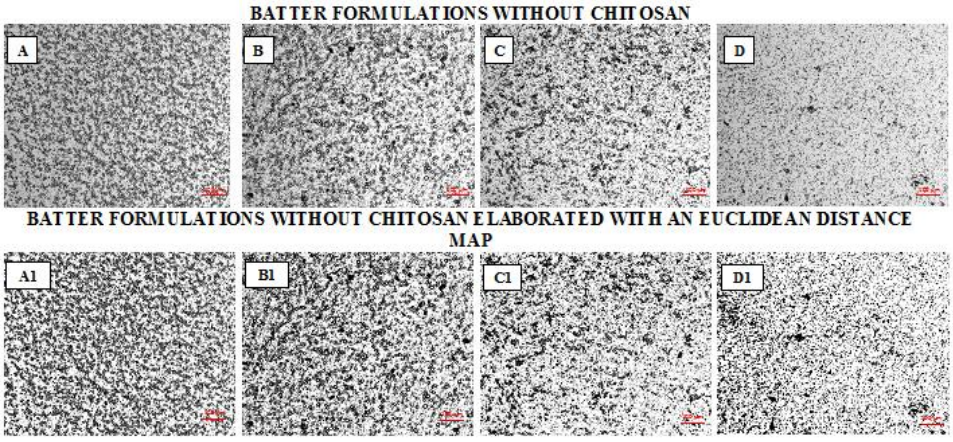

Figure 4.13. *Micrographs of different batter formulations without chitosan, acquired at 10% of magnification. Samples A, B, C, D correspond to formulations respectively made of: 100% wheat flour; 70% wheat flour + 30% rice flour; 30% wheat flour + 70% rice flour; 100 % rice flour. Samples A1, B1, C1, D1 represent respectively the same formulations elaborated with an Euclidean Distance Map.*

In order to better highlight the state of aggregation of the batter matrices, Euclidean distance maps (EDM) were obtained (Figure 4.12: A1, B1,C1, D1). By using an EDM it was possible to highlight the distribution of particles (black areas)

and void spaces (white areas) and to evaluate the minimum distance between particles and therefore their state of aggregation related to their interactions (Glicerina et al., 2016). In Table 4.9, the particle Feret diameters and the minimum distance between particles of the batter formulations with and without chitosan are reported.

Table 4.9*. Particles size (Feret diameter) and minimum distance between particles of different batters with 1% and without chitosan formulated with 100% wheat flour (100%WF); 70% wheat flour + 30% rice flour (70%WF/30%RF); 30% wheat flour + 70% rice flour (30%WF/70%RF) and 100 % rice flour (100%RF). Values (mean ± standard deviation) in the same column followed by different letters differ significantly at a p < 0.05 level.*

Formulation	***Chitosan content (%)***	***Feret diameter (µm)***	***Minimum distance between particles (µm)***
100% WF	0	39.12±3.48^{d}	12.12±1.12^{d}
70%WF/30%RF		31.12±2.14^{c}	16.66±1.18^{c}
30%WF/70%RF		19.78±2.19^{b}	28.44±2.74^{b}
100% RF		9.12±0.57^{a}	37.11±3.12^{a}
100% WF	1	36.10±2.57c	10.12±0.12^{c}
70%WF/30%RF		32.19±2.18^{c}	11.66±2.18^{c}
30%WF/70%RF		17.42±2.03^{b}	5.18±2.74^{b}
100% RF		7.96±0.45^{a}	1.98±0.07^{a}

It is possible to notice that 100%WF and 70%WF/30%RF samples have greater particles size compared to 30%WF/70%RF and 100%RF did with higher amount of rice flours. However, despite as expected from literature, (Afoakwa, Paterson, Fowler, & Vieira, 2009; Prasad et al., 2003), the minimum distance between particles increased as he particle size decreased. These results confirm rheological ones. In fact, samples characterized by a less aggregate structure and more distance between particles (30%WF/70%RF and 100%RF) had a lower consistence index (K) and yield values (τ_0) compared to 100%WF and 70%WF/30%RF samples. This means that the amount of energy needed to allow

the sample to start flowing was lower in the two former samples. In Figure 4.14 (E, F, G, H) are shown the micrographs of the different batter formulations with of 1% of chitosan and the same pictures elaborated by using EDM (E1, F1, G1, H1). Adding 1% of chitosan in the batter formulations it was highlighted an increase in the structure aggregation from sample E to H. As previously shown for batter mixtures made up without chitosan, a reduction in particle size was noticed as the rice flour amount increased (Table 4.9). However, the presence of chitosan induced a reduction in particle size proportional to a decrease in the distance between them. An increase in the contact point between particles was observed from sample E (100% WF)) to H (100% RF), and the presence of high agglomeration areas was highlighted as rice amount increased (Fig 4.14).

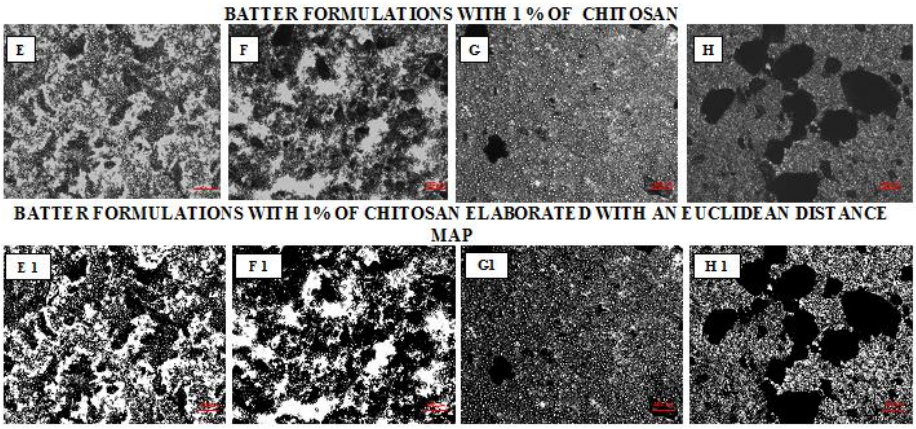

Figure 4.14. *Micrographs of different batter formulations with 1% of chitosan, acquired at 10x of magnification. Samples E, F, G and H correspond to formulations made of 100% of wheat flour; 70% wheat flour + 30% rice flour; 30% wheat flour + 70% rice flour; 100 % of rice flour, respectively. Samples E1, F1, G1, H1 represented respectively the same formulations elaborated with an Euclidean distance map.*

As known by literature, hydrocolloids, such as chitosan, are hydrophilic compounds, that can dramatically increase the viscosity of products in which are presents, due to their interactions with the water molecules through hydrogen –

bonding (Kapoor, Khandal, Seshadri, Aggarwal, & Kumar Khandal, 2013). At sufficiently high concentrations, the hydrocolloids become entangled with each other, forming loose networks that change the flow properties of the solution (Cassiday, 2012). For these reasons, probably, the structure of samples with 1% chitosan were more aggregate than in batter mixtures without this compound.

Moreover, hydrocolloids such as pectin, guar gum, arabic gum, galactomannans, methylcellulose, etc, are frequently used in gluten free baked product in order to form structural equivalent of gluten network in wheat dough (Ahlborn, Pike, Hendrix, Hess, & Huber, 2005; McCarthy, Gallagher, Gormley, Schober, & Arendt, 2005; Sanchez, Osella, & Torre, 2002). Many characteristics of gluten-free bread depend on the amount and type of non-starch hydrocolloids used as gluten replacers (Eidam, Kulicke, Kuhn, & Stute, 1995; Funami et al., 2005). For this reason, one of the most important goal for researchers is to evaluate the optimum proportion of hydrocolloids for the production of gluten-free bread. As demonstrated here from the rheological and microstructural obtained results, the addition of 1% of chitosan to the different batter formulations give arise to a product with high yield stress values and aggregation state, that become limiting factors, especially in the case of 100% of rice flours. The presence or the addition of protein in rice flour coupled to the hydrocolloids (in right proportions) give arise to more compact structures compared to wheat flour matrices with high moisture content (Nammakuna, Barringer, & Ratanatriwong, 2016). However, the difference in the microstructural characteristics and aggregation state, observed between batters made with the same chitosan amount can be probably attributed to the different amount of gluten in samples (higher in 100% WF and 70%WF/30%RF). Gluten, in fact, competing with hydrocolloids for water absorption, could retain a part of water that cannot be bound by chitosan. In the mixtures made with 70 % and 100% of rice flour, instead, the low amount or the absence of gluten, make available water for chitosan, creating intra or inter –hydrogen bonding, give arise to very aggregate structures (Figure 4.14: E, F, E1,F1) (Xue & Ngadi, 2007b).

3.3. Effect of wheat-rice flours combinations and chitosan on thermal properties of batters

In Table 4.10 the different parameters related to the starch gelatinization of the studied wheat-rice-chitosan flours mixtures are reported: the peak temperature (T_p) ranging from 70.25 to 83.4ºC, the onset temperature (T_o) from 62.5 to 74.5ºC and the corresponding enthalpy (AH_G) varied from 3.8 to 6.2 J/g.

***Table 4.10**. Mean values (and standard deviation) of gelatinization peak temperature (Tp), onset temperature (To) and enthalpy (ΔHG) of the studied wheat-rice mixtures: 100% wheat flour (WF), 100 % rice flour (RF), 70% WF + 30% RF and 30% WF + 70% RF, with 0 (control), 0.25, 0.5 and 1% of chitosan.*

Formulation	***Chitosan content (%)***	***Gelatinization temperature T_p (ºC)***	***T_o (ºC)***	***Gelatinization enthalpy ΔH_G (J/g)***
100% WF	0	70.25 (0.17)b	62.59 (0.15)c	4.1 (0.2)b
70%WF/30%RF		71.0 (0.4)b	62.7 (0.2)c	4.91 (0.13)b
30%WF/70%RF		78.0 (1.8)a	67.1 (1.4)b	6.1 (0.8)a
100% RF		81.4 (0.2)a	74.5 (0.5)a	5.4 (0.7)a
100% WF	0.25	70.33 (0.06)b	62.5 (0.2)c	4.2 (0.5)b
70%WF/30%RF		70.9 (0.3)b	62.75 (0.05)c	4.77 (0.09)b
30%WF/70%RF		76.36 (1.19)a	63.6 (0.5)c	5.14 (0.01)a
100% RF		82.9 (0.5)a	74.1 (0.5)a	4.4 (0.5)a
100% WF	0.5	70.6 (0.5)b	62.5 (0.4)c	4.23 (0.10)b
70%WF/30%RF		70.8 (0.3)b	62.7 (0.3)c	4.6 (0.8)b
30%WF/70%RF		77.97 (1.17)a	63.4 (0.2)c	5.7 (0.3)a
100% RF		83.4 (0.5)a	73.7 (0.7)a	6.1 (0.7)a
100% WF	1	71.1 (0.6)b	62.5 (0.3)c	4.14 (0.10)b
70%WF/30%RF		70.8 (0.2)b	62.63 (0.12)c	3.8 (0.6)b
30%WF/70%RF		78.8 (1.4)a	64.0 (0.8)c	5.5 (1.0)a
100% RF		78.1 (1.6)a	65.7 (0.3)bc	6.2 (0.2)a

Homogenous groups are represented by the same letter.

Control samples (without chitosan) with rice as main flour (30%WF/70%RF and 100% RF) exhibited the highest gelatinization temperatures (T_p and T_o) and

enthalpy values. The reduction of gluten presence in the formulations basically based in rice flour increases the amount of available water able to interact with starch (X. Wang, Choi, & Kerr, 2004). Xue & Ngadi, (2007b) reported similar results in batter systems formulated with different blends of wheat and rice flours and also, in corn and wheat flour mixtures. Chitosan incorporation to blends did not significantly modify either gelatinization temperatures (T_p and T_o) or enthalpy (ΔH_G). Chitosan presence, however, contributed to homogenate the onset temperature in formulations with wheat flour as main ingredient (100%WF and 70%WF/30%RF). Chitosan might have contributed to a better transfer and control of water in the gluten net, managing the starch hydration process. In addition, the incorporation of 1% of chitosan to 100% RF notably reduced the T_o. In 100% RF without chitosan, a visible syneresis took place due to the lack of interaction between water and rice flour; while in 100% RF-1% chitosan, it was more stable and phase-separation did not occur, evidencing chitosan addition facilitating starch hydration. However, other hydrocolloids such as hydroxypropyl methylcellulose (HPMC), pectin, alginate, guar and xanthan gum, added in similar concentrations (1%) to wheat flour batters increased T_o and decreased gelatinization enthalpy (AH_G). Apparently, the strong interaction between the hydrocolloids and the starch induces a stable structure that requires higher temperatures to start starch gelatinization (Rojas, Rosell, & Benedito de Barber, 1999).

Glass transition temperature (T_g') was analyzed during thawing step, appearing close to water melting endothermic transition (Figure 4.15). Obtained results showed that the replacement of wheat-flour by rice-flour, with the consequent gluten reduction in batters, increased the T_g' of the batters due to an increase of available water in the mixing compared to 100% wheat-flour batters. In addition, rice-starch granule size is smaller than the wheat-starch granule size, contributing negatively to the water retention (Xue & Ngadi, 2007b). However, water retention in batters seemed to increase when chitosan was added to the formulations, being this effect more noticeable in 100% of rice flour batters (100% RF) with an increase of T_g' from -12.42 to -10.66 ºC.

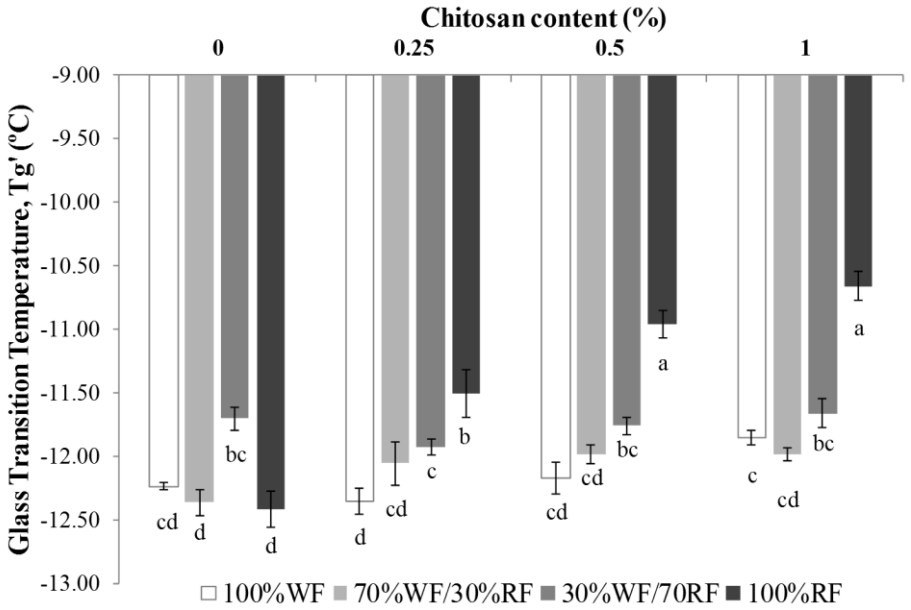

***Figure 4.15**. Glass transition temperature (Tg') corresponding to formulations of wheat flour (WF), rice flour (RF) and their combinations, 70% wheat flour + 30% rice flour and 30% wheat flour + 70% rice flour; with 0 (control), 0.25, 0.5 and 1% of chitosan.*

Homogenous groups are represented by the same letter.

Data related to melting transition (melting enthalpy, melting temperature and non freezable water content are reported in table 4.11. Melting temperature (T_m) was, in general, non-dependent on the type of flour or chitosan percentage in the batter. The influence of chitosan presence in batters on melting enthalpy (ΔH_m), melting temperature (T_m), and thus non-freezable water content, was only noticeable in 100% of rice-flour batters. In particular, the incorporation of chitosan at contents of 0.5 and 1% gradually decreased ΔH_m, T_m, and increased the bonding water content. These results pointed out the relevance of the interactions between chitosan and water molecules when rice flour is present in high quantity or is the only flour in batter formulations 30%WF/70%RF and 100% RF), that increased non freezable water percentage.

***Table 4.11**. Mean values (and standard deviation) of melting temperature and enthalpy, and non-freezable water content corresponding to formulations of wheat flour (WF), rice flour (RF) and their combinations, 70% wheat flour + 30% rice flour and 30% wheat flour + 70% rice flour; with 0 (control), 0.25, 0.5 and 1% of chitosan.*

Formulation	***Chitosan content (%)***	***Melting enthalpy ΔH_m (J/g)***	***Melting temperature T_m (ºC)***	***Non freezable water (%)***
100% WF	0	143 (3)b	1.4 (0.3)a	24.0 (1.8)b
70%WF/30%RF		142 (3)b	1.5 (0.5)a	24.8 (1.9)b
30%WF/70%RF		140.6 (1.1)b	0.9 (0.3)b	25.9 (0.7)b
100% RF		154 (11)a	1.4 (0.4)a	17 (4)c
100% WF	0.25	144 (7)b	1.2 (0.2)b	24 (4)b
70%WF/30%RF		144.3 (1.1)b	1.1 (0.3)b	23.5 (0.7)b
30%WF/70%RF		141.8 (0.5)b	0.97 (0.14)b	25.1 (0.3)b
100% RF		143.0 (0.1)b	1.11 (0.13)b	24.3 (0.1)b
100% WF	0.5	142.2 (0.6)b	1.03(0.04)b	24.8 (0.4)b
70%WF/30%RF		140.9 (0.6)b	0.9 (0.3)b	25.7 (0.4)b
30%WF/70%RF		141 (3)b	1.03 (0.13)b	25.7 (1.9)b
100% RF		125.0 (1.6)c	0.55 (0.13)c	33.3 (1.0)a
100% WF	1	140 (4)b	1.5 (0.3)a	26 (2)b
70%WF/30%RF		145 (3)b	1.06 (0.14)b	23.1 (1.7)b
30%WF/70%RF		128.9 (0.2)c	1.2 (0.5)b	33.5 (0.1)a
100% RF		131 (3)c	1.3 (0.4)b	32 (2)a

Homogenous groups are represented by the same letter.

4. Conclusions

Wheat flour replacement by rice flour in batters gave as a result a decrease of consistency (K) and the yield stress (τ_0) and an increase of the flow behavior index (n) because of the absence or lower gluten content. The addition of 0.25% chitosan to rice-flour formulation enhanced viscosity of the batters and therefore it could be considered a new strategy to produce gluten-free batters based on rice-flour. From a microstructural point of view, a reduction in structure

aggregation was observed when rice-flour was replaced by wheat flour in battering formulation. Newly, chitosan addition improved the presence of high agglomeration areas, particularly in batters with high rice flour proportion.

Chitosan incorporation to blends did not significantly modify either gelatinization temperatures (T_p and T_o) or enthalpy (ΔH_G); however, batters formulated with rice flour presented higher T_g' than those of 100% of wheat flour.

Melting temperature (T_m) was, in general, non-dependent on neither the type of flour nor the chitosan percentage in the batter. The influence of chitosan presence in batters on melting enthalpy (ΔH_m), melting temperature (T_m), and thus non-freezable water content, was only noticeable in 100% of rice-flour batters, reducing ΔH_m, T_m, and increasing the bonding water content.

Acknowledgements

The authors want to acknowledge the financial support of Universidad Politécnica de Valencia (Spain) for the scholarship to support Mariola Sansano Tomás' PhD studies and the mobility grant to University of Bologna (Italy).

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4.5. DIETARY ACRYLAMIDE: WHAT HAPPENS DURING DIGESTION

Mariola Sansano, Ana Heredia; Irene Peinado, Ana Andrés

Food Chemistry, 237 (2017), 58–64

http://dx.doi.org/10.1016/j.foodchem.2017.05.104

Instituto de Ingeniería de Alimentos para el Desarrollo
Universitat Politècnica de València, Valencia, Spain

DIETARY ACRYLAMIDE: WHAT HAPPENS DURING DIGESTION

Mariola Sansano; Ana Heredia; Irene Peinado; Ana Andrés

It is generally assumed that the bioaccesible fraction of acrylamide is the same as its intake amount, because of its water solubility. However, specific changes in its chemical structure and/or soluble fraction because of mechanical and physico-chemical processes acting during gastrointestinal digestion, such as chewing, dilution, variation in pH and the action of digestive enzymes, could modify its bioaccesibilty, and thus its bioavailability. In this sense, few studies have been published about the changes undergone by acrylamide along gastrointestinal digestion. Therefore, the purpose of this work was to study the effect of *in vitro* digestion on acrylamide coming from food products with noteworthy levels of acrylamide. The following specific objectives were as follows:

(a) to analyze the effect of gastric digestion on acrylamide in nine food products;

(b) to study the Kinetics of acrylamide content during gastric and intestinal digestion of French fries and chips; and

(c) to evaluate if the effectiveness of blanching and air-frying on acrylamide reduction during frying remains after gastric and intestinal digestion.

The recent standardized protocol published by Minekus et al. (2014) was used in order to mimic oral, gastric and intestinal digestion of the following foodstuffs: potato chips, French fries, onion rings, chicken nuggets, instant coffee, coffee substitute, crackers, sweet biscuits and breakfast cereals. Acrylamide was extracted from food products, and from freeze-dried digested samples by QuEChERS method and quantified by LC/MS/MS.

Results showed a significant increase of acrylamide after the gastric digestion of all the studied products, except for breakfast cereals. This fact evidenced the role of the acid pH of gastric digestion on acrylamide solubility, and therefore on its bioavailability. Potato based products (French fries and chips), instant coffee and coffee substitute were the food products with the greatest acrylamide content

before digestion. However, sweet biscuits, crackers, chicken nuggets and onion rings experimented the biggest increase of acrylamide after gastric digestion. Chips, French fries, and chicken nuggets increased acrylamide amount within 120 minutes of gastric digestion, while instant coffee, coffee substitute, onion rings, sweet biscuits and crackers increased acrylamide levels at the beginning of the gastric stage. The kinetic study of acrylamide changes in French fries and chips during gastrointestinal digestion resulted in a decrease of acrylamide content at the end of the intestinal stage, until similar amounts as in the food product (before digestion). Finally, the evaluated acrylamide mitigation strategies, blanching and air-frying, were also effective in reducing acrylamide content, after the gastrointestinal digestion, compared to control (without blanching and fried by conventional frying technique).

In conclusion, food composition and gastrointestinal conditions should be considered because these factors may modulate acrylamide bioavailability.

DIETARY ACRYLAMIDE: WHAT HAPPENS DURING DIGESTION

Mariola Sansano; Ana Heredia; Irene Peinado; Ana Andrés

Abstract

Acrylamide is a well-known potentially carcinogen compound formed during thermal processing as an intermediate of Maillard reactions. Three objectives were addressed: the impact of gastric digestion on acrylamide content of French Fries, chips, chicken nuggets, onions rings, breakfast cereals, biscuits, crackers, instant coffee and coffee substitute; the acrylamide content evolution during gastrointestinal digestion of French fries and chips; and the effectiveness of blanching and air-frying on acrylamide mitigation after gastrointestinal digestion.

A significant increase (p-value < 0.05) in acrylamide content was observed for most of the products after gastric digestion (maximum registered for sweet biscuits, from 30 ± 8 to 150 ± 48 µg/kg,). However, at the end of the intestinal stage, acrylamide values were statistically similar (p-value = 0.132) for French fries and lower than the initial values (before digestion) in potato chips (p-value = 0.027). Finally, the low acrylamide content found in blanched and air-fried samples, remained still lower than for deep fried samples even after gastrointestinal digestion.

Keywords: acrylamide, *in vitro*, digestion, kinetics, bioaccessibility

1. Introduction

Acrylamide is a soluble compound with low molecular weight formed during thermal processing as an intermediate product of the Maillard reactions, mainly through the reaction between the amino acid asparagine and some reducing sugars (Stadler et al., 2002). It is considered a neurotoxic and potential carcinogenic compound (IARC, 1994) and glycidamide, an acrylamide metabolite, has also a genotoxic character (Blank, 2005). Temperature above 120 ºC is required to generate acrylamide, this occurring frequently in carbohydrates rich-foods subjected to frying, roasting or baking (Matthäus, Haase, & Vosmann, 2004; Tareke et al., 2002). The highest concern about

acrylamide intake comes from the cereal grains based products (such as biscuits, crackers or bread), breakfast cereals, coffee and more especially, potato products (French fries and chips).

Since the first announcement of acrylamide presence in foods, health institutions and food industries have put together some effort in order to reduce consumer exposure. For that purpose, the European Food and Drink Federation published the 'Acrylamide Toolbox'. A useful document where the last scientific and technological developments are gathered and periodically updated (Food Drink Europe, 2013). It summarizes the most effective low-acrylamide strategies for each defined food group ((I) potato based snacks, (II) French Fries & Other Cut Potato Products, (III) Cereal/Grain Based Products, (IV) Coffee, Roasted Grain & Substitutes and (V) Baby Biscuits, Infant Cereals & Baby Foods Other than Cereal Based Foods), and specifies in which step of the process they might be applied (raw material selection, recipe design or process design). Among the proposed strategies, blanching has been widely studied as potential procedure to mitigate acrylamide in French fries (Pedreschi et al., 2010, 2004), being its application strongly recommended at domestic and industrial levels. As an alternative to deep fryers, air fryers have recently been introduced domestically, due to its capability to produce healthier fried products (low fat). In fact, a recent study showed reductions in acrylamide formation up to 90 % in fried potatoes obtained by air frying compared to conventional deep oil-frying (Sansano et al., 2015).

Besides the scientific evidence of the influence of process variables and food composition on acrylamide generation, numerous studies have been published in relation to acrylamide distribution, metabolism and excretion in animal and human assays (Doerge, Young, McDaniel, Twaddle, & Churchwell, 2005a, 2005b; Shipp et al., 2006; Zödl et al., 2007). Distribution studies showed that acrylamide is rapidly distributed to all tissues without evidence of accumulation; it might be found in breast milk being even capable of penetrating the placenta (Schettgen et al., 2004; Sörgel et al., 2003). In addition, the presence of other

Maillard products should be considered because they could enhance or suppress acrylamide toxicity (Friedman, 2005; Somoza, 2005). Concretely, antiallergenic/allergenic, antibiotic, anticarcinogenic/carcinogenic, antimutagenic/mutagenic, antioxidative/oxidative, clastogenic, and cytotoxic activities have been attributed to certain Maillard products.

In the organism, acrylamide is mainly metabolized through the cytochrome P450 2E1, action that catalyzes the formation of glycidamide, a reactive epoxide metabolite (Blank, 2005). Both, acrylamide and glycidamide, have genotoxic effects though only glycidamide forms adducts with proteins and DNA in vivo (M. Friedman, 2003). Nevertheless, few studies have been published about the effect of digestion process on acrylamide (Eriksson & Karlsson, 2006; Hamzalıoğlu & Gökmen, 2015; Schabacker, Schwend, & Wink, 2004). Eriksson & Karlsson (2006) studied the effect of digestive enzymes and pH on acrylamide extraction. Pepsin extraction in acid conditions at 37ºC during 72 h (62.5 FIP-U/g) showed no differences in acrylamide content compared to normal water extraction. Schabacker, Schwend, & Wink, (2004) showed that acrylamide binds to egg albumin through Caco-2 cells (human intestine model), under simulated intestinal conditions, revealing that protein intake could attenuate acrylamide content in the organism. Hamzalıoğlu & Gökmen (2015) studied the influence of gastrointestinal digestion of biscuits and fried potato products on the acrylamide content and reported different results depending on the food product. Though acrylamide content was notably reduced after the gastrointestinal digestion, acrylamide from French fries experimented a noteworthy increase after gastric digestion (Hamzalıoğlu & Gökmen, 2015).

Due to its solubility in water, acrylamide bioavailable content is assumed to be the same as the intake amount (Eriksson, 2005; Eriksson & Karlsson, 2006). However, as any other contaminant, the total amount present in the ingested food does not necessary correspond to the bioavailable content. After the intake of any food product, an alteration of its structure and chemical composition may take place by multiple mechanical and physicochemical factors, such as chewing,

dilution, variation in pH and/or the action of the different enzymes present in the mouth, the stomach and intestine, among others. The effect of different digestive stages on the compounds present in food varies considerably between individuals, and *in vivo* studies would be the ideal ones. However, *in vivo* studies are intrusive, and imply ethical and costs constraints. Based on these limitations, *in vitro* models represent an alternative methodology scientifically validated to evaluate the influence of digestion on different compounds. Some of the advantages of *in vitro* models are the quick results, lower cost, lack of ethical constraints and reproducibility, selection of controlled conditions or to facilitate sampling at different times of the digestion process (Minekus et al., 2014).

In this scenario, there is a lack of information related to acrylamide changes during gastrointestinal digestion in different food matrices. The objective of this study was multiple: (a) to analyse the effect of gastric digestion on acrylamide content in nine food products with noteworthy levels of this toxic; (b) to study the kinetics of acrylamide variation along digestion (gastric and intestinal stages) of French fries and chips and (c) to evaluate whether the effectiveness of blanching and air-frying as mitigating strategies of acrylamide persists during gastrointestinal digestion.

2. Materials and methods

2.1. Reagents

Potassium chloride, sodium chloride, magnesium chloride, hexane and methanol were purchased from Panreac (Barcelona, Spain). Ammonium bicarbonate, potassium dihydrogen phosphate, porcine pepsin (3200-4500 U/mg), α-amylase from human saliva (500 U), pancreatin (8 × USP) from porcine pancreas and bovine bile extract, were from Sigma-Aldrich (Deisenhofen, Germany). The standard acrylamide (≥ 99 %) was obtained from Merck (Darmstadt, Germany) and $^{13}C_3$-labelled acrylamide (99 %) from Cambridge Isotope Laboratories (Andover, MA). Sodium carbonate hydrogen was purchased from Scharlau (Barcelona, Spain). Acetonitrile, formic acid (99-100 % purity) and magnesium sulphate were purchased from VWR (Fontenay-sous-Bois, France).

PSA (Primary Secondary Amine) was obtained from Supelco (Bellefonte, PA). All solvents used for the determination of acrylamide were HPLC grade and all other analytical grade. Bidistilled water was used for chromatographic analysis (Milli-Q, Millipore Corp., Bedford, MA). Acrylamide and $^{13}C_3$-acrylamide solutions (1 mg/mL) were prepared daily from stock solutions (100 mg/mL in acetonitrile). Standard solutions were stored at -20 °C.

2.2. Food samples

Nine different food products representatives of the food groups defined in Acrylamide Toolbox (Food Drink Europe, 2013) were selected. Concretely, fried potatoes (French Fries), chicken nuggets and fried onions rings purchased in a fast food restaurant in Valencia (Spain); and chips, breakfast cereals (based on rice, wheat and barley), sweet biscuits, crackers, instant coffee and coffee substitute (cereals basis) bought in a supermarket. In all cases, except in coffee and coffee substitute, a visual selection was performed to discard excessively brown samples, in as much as the relationship between the non-enzymatic browning degree and acrylamide content.

Potato of Agria variety was bought in a local market for the study of the effect of gastrointestinal digestion on acrylamide from French fries obtained by conventional deep-oil frying (control) or hot-air frying without pre-treatment, and subjected to blanching and subsequent deep-oil frying.

2.3. In vitro digestion

The digestive process (oral, gastric and intestinal stages) and the simulated fluids (salivary, gastric and intestinal) were prepared as the internationally agreed protocol, published by Minekus et al., (2014) with slight modifications. The simulated salivary fluid (SSF), the simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared daily from stock solutions prepared weekly (Table 4.12).

***Table 4.12**. Electrolyte composition of salivary, gastric and intestinal fluids prepared from stock solutions (salivary, gastric and intestinal). Final volume was adjusted with distilled water after adjusting the pH.*

	SSF	***SGF***	***SIF***
	mmol/ L	***mmol/ L***	***mmol/ L***
KCl	15.1	6.9	6.8
KH_2PO_4	3.7	0.9	0.8
$NaHCO_3$	13.6	25	85
NaCl	-	47.2	38.4
$MgCl_2(H_20)_6$	0.15	0.1	0.33
$(NH_4)_2CO_3$	0.06	0.5	-
$CaCl_2(H_20)_2$	1.5	0.15	0.6

i. Influence of gastric digestion on acrylamide from different food matrices

Samples of food products (French Fries, chips, chicken nuggets, fried onions rings breakfast cereals, sweet biscuits and crackers) were mixed with SSF (75 U α-amylase/mL SSF) in a ratio 50:50 w/v during 2 min (hand blender, Ufesa 600W, Slovenia). For instant coffee and coffee substitute, foods were dissolved with water in a ratio 50:50 (w/v) before and then mixed with SSF in the same ratio as the other foodstuff (50:50 w/v). 10 g of the simulated bolus was weighed in a Falcon tube and 10 mL of SGF (2000 U pepsin/mL in the final mixture) was added (ratio 50:50 w/v) and pH adjusted to 3.0 ± 0.1 with HCl 1M (pH- meter Mettler Toledo, Schwerzenbach, Switzerland). Porcine Pepsin activity was previously determined according to the EC 3.4.23.1, described by Minekus et al. (2014), to achieve 2000 U/mL in the final digestion mixture. Subsequently, the mixture was placed in a thermostatically controlled chamber (Selecta, Spain) at 37 °C coupled to a shaker (Intelli-Mixer RM 2, Elmi Ldt., Baltics and Russia) at a speed of 55 rpm and head-over-heels movements. The total duration of this stage was 120 min, and every 30 min, the pH was measured and adjusted to 3 (if necessary). To stop enzymatic reactions, right after the gastric digestion, tubes were immersed in ice and samples were frozen to - 40 ºC (model CVN-40/105, Matex,

Barcelona) for subsequent freeze-drying (-40 °C and 1.25 mbar, Telstar, Terrassa, Spain). Sampling was performed at the beginning (just after 5 min of the SGF addition and pH adjustment) and at the end of the gastric stage (120 min). Samples were always independent tubes and tested in triplicate.

ii. Kinetics of acrylamide variation during gastrointestinal digestion of commercial French fries and chips

Acrylamide content was determined along gastric (at 0, 15, 30, 60 and 120 min) and intestinal digestion (15, 30, 60 and 120 min) of French fries and chips. There independent tubes for each sampling time were subjected to digestive reactions. Simulation of oral and gastric stages was proceeded as for the previous section. For the intestinal stage, 20 mL of SIF was added to the gastric chime (ratio 50: 50 (v/w)), and pH was adjusted to 7.0 ± 0.1 with NaOH 1M, right after 120 min of gastric stage. The final bile salts and pancreatin concentrations were 10 mM and 1260 LU (Lipase Units or FCC), respectively, in each tube. To stop the enzymatic reactions (after the different sampling times along the gastric and intestinal simulated digestion) the tubes were placed in ice for 10 minutes, and then stored at -40 °C until they were freeze-dried.

iii. Influence of blanching and air frying on acrylamide changes along gastrointestinal digestion of French fries.

Potatoes were sliced with a commercial cutter (Taurus kitchenline, New Wulmstorf, Germany) into strips (10 mm x 10 mm x 50 mm). Strips were introduced in tap water until pre-treatment or frying step, and they were divided in 3 groups that corresponded to each treatment: A: control (deep oil-frying); B: blanching (85 ºC, 5 min) + deep oil-frying; C: hot air-frying. Frying step was carried at 180 °C during 6.5 min in a commercial deep-oil-fryer (model: FM 6720 Ideal 2000 Professional, Solac, with a nominal power of 2000W); and air-frying in a hot-air-frying equipment for 21 min (model: AH-9000 Actifry, Tefal with a nominal power of 1400 W). The frying time was that required to achieve the same superficial color as under deep-oil fried samples. Commercial sunflower oilseed

was used with a potato-to-oil ratio of 1:20 (w/v) in deep oil-frying, and 0.3 g of oil per 100 g of potatoes in air-frying, according to the equipment specifications.

Samples were subjected to oral, gastric and intestinal digestion as explained in the previous sections. Sampling was performed at the beginning (just after the SGF addition and pH adjustment) and at the end of the gastric stage (after 120 min), as well as after the intestinal stage (120 min more). Samples were always independent tubes and tested in triplicate.

2.4. Analytical determinations

Water content of the food products was analyzed by vacuum drying at 60 ºC until constant weight was achieved (AOAC, 2000).

Acrylamide content was analyzed in food products previous digestion as well as in lyophilized samples after the different digestion conditions. Acrylamide extraction was carried out as Sansano et al., (2015), with minor modifications. Sample preparation procedure has been tested and resulted fitted for different food matrixes (coffee, chocolate, peanuts butter, crackers and potato chips). It includes a dispersive solid phase extraction (dSPE) cleanup, named QuEChERS, and uses an internal standard ($^{13}C_3$-acrylamide). Concretely, 1g of food product or lyophilized (digested samples) was weighted in a Falcon tube. 0.5 mL of internal standard and 5 mL of n-hexane were added and mixed in a vortex for 30 s. Then, 10 mL of Mili-Q water and 10 mL of acetonitrile were added as well as the salt packet included in QuEChERS 1 (4g of $MgSO_4$ and 0.5g of NaCl), and the mixture was stirred for 1 min in the vortex. Centrifugation (2026 RCF; Centronic BL II, Selecta, Spain) for 5 min) was performed to properly separate all the layers, and after discarding the hexane layer, 1 mL of acetonitrile phase was transferred to a 2 mL tube that contained 50 mg of PSA (Primary Secondary Amine) and 150 mg of $MgSO_4$ (QuEChERS 2). The acetonitrile phase was then vortexed during 1 min and centrifuged (2697 RCF; Labofuge 200 Heraeus, Germany). The supernatant was filtered and transferred to a vial for the LC/MS/MS analysis.

The acrylamide analysis was performed with an Agilent 1200 Series HPLC system coupled to an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies Inc., CA, USA) with an electrospray type ionization source. The column used was a Zorbax Eclipse XDB C-18 (2.1mm x 50mm, 1.8µm). The mobile phase used consisted of 2.5 % methanol/ 97.5 % of 0.1 % formic acid (A) and methanol (B). The elution gradient was as follows: 0-3 min 100 % of A; 3.1-3.5 min 70 % A; 3.6 min 100 % A, with 1 min post-time to equilibrate the column. The column oven temperature was set at 30 °C, the flow was maintained at 0.4 mL/minute and the injection volume was 10µL. The electrospray was operated in positive ion mode. The conditions used in the ionization source were: 350 ºC at 12 L/min for the drying gas (N_2), a nebulizer pressure of 40 psi and a capillary voltage of 4000 V. Acrylamide content was performed using the multiple reaction monitoring mode (MRM), monitoring the ions m/z 72 > 27 and m/z 72 > 55.2, and also m/z 75>58 for $^{13}C_3$-acrylamide (internal standard). Five different levels of acrylamide (10, 20, 50, 100 and 200 µg/L) and 50 µg/L of internal standard, with six replicates for each level (n=6) were studied, being 10 µg/L of acrylamide the limit of quantification.

2.5. Statistical analysis

Analysis of variance (one-way-ANOVA), using the Statgraphics Centurion software, with a confidence level of 95 % (p-value ≤ 0.05) was performed to analyze the statistical influence of the digestion process on the changes undergone by dietary acrylamide coming from the different studied food matrices.

3. Results and discussion

3.1. Changes in acrylamide content of different food products because of gastric digestion

Figure 4.16 shows the acrylamide content (µg/kg) of the studied foods prior to digestion, as well as at the beginning and at the end of their gastric stage.

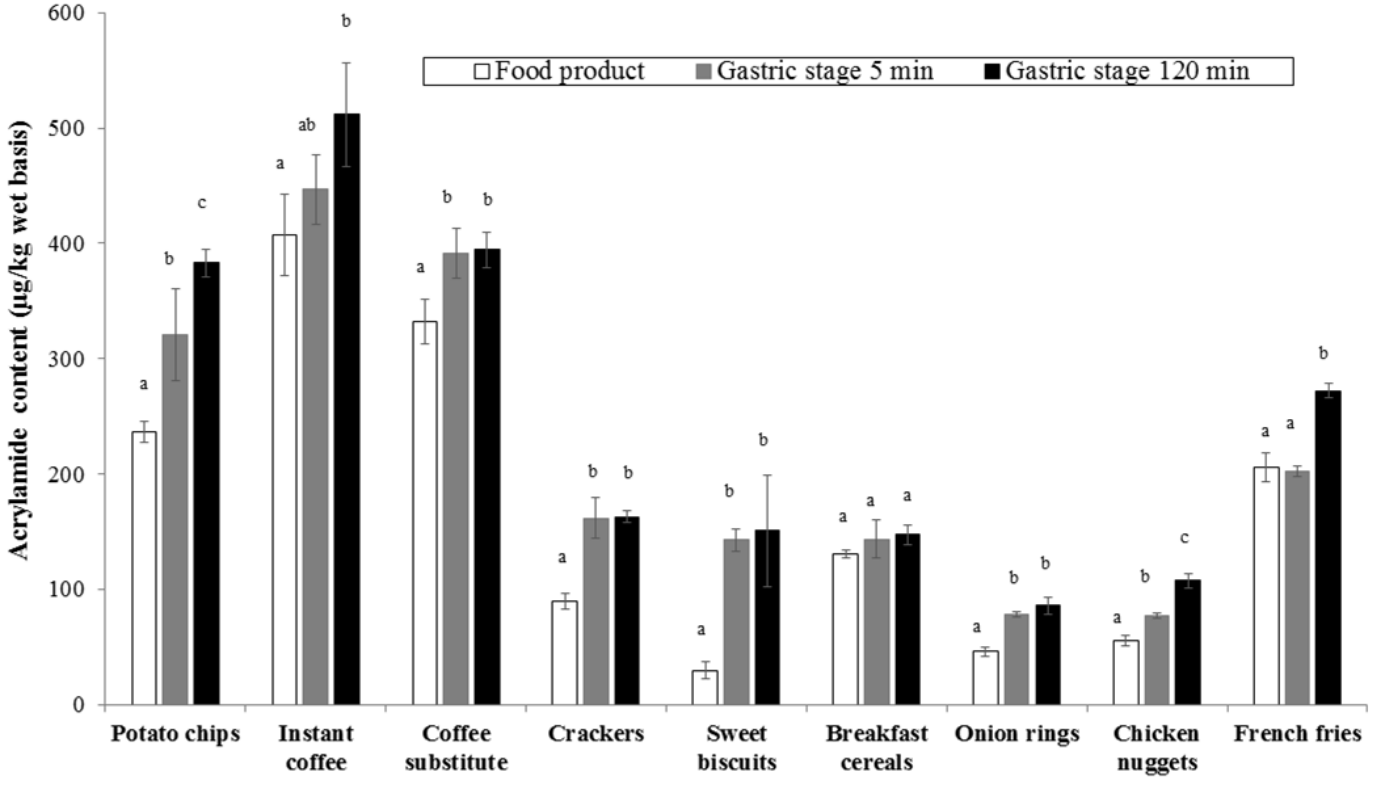

***Figure 4.16**. Acrylamide average content and standard deviation (µg/kg in wet basis) of food products before digestion and at 5 and 120 min of gastric digestion.*

Homogenous groups are represented by the same letter in each food product (One way ANOVA).

According to the obtained results, the major contribution of acrylamide throughout the diet comes from instant and substitute coffees together with potato-based products (chips and French fries), followed by breakfast cereals, crackers, chicken nuggets, onion rings and sweet biscuits. This could be due to the differences existing in terms of macronutrients, processing technology and moisture content among the studied food products. It is well known that besides the presence of native precursors in the food (reducing sugars and asparagine) and/or the process temperature, the low availability of water in food represents a key factor that triggers acrylamide formation (Matthäus et al., 2004). Acrylamide is mainly formed on the external surface of the product where moisture content is much lower than in the inner part of the food. This lower moisture favors an important increase on the surface with an exponential acrylamide generation (Bråthen & Knutsen, 2005). In this sense, foods with lower moisture content and rich in acrylamide precursors (reducing sugars and free asparagine), and those subjected to the highest processing temperatures were the ones with the highest acrylamide content. Table 4.13 illustrates the influence of moisture and acrylamide precursors on acrylamide content for chips and French fries (Biedermann-Brem et al., 2003), while the effect of roasting in acrylamide content was evident in coffee-products.

Table 4.13. *Moisture (%) of studied food products (wet basis).*

Food product	***Moisture content (%)***
Potato chips	1.04 ± 0.02 ab
Instant coffee	2.69 ± 0.07 bc
Coffee substitute	2.72 ± 0.06 bc
Sweet biscuits	0.20 ± 0.02 a
Crackers	0.20 ± 0.03 a
Breakfast cereals	3.2 ± 0.3c
Onion rings	40 ± 3d
Chicken nuggets	51 ± 1g
French fries	43.7 ± 0.9 e

Homogenous groups are represented by the same letter.

The effect of gastric digestion on acrylamide was marked, with a significant increase in all digested samples excepting in breakfast cereals. The magnitude of this increase seems to depend on the food product. It is worthy of note that the highest increase of acrylamide during the gastric digestion took place in those products previously fried and baked. Particularly, a relative increase of acrylamide content was registered in French fries (32 ± 3 %), potato chips (62 ± 5 %), crackers (83 ± 1 %), onion rings (87 ± 16 %), chicken nuggets (94 ± 11 %) and sweet biscuits (410 ± 163 %). Acrylamide content in heated foodstuffs is the net result of complex reactions leading to the formation and degradation of this compound during Maillard reactions (Luning & Sanny, 2016). Both, intermediate products such as Schiff bases and final acrylamide are accumulated in heated products and their final content depends on the heating conditions among other factors. Gastric pH seems to favor the conversion of intermediate products (Schiff bases formed during thermal process) into acrylamide (Hamzalıoğlu & Gökmen (2015). Moreover, the proteolysis occurring due to the pepsin activity together with the mechanical forces in the stomach might favor the release of acrylamide because of matrix degradation. Additionally, acrylamide increase rapidly occurred at the beginning of the gastric stage in some products (coffee substitute, crackers, sweet biscuits, breakfast cereals and onion rings), while it was observed after a longer gastric digestion time for other products (instant coffee, potato chips, French fries and nuggets) the acrylamide increase. This, suggests that the different kinetics of acrylamide changes might be related to the matrix degradation rate. The enzymatic activity of pepsin, which contributes to the food matrix degradation, plays an important role in acrylamide' release from the food matrix into the gastric fluid. It can be said therefore, that the acrylamide increase observed at short times is related to the acid pH (enhancing Schiff bases conversion to acrylamide), while matrix degradation would explain the acrylamide increase observed at the end of the gastric stage.

Other mechanisms can be implied in acrylamide increase during digestion. Pastoriza, Rufián-Henares, & Morales, (2012) reported the formation of melanoidin-bound-acrylamide based on Michael addition reactions. This

melanoidin-bound-acrylamide can be formed during heating processes (such as roasting of coffee-grains), and probably broken later on, under the acid conditions during the gastric stage explaining the observed acrylamide increase.

Finally, it is important to point out that the highest final content of soluble acrylamide (µg/kg) in the stomach is achieved after the complete gastric digestion of French fries, chips, instant coffee and substitute coffee, in spite of the partial increase after gastric digestion was higher in other products, such as sweet biscuits, crackers and battered foods.

3.2. Kinetics of acrylamide changes during gastrointestinal digestion of commercial French fries and potato chips

Due to the important consumption of potato products among populations of different ages and their contribution to acrylamide in the diet, a kinetic study of acrylamide changes during gastrointestinal digestion of French fries and chips was performed. Figure 4.17 gathers acrylamide content (µg/kg), at different times of the gastric and intestinal digestion. Results showed that the kinetics of acrylamide conversion from Schiff bases during gastric stage took place faster for chips compared to French fries; a maximum increase of acrylamide was reached after 60 min of gastric digestion for French fries and after 15 min for chips. From this point onwards an acrylamide decrease was observed for both products. These results evidenced that solubilization and conversion of Schiff base intermediates into acrylamide occurred mainly at the beginning of the gastric stage, being the acidic pH responsible for the observed changes.

In order to confirm the role of gastric pH on the acrylamide increase, a parallel *in vitro* digestion was performed without pepsin addition. This time, sampling took place only after 120 min of gastric digestion. The relative variation of acrylamide after 120 min of gastric digestion with and without pepsin was similar, this confirming the role of pH on this phenomenon. Hamzalıoğlu & Gökmen (2015) reported an increase in acrylamide of 295 % and 20-45 % after gastric digestion of French fries and chips, respectively. However, in this work 45 % and 35% increase, referred to acrylamide content before digestion, was found in French

fries and chips, respectively. Since these differences could be due to the different pH used in the *in vitro* simulation (pH 3 in this work and pH 2 in Hamzalıoğlu & Gökmen (2015) study), an additional experiment was performed. The obtained results confirmed that the differences found between our study and the one carried out by Hamzalıoğlu & Gökmen (2015) could be attributed to differences in gastric pH used in the simulation. Acrylamide content was found to be higher at pH 2 when compared to pH 3 reaching values of 534 ± 36, 736 ± 41 and 556 ± 39 µg/kg at 0, 60 and 120 min of gastric digestion of French fries.

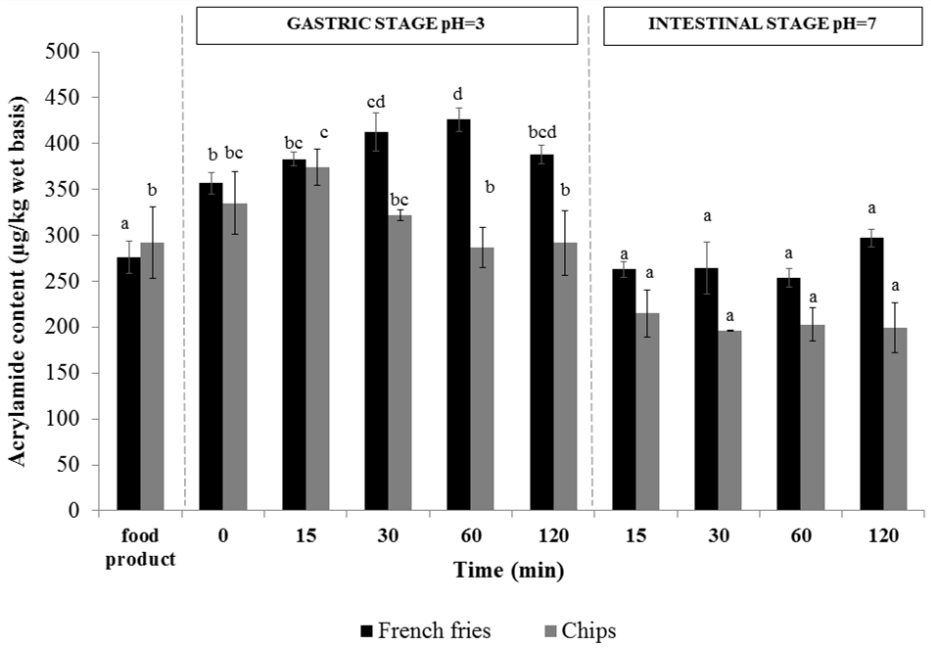

***Figure 4.17**. Acrylamide content (µg/kg in wet basis) before digestion and during in vitro gastric and intestinal digestion of French fries and chips.*
Different letters mean significant differences (95% confidence level) between digestion times for each food matrix (One-way ANOVA).

During the subsequent intestinal stage, acrylamide content decreased to a minimum value after 15 minutes of digestion, and remaining constant till the end

of the experiment. This reduction could be explained by the formation of Michael adducts in which acrylamide would be involved. This in fact, is a mechanism proposed as a potential strategy to reduce acrylamide content in foods (Hamzalıoğlu & Gökmen, 2015; Hidalgo, Delgado, & Zamora, 2010; Zamora, Delgado, & Hidalgo, 2010). During gastric digestion, pepsin hydrolyses the protein releasing short peptides and free amino acids such as cysteine or lysine with nucleophilic character (-SH and -NH_2). These small peptides and amino acids are capable to interact with acrylamide, forming adducts and causing thus, an apparent reduction in its bioavailability during the intestinal stage (Hamzalıoğlu & Gökmen, 2015). Hamzalıoğlu & Gökmen, (2015) studied model systems composed of acrylamide and lysine or cysteine and obtained slight reductions of the acrylamide content after simulating gastric digestion, compared to the control (without amino acids). In other studies, a rapid decrease of acrylamide content was observed after a heating process in the presence of N-acetyl-cysteine or lysine, resulting from Michael addition, between the nucleophilic groups of these amino acids (-SH and-NH_2) and the double bond (C = C) of acrylamide (Hidalgo et al., 2010; Zamora et al., 2010). Hoenicke & Gatermann, (2005) observed an acrylamide reduction after long storages in specific foodstuff, reporting the apparent reactive effect of SH-group-containing substances. The results obtained in this study are in agreement with those found in literature, confirming the possible interaction of amino acids from the proteolytic activity and acrylamide in the gastrointestinal tract, specifically under intestinal conditions.

3.3. Acrylamide bioaccessibility of French fries subjected to blanching and air-frying

French fries were prepared in lab-scale to specifically study the influence of blanching and air-frying on acrylamide changes during *in vitro* digestion. Fig. 4.18 shows the acrylamide content (μg/kg) corresponding to the different French fries samples, at the beginning and at the end of the gastric stage (5 and 120 min) and

after the intestinal stage (120 min), compared to acrylamide content before digestion.

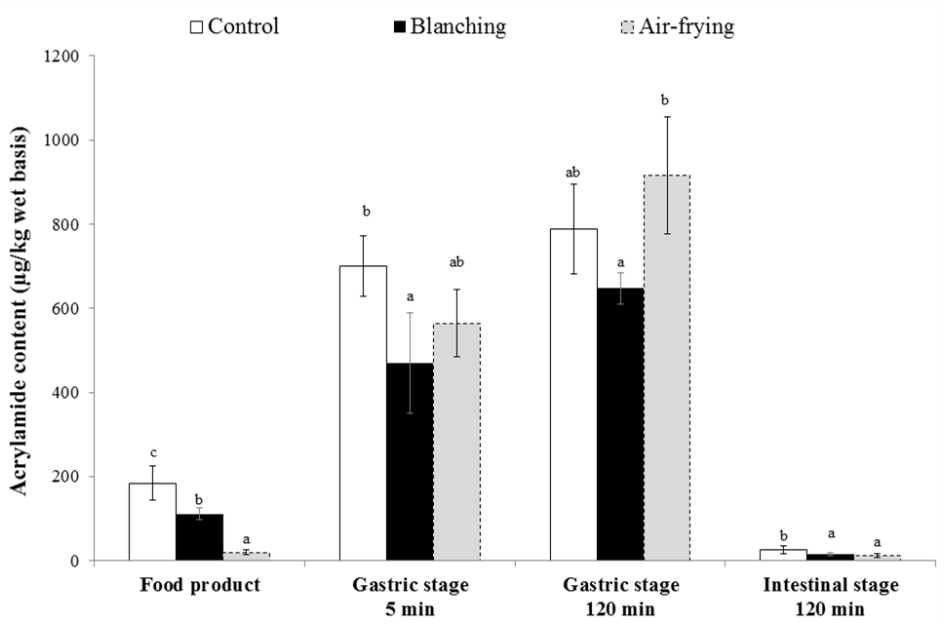

Figure 4.18. *Acrylamide content (µg/kg in wet basis) of deep-oil fried French fries with or without blanching pre-treatment (control), and fried by air-frying before digestion and after 5 and 120 min of gastric digestion and after 120 min of intestinal digestion.*

Different letters mean significant differences in each digestive stage (One-way ANOVA).

As expected, acrylamide content was lower in blanched and air fried samples (Pedreschi, Kaack, Granby, & Troncoso, 2007; Pedreschi et al., 2011; Sansano et al., 2015). Air-frying was the most effective technique to reduce acrylamide formation (88% reduction) similarly to the results reported in a previous study (Sansano et al. 2015). Blanching has been commonly used not only due to its effectiveness in reducing acrylamide content, but also to improve the final color and texture of French fries (Pedreschi et al., 2004). This pre-treatment promotes

the lixiviation of the main precursors of acrylamide formation (reducing sugars and asparagine).

After gastric digestion (120 min) a high acrylamide increment was observed in all samples. This increment was observed since the beginning of the gastric stage and progressed, especially in French fries obtained from air-frying. These results prove again the important role of pH on acrylamide conversion. Blanching lead to lower levels of acrylamide, from the beginning to the end of the gastric stage. This suggests that the decrease of reducing sugars and asparagine during the pre-treatment also reduces the formation of Schiff bases and thus, less acrylamide appears at the end of the gastric stage. On the other hand, air-frying was used as a strategy to obtain French fries with low acrylamide content. However, these samples were the ones that experienced the higher increase of acrylamide after gastric digestion. These results suggest that air frying would enhance the formation of Schiff basis, this explaining the acrylamide conversion observed in gastric digested samples. After that, a reduction of acrylamide content was observed at the end of the intestinal digestion (values below 100 µg/kg in all samples), although blanched and air-fried samples showed lower values than the control samples. These results suggest that regardless the different increments of acrylamide during gastric digestion, the strategy to reduce acrylamide is useful after the intestinal stage. Specific products originated from digestive enzymes reactions, such as amino acids (cysteine or lysine), sulfhydryl compounds, or melanoidins, with nucleophilic properties, could react with acrylamide, reducing its bioavailability (Hoenicke & Gatermann, 2005; Lineback et al., 2012). Acrylamide content in lab-scale French fries was found to decrease after intestinal stage, compared to French fries from the fast food restaurant.

4. Conclusions

Our findings showed that the gastric *in vitro* simulation of the studied food matrices resulted into a significant increase of the soluble fraction of acrylamide, for all the tested products except for breakfast cereals. The maximum increase was found in crackers and sweet biscuits, followed by battered foods and potato

products (Chips and French fries). Despite the acrylamide increment observed after gastric digestion, acrylamide bioaccessibility (estimated from acrylamide content after the intestinal digestion), was similar in French fries and lower in potato chips than before digestion. Finally, the evaluated mitigation strategies, blanching and air-frying, were effective in reducing acrylamide content, even after the gastrointestinal digestion, compared to control.

In vitro simulated digestion can be considered then as a useful tool to obtain the bioavailable acrylamide content. It takes into account not only the influence of food intrinsic factors (structure, composition, nutrients interactions, etc.) but also extrinsic factors associated to the physiological process (gastric and intestinal pH, transit time, enzyme activities, etc.).

Acknowledgments

The authors would like to thank the Universitat Politécnica de València for the PhD scholarship given to Mariola Sansano Tomás.

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5. CONCLUDING REMARKS

5. CONCLUDING REMARKS

The growing public concern about the presence of acrylamide in food and its harmful effects on health has been tackled in this research. Concretely, the work developed in this doctoral thesis has provided some knowledge about new strategies to reduce acrylamide and the influence of the *in vitro* digestion process on acrylamide content have been studied. Some final considerations and future recommended research have been exposed below, based on the main conclusions obtained from each specific objective:

i. Influence of pre-frying treatments and frying process variables on the formation of acrylamide in fried potatoes.

Air-frying technology allows to obtain fried products with less oil content, and as have been proved in *paper I*, it is also possible to produce potatoes with a considerably low content of acrylamide (about ≈ 90%) compared with deep oil-frying conditions. Both evidences permit to affirm that air-frying is a promising technology for obtaining healthy fried products.

The application of a pre-treatment such as, dipping the potatoes into a solution of nicotinic acid, citric acid, glycine or NaCl might be a viable approach for the minimization of acrylamide content. However, the sensory repercussion of any strategy to reduce acrylamide generation should be evaluated before application.

ii. Functionality of chitosan as ingredient to mitigate acrylamide formation in batter formulations.

Adding small amounts of chitosan in model and fried batter systems has been proven to be a new way to mitigate the generation of acrylamide. The proposed mechanism of acrylamide reduction is based on the richness of amino groups of chitosan, which compete with asparagine amino groups to bind carbonyls (e.g. reducing sugars), the first stage of acrylamide formation. For this reason, chitosan has a high potential to provide consumers with lower acrylamide content if it is incorporated into batters on a commercial scale. However, some limitations must be considered to achieve this objective:

-Chitosan is classified as GRAS compound (Generally Recognized as Safe) and its use as a dietetic supplement is approved. Some countries (Japan, Corea…) have accepted chitosan use as a food additive, but Europe has not approved yet its use.

-In addition, chitosan is a rather expensive ingredient, but results showed that less than 0.5% in battering formulations is enough to reduce by half acrylamide formation. However, it was done a collaborative study with economics students of Universitat de Valencia, focused on analyzing the financial viability of a fictitious company that would include this formula to pre-fried squid rings production. The resulted economic indicators showed positive values of profitability.

-As have seen in Paper III, both deacetylation degree and molecular weight conditioned the acrylamide mitigation capability, and thus, the specifications of chosen chitosan would have to be analyzed and the effectiveness in reducing acrylamide content might be verified.

-There were not significant modifications of the color and texture but consistency was affected and this fact should be considered. In addition, the feasibility of using rice-flour batters with chitosan as coating in different food matrices should be evaluated.

-Finally, the data generated can be used in optimizing the production of gluten-free batters made of rice flour and chitosan. However, resulted batters should be analyzed also in fried samples, in order to guarantee the persistence of the proper consistency and final quality.

iii. Evolution of acrylamide content during gastrointestinal digestion of different thermally processed foods.

It is important to mention that, despite the increment of acrylamide content observed after gastric digestion, at the end of the intestinal stage, and thus the bioaccessible acrylamide amount, is similar or lower than the content before digestion, at least in potato chips and French fries.

The increment observed after gastric digestion is probably due to the conversion of Schiff basis to acrylamide, as mentioned in the bibliography. However, monitorization of both, Schiff basis and acrylamide would give an approach to confirm the specific routes of acrylamide formation. In addition, the Michael adducts formed during the intestinal stage from amino acids released from pepsin activity (during gastric digestion), should be also measured to identify the specific reaction ways that involve acrylamide formation and decomposition.