Universidad Autónoma de Madrid
Facultad de Ciencias
Departamento de Química-Física Aplicada

Biological Activity and Nutritional Properties of Processed Onion Products

María Eduvigis Roldán Marín
PhD Thesis

Spanish National Research Council (CSIC)
Instituto del Frío
Department of Plant Food Science and Technology

This European Doctorate is based on a literature review and five scientific publications

A mis padres,

A mi hermano.

'Biological Activity and Nutritional Properties of Processed Onion Products'

PhD Thesis by **María Eduvigis Roldán Marín**

Supervisors

M. Pilar Cano Dolado
Profesor de Investigación del CSIC

Concepción Sánchez-Moreno González
Científico Titular del CSIC

Department of Plant Food Science and Technology. Instituto del Frío
Spanish National Research Council (CSIC). Madrid. SPAIN

TABLE OF CONTENTS

Accompanying papers

Paper I

Roldán E, Sánchez-Moreno C, de Ancos B, Cano MP (2008) Characterisation of onion (*Allium cepa* L.) by-products as food ingredients with antioxidant and antibrowning properties. *Food Chemistry*, 108:907-916.

Paper II

Roldán-Marín E, Sánchez-Moreno C, Lloría R, de Ancos B, Cano MP (2009) Onion high-pressure processing: Flavonol content and antioxidant activity. *LWT-Food Science and Technolology*, 42:835-841.

Paper III

Roldán-Marín E, Krath BN, Poulsen M, Binderup M-L, Nielsen TH, Hansen M, Barri T, Langkilde S, Cano MP, Sánchez-Moreno C, Dragsted LO (2009) Effects of an onion by-product on bioactivity and safety markers in healthy rats. *British Journal of Nutrition*, in press (doi:10.1017/S0007114509990870)

Paper IV

Winning H, Roldán-Marín E, Dragsted LO, Viereck N, Poulsen M, Sánchez-Moreno C, Cano MP, Engelsen SB (2009) An exploratory NMR nutri-metabonomic investigation reveals dimethyl sulfone as a dietary biomarker for onion intake. *Analyst*, 134: 2344-2351.

Paper V

Roldán-Marín E, Jensen RI, Krath BN, Poulsen M, Kristensen M, Cano MP, Sánchez-Moreno C, Dragsted LO (2009) Effects of an onion by-product on plasma lipids and platelet aggregation in healthy rats. *Journal of Agricultural and Food Chemistry* (under review)

Preface

This PhD Thesis has been conducted at the Department of Plant Food Science and Technology at Instituto del Frío, Spanish National Research Council (Consejo Superior de Investigaciones Científicas-CSIC), Madrid (Spain); the Department of Toxicology and Risk Assessment, National Food Institute, Technical University of Denmark (DTU), Søborg, Denmark; and the Department of Human Nutrition, Faculty of Life Sciences, University of Copenhagen, Frederiskberg, Denmark.

During my PhD Thesis period I was granted by the Spanish Ministry of Science and Innovation with a Predoctoral Fellowship. My main workplace was Instituto del Frío-CSIC (Madrid) under the supervision of Dr. M. Pilar Cano and Dr. Concepción Sánchez-Moreno. I also spent one valuable year in Denmark in short 'winter' periods during three following years (2006-2008). During those periods I had the opportunity of working at two universities in Denmark with Professor Lars Ove Dragsted and his research team whom I am very grateful too, not only for their warmly welcome but also for their inexhaustible help, friendship and humility. There, I got the chance of learning everyday something new and discussing all the 'onion' results in a highly inspiring and nicely environment.

These studies were supported by funding from the Spanish Ministry of Science and Innovation AGL2003-09138-C04-01; 200670I08; Program Consolider-Ingenio 2010, FUN-C-FOOD CSD2007-00063; and AGL2008-04798-C02-01/ALI, and from the Danish Ministry of Food, Agriculture and Fisheries (NuBI, 3304-FVFP-060696-01; and 3304-FVFP-060706-01).

María Eduvigis Roldán Marín

Madrid, 26 October 2009

Summary

Nowadays, there is a constant and increasing social concern about the food we daily eat and particularly about vegetables. There is a great demand of minimally processed food or food ingredients with functional properties as a consequence of the current changing healthy and environmental sustainable living. Onion (*Allium cepa* L.) is an important vegetable traditionally used as a food ingredient in the Mediterranean diet that has a high production, domestic, and foreign trade worldwide. It is consumed raw, cooked or processed into different onion products in the daily diet of many subjects. This *Allium* vegetable has been proven to have interesting technological properties and beneficial health effects including antioxidant, anticarcinogenic, antimicrobial, prebiotic, hypolipidemic, and antithrombotic properties. Onion biological properties have been related with its bioactive compounds such as flavonols (quercetin and its glucosides), dietary fibre (fructans and fructooligosaccharides (FOS)), and organosulfur compounds (OSCs).

The main aim of this PhD Thesis was to study the biological activity and nutritional properties of processed onion products in order to evaluate a possible development of novel and innovative functional onion ingredients that could substitute available synthetic ingredients according to the current consumers demand and global concern towards a healthier diet and living. To achieve this objective, *in vitro* and *in vivo* studies have been carried out with onion products of three Spanish onion cultivars: *Allium cepa* L. var. *cepa*, 'Recas', *Allium cepa* L. var. *cepa*, 'Figueres', and *Allium cepa* L. var *cepa*, 'Grano de Oro'.

The first part of the PhD Thesis aimed to evaluate *in vitro* effects of food processing and preservation technologies on onion nutritional and technological properties. The first *in vitro* study analyzed 'Figueres' and 'Recas' onion by-products (juices, pastes and bagasses) stabilized by sterilization, pasteurization, and freezing technologies. Results demonstrated that processing 'Recas' onion wastes to obtain onion pastes and the subsequent stabilization with pasteurization trigger to safe onion by-products with a good bioactive compound content and excellent antibrowning properties. In parallel, a second *in vitro* study showed that processing fresh 'Grano de Oro' onion with a nonthermal technology that combines high hydrostatic pressure with low temperature

enhances flavonol (quercetin and its glucosides), total phenol extractabilities and maintains the antioxidant activity compared with the unprocessed onion.

The second key topic of the investigation was the *in vivo* evaluation of the biological activity and nutritional properties of 'Recas' onion by-products. For that purpose, we studied the pasteurized 'Recas' onion paste chosen in the previous *in vitro* study, and its two derived onion fractions, an onion extract (rich in fructans and FOS) and an onion residue. The biological responses shown in healthy rats fed 'Recas' onion by-products revealed that these by-products are not genotoxic and that they exert interesting antioxidant and prebiotic properties. Moreover, they showed antithrombotic effects exerting a potential usefulness of these by-products in a cardiovascular disease (CVD) preventive diet. Onion OSCs and FOS seem to be the bioactive compounds responsible of some of the effects shown. Furthermore, the dimethyl sulfone was identified as a dietary biomarker for onion intake in a nutri-metabonomic study. Being able to detect and quantify specific onion intake biomarkers is highly beneficial in control of nutritionally enhanced functional foods and in human intervention studies.

To go deep inside the biological activity evaluation of processed onion products, a human intervention study with overweighed subjects was also conducted using a 'Recas' onion product which was added to two four precooked dishes as a food ingredient. Currently, the main health effects are being evaluated. In the future, *in vitro* and *in vivo* studies would be required including different onion products processed by high hydrostatic pressure or other nonthermal technologies in order to study in depth the potential improvement of onion bioactive compounds bioaccessibility that could promote preventive or protective effects on CVD and obesity, two of the more prevalent diseases nowadays.

In conclusion, the current PhD Thesis shows that pasteurizating onion pastes obtained from onion wastes and high-pressure processing fresh onion are two valuable processing food technologies for the food industry nowadays. These processed onion products studied have good technological and nutritional quality as added value. Therefore, there is a new challenge for the food onion industry using processed onion products as novel and innovative ingredients for the potential functional food design and development.

Resumen

Actualmente, existe una creciente y constante preocupación social por los alimentos que ingerimos diariamente y en particular por la ingesta de frutas y hortalizas. Como consecuencia del actual cambio de estilo de vida hacia otro más saludable y a la vez sostenible con el medio ambiente, existe una gran demanda de alimentos mínimamente procesados o ingredientes alimentarios con propiedades funcionales. La cebolla (*Allium cepa* L.) es un importante producto vegetal utilizado clásicamente como ingrediente alimentario dentro del marco de la dieta Mediterránea, con una elevada producción y comercialización a nivel mundial. Este vegetal del género *Allium* se caracteriza por sus interesantes propiedades tecnológicas así como por sus efectos beneficiosos para la salud incluyendo sus propiedades antioxidantes, anticancerígenas, antimicrobianas, prebióticas, hipolipidémicas y antitrombóticas. Las propiedades biológicas de la cebolla han sido relacionadas con sus compuestos bioactivos como los flavonoles (quercetina y sus glucósidos), fibra dietética (fructanos y fructooligosacáridos) y compuestos organosulfurados.

El principal objetivo de esta Tesis Doctoral fue el estudio de la actividad biológica y propiedades nutricionales de productos de cebolla procesados para evaluar un posible desarrollo de nuevos e innovadores ingredientes funcionales de cebolla que puedan sustituir a los ingredientes sintéticos disponibles, de acuerdo con la actual demanda de los consumidores y conciencia global hacia una dieta y un estilo de vida más saludable. Para ello, se llevaron a cabo estudios *in vitro* e *in vivo* realizados con diferentes cultivares de cebolla españoles: *Allium cepa* L. var. *cepa*, 'Recas', *Allium cepa* L. var. *cepa*, 'Figueres', y *Allium cepa* L. var *cepa*, 'Grano de Oro'.

El objetivo de la primera parte de la Tesis Doctoral fue la evaluación *in vitro* de los efectos de tecnologías de procesado y conservación en las propiedades tecnológicas y nutricionales de la cebolla. El primer estudio *in vitro* consistió en el análisis de los subproductos de cebolla 'Figueres' y 'Recas' (zumos, pastas y bagazos) estabilizados mediante tecnologías de esterilización, pasteurización y congelación. Los resultados demostraron que el procesado de excedentes y residuos de cebolla 'Recas' para la obtención de pastas y la siguiente estabilización de éstas mediante la tecnología térmica de pasteurización da lugar a subproductos de cebolla seguros con un buen contenido en compuestos bioactivos y excelentes propiedades de antipardeamiento enzimático.

Paralelamente, el segundo estudio *in vitro* realizado mostró que el procesado de cebolla fresca 'Grano de Oro' con una tecnología no térmica que combina la alta presión hidrostática con bajas temperaturas incrementa la extractabilidad de flavonoles (quercetina y sus glucósidos) y fenoles totales, además de mantener la actividad antioxidante respecto a la cebolla no procesada.

El segundo aspecto de la investigación desarrollada fue la evaluación *in vivo* de la actividad biológica y propiedades nutricionales de subproductos de cebolla 'Recas'. Para llevar a cabo este propóstito se estudió la pasta pasteurizada 'Recas' que fue seleccionada en el estudio *in vitro* previo y dos fracciones de cebolla derivadas de ella: un extracto de cebolla rico en fructanos y fructooligosacáridos y un residuo. Las respuestas biológicas que mostraron las ratas sanas alimentadas con los subproductos de cebolla 'Recas' revelaron que estos subproductos no son genotóxicos y tienen interesantes propiedades antioxidantes y prebióticas *in vivo*. Además, los efectos antitrombóticos que mostraron las ratas alimentadas con los subproductos dan un valor positivo a estos productos de cebolla para su potencial utilización en una dieta preventiva de enfermedades cardiovasculares. Los compuestos organosulfurados y los fructooligosacáridos parecen ser los compuestos bioactivos responsables de algunos de los efectos mostrados. Además, la dimetil sulfona fue identificada como un biomarcador dietético para la ingesta de cebolla en un estudio nutri-metabonómico. La capacidad de detectar y cuantificar biomarcadores específicos de la ingesta de cebolla es altamente beneficioso para el control de alimentos funcionales suplementados nutricionalmente y en estudios de intervención en humanos.

Como continuación a la evaluación de la actividad biológica de productos de cebolla procesados, se llevó a cabo un estudio de intervención en humanos con sujetos con sobrepeso con un producto de cebolla 'Recas' que se incorporó a cuatro platos precocinados como ingrediente alimentario. En la actualidad están siendo evaluados los principales efectos en salud. En el futuro, se hace necesaria la ejecución de diversos estudios *in vitro* e *in vivo,* en los que se haga una valoración de diferentes productos de cebolla procesados mediante alta presión hidróstática u otras tecnologías no térmicas para el estudio en profundidad de la mejora en la bioaccesibilidad de compuestos bioactivos de cebolla que potencien los efectos preventivos o protectores de enfermedades cardiovasculares y obesidad, dos de las patologías con mayor prevalencia en la actualidad.

En conclusión, la presente Tesis Doctoral muestra que la pasteurización de pastas de cebolla obtenidas de excedentes y residuos de cebolla y el procesado con alta presión hidrostática de cebolla fresca son dos tecnologías de procesado que pueden ser valoradas en la industria alimentaria actual. Estos productos de cebolla procesados presentan una buena calidad tecnológica y nutricional como valor añadido. Por tanto, existe un elevado potencial en el uso de estos productos como nuevos e innovadores ingredientes en el desarrollo y diseño de alimentos funcionales.

Abbreviations

AA	Arachidonic acid
ACSO	Alk(en)yl cysteine sulphoxide
AE	Antiradical efficiency
Alas1	5-Aminolevulinate synthase 1 *gene*
ALAT	Alanine aminotransferase
AlP	Alkaline phosphatase
BGL	ß-glucosidase
CAT	Catalase
COX	Cyclooxygenase
CV	Cross-validation
CVD	Cardiovascular disesase
CYP7A1	Cholesterol 7-alfa-hydroxylase
CYPs	Cytochromes P450
DMSO	Dimethyl sulfoxide
$DPPH^{\bullet}$	2, 2-diphenyl-1-picrylhydrazyl radical
dw	Dry weight
EC_{50}	Half maximal effective concentration
FOS	Fructooligosaccharides
Gclc	γ-glutamate-cysteine ligase catalytic subunit *gene*
GGT	Gamma glutamyl transferase
GPx1	Glutathione peroxidase 1
GR	Glutathione reductase
GSH	Glutathione
GUS	ß-glucuronidase
^{1}H NMR	^{1}H Nuclear magnetic resonance
H_2S	Hydrogen sulphide
Hb	Haemoglobin
HDL	High density lipoprotein
HHP	High hydrostatic pressure
Hmgcr	3-hidroxy-3-methylglutaryl-coenzyme A reductase *gene*
HP	High-pressure
HPLC	High performance liquid chromatography

HPP	High-pressure processing
HSQC	Heteronuclear single quantum coherence
iECVA	Interval extended canonical variates
iPLS	Interval partial least squares regression
LDL	Low density lipoprotein
LV	Latent variable
OSCs	Organosulfur compounds
OTM	Olive tail moment - Comet assay parameter
Nqo1	NAD(P)H:quinone oxidoreductase *gene*
PPO	Polyphenol oxidase
PCA	Principal component analysis
Q_{DG}	Quercetin-3,4'-diglucoside
Q_{MG}	Quercetin-4'-glucoside
RBC	Red blood cells
RMSE	Root mean square error
RSM	Response surface methodology
SCFA	Short chain fatty acids
SD	Standard deviation
SMSCS	S-methyl cysteine sulfoxide
SREBP	Sterol response element-binding protein
TAG	Triacylglycerides
TC	Total cholesterol
T_{EC50}	Time needed to reach the steady state to EC_{50}
TEM	Tail extent moment - Comet assay parameter
TL	Tail length - Comet assay parameter
TOCSY	Total correlation spectroscopy
TP receptor	Thromboxane-prostanoid receptor
TP	Total phenols
TQ	Total quercetin
Txas	Thromboxane A_2 synthase *gene*
VLDL	Very low density lipoprotein
WBC	White blood cells
% tail DNA	Percentage of tail DNA - Comet assay parameter

XIV

Chapter 1

General introduction

Chapter 1. General introduction

The Mediterranean diet which traditionally includes generous amounts of fruits and vegetables has been associated with health benefits such as lower risks for cardiovascular disease (CVD) and cancer. There is epidemiologic evidence that frequent consumption of *Allium* vegetables such as garlic, scallions, onions, chives, and leeks is protective against cancer. Therefore, *Allium* vegetables should be considered to be an important component of a healthy, cancer and CVD resistant diet.

Nowadays, there is a constant and increasing social concern about the food we daily eat and particularly about vegetables. Moreover, there is a worldwide great demand of minimally processed food or food ingredients with functional properties as a consequence of the changing healthy and environmental sustainable living that is becoming to spanning almost the totality of the world's cultures.

Therefore, we considered that it is needed to study in depth not only the technological but also the biological and nutritional properties of vegetables products in order to better understand their role in our daily diet and lifestyle. In the present PhD Thesis onion was chosen as a model of an *Allium* vegetable due to its good technological and nutritional properties reported and to its presence in the daily diet worldwide.

1. Onion

1.1. Onion production

Onions (*Allium cepa* L.) are bulbous vegetables from the *Liliaceae* family, important in terms of domestic consumption and export (Figure 1). Onions are grown mainly as food materials. They are highly valued for their flavour and for their nutritional value. Onion bulb which may be red, white or yellow in colour, is consumed in its tender state, raw, ripe, pickled or in form of powder. The bulbs are boiled and used in soups and stews, fried or eaten raw. They are also preserved in the form of pickles. Onion leaves are also used in salads and soups (FAO, 2009).

According to the Food and Agriculture Organization (FAO) of the United Nations, onions are grown in at least 175 countries. Of those countries, two of the leading producers are China, which harvested 2.2 million acres of onions in 2005, and India, which harvested 1.3 million acres (Onions, Vegetables, NASS, USDA, April 2008).

In Spain the provisional onion cultivated area and production for the year 2007 is 22. 4 and 1190.3 miles of onion cultivated hectares and tons of onion, respectively. In the

year 2006, among the more important Spanish onion varieties, onion ‘grano o valenciana’ was the onion ranking the higher cultivated area and production (10.9 and 666.3) followed by other varieties of onions (5.7 and 233.7), onion ‘babosa’ (3.1 and 138.2) and onion ‘medio grano o Liria’ (1.4 and 61.3 miles of onion cultivated hectares and tons of onion) (MAPA, 2008).

Figure 1. *Allium cepa* L. var. *cepa*

1.2. Onion processing

Growing onions is expensive, requires a lot of water, and like most agriculture, is easily disrupted by extreme weather. Most commercial operations are large-scale, integrated production-processing-packing systems having ample irrigation and processing water, as well as specialized processing and storage equipment.

Harvesting onions may be difficult. Some varieties can easily be mechanically handled, while others suffer dramatically from inappropriate mechanical treatment. Standard practices in onion harvesting include undercutting the onions and allowing them to cure (air dry) for two to three days, clipping the tops and roots, bagging the onions in burlap sacks, transporting them to a warehouse, drying, grading, bagging or boxing and shipping.

Onions also need an appropriate "curing period" where the neck opening closes. Inadequate curing will lead to onion rot and loss of the bulbs; prolonging curing can lower bulb quality.

Additional processing may include washing, peeling, coring and cutting for special packaged products or ingredients for the prepared foods industry.

1.2.1. Onion by-products

More than 450000 tons of onion wastes are produced annually in Europe, mainly in the United Kingdom, Holland, and Spain. There is considerable industrial pressure to come up with a way to convert the waste into useful products. This attempt would reduce the environmental impact of onion waste disposal by converting waste streams into useful products resulting in low-waste food production (Waldron, 1999; Waldron, 2001).

By-products are promising sources of compounds which may be used because of their favorable technological or nutritional properties (Schieber *et al*., 2001).

Onion by-products obtained from onion food processing or from the surplus of onion not commercially available represent a major disposal problem for the industry concerned. Jaime *et al*. (2001) reported that the onion tissues richest in fructans were the fleshy layers, so that the outer two fleshy layers turn out to be the best onion by-product as a possible fructan source.

Processing and stabilizing onion wastes (residues and surpluses of onion) could represent both advantages: a solution for the environmental problem derived from the great onion wastes disposal and the obtaining of stabilized onion by-products as natural antioxidant food ingredients (Roldán *et al*., 2008). Onions wastes can be processed by grinding and pressing (Figures 2-4) to obtain several onion by-products such as onion juices, pastes, and bagasses. In order to obtain safe onion products, these onion by-products can be stabilized by different preservation technologies such as freezing (Figure 5), pasteurization, or sterilization (Figure 6).

Moreover, new vinegar production and fertilizer (compost) from worthless onions has also been investigated. Different fermentation systems have been used to obtain onion vinegar from onion juices and onion bioethanol. Onion vinegar could be also a new valuable product from onion wastes and by-products (Horiuchi *et al*., 2004; Gonzalez-Saiz *et al*., 2008).

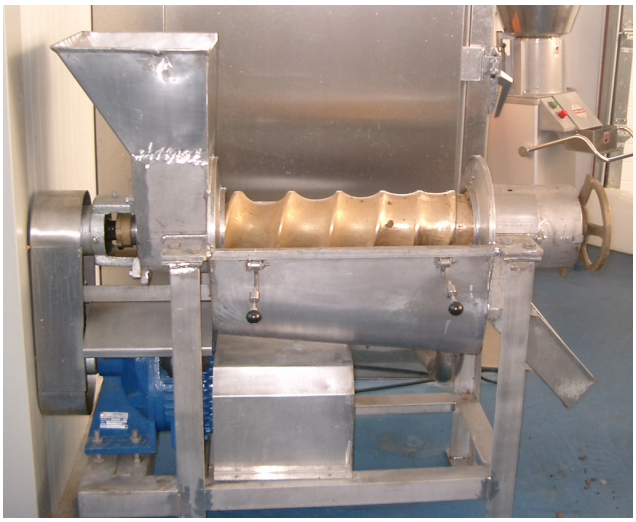


Figure 2
Pressing machine of "tornillo"

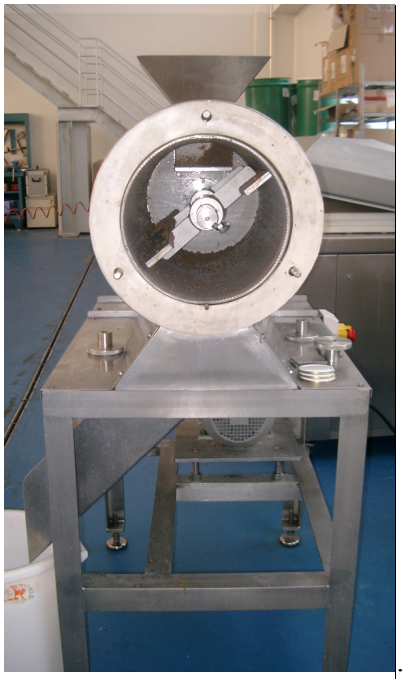


Figure 3
Sieve (front)

Figure 4
Sieve (lateral)

Figure 5
Cryogenic cupboard

Figure 6
Steam pressure sterilizer (autoclave)

Pictures from the National Center for Technology and Food Safety (CNTA). San Adrián (Navarra-Spain)

The exploitation of by-products of onion processing as a source of functional compounds and their application in food is a promising field which requires interdisciplinary research of food technologists, food chemists, nutritionists and toxicologists. Active participation of the food and allied industries with respect to sustainable production and waste management is required.

1.2.2. High-pressure processing

High-pressure processing (HPP) is an industrially tested technology that offers a natural alternative for the processing of a wide range of different food products.

It is a technology that can achieve the food safety of heat pasteurization whilst meeting consumer demand for fresher-tasting minimally processed foods. Application of high-pressure (HP) can inactivate microorganisms and enzymes and modify structures whilst having little or no effects on nutritional and sensory quality aspects of foods. The key advantages of HP applications to food systems are the independence of size and geometry of the sample during processing, possibilities for low temperature treatment, and the availability of a waste-free environmentally friendly technology (Norton & Sun, 2008).

Variations of the process depend on specific products, temperature and pressure transmitting fluid (gas or water). The high hydrostatic pressure process is quite simple.

As its name suggests, the food (liquid or solid) is subjected to pressures above 100 MPa up to 900 MPa, with pressures used in commercial systems between 400 and 700 MPa. The pressurization is carried out for the duration of the treatment in a confined space (pressure vessel) containing a fluid (usually water) that acts as the pressure transmitting medium. Pressure is applied isostatically that is equally applied in all directions, which allows solid foods to retain their original shape. The pressure is held for the desired treatment time and then released. The applied pressure and the holding time will depend on the type of product treated and the expected final result (San Martín *et al*., 2002).

1.3. Onion technological properties

From a technological point of view onion has interesting properties. Among them, onions have been reported to have antibrowning, antioxidant, and antimicrobial properties. These onion technological properties represent an added value to onion that would give a new challenge for the food industry nowadays.

1.3.1. Onion antibrowning properties

The browning reaction is a widespread phenomenon in fruits and vegetables, resulting from mechanical or physiological injury during post-harvest storage or processing. It is a major factor contributing to quality loss in foods and beverages. The polyphenol oxidase (PPO) enzyme mainly caused the enzymatic oxidation of endogenous phenols into quinones, which then polymerize into brown products. Thus, polyphenolic compounds and PPO are directly responsible for the enzymatic browning.

The use of sulfiting agents is the most widespread chemical approach for controlling browning. However, consumer awareness of the risks associated with sulfites and increased regulatory scrutiny have created the need for substitutes (Iyengar & McEvily, 1992). Among the compounds that have been shown to inhibit the PPO activity are sulfites, ascorbic acid and its derivatives, and thiol compounds such as cysteine (Martínez & Whitaker, 1995; Negishi & Ozawa, 2000; Ding *et al*., 2002; Jang *et al*., 2002) (Figure 7).

Onion has been reported to inhibit PPO of fruits such as pear or banana. Kim *et al*. (2005) reported that thiol compounds in onion might be the active components responsible for the inhibitory effect of onion extract. When the onion extract was dialyzed, the inhibitory effect against pear PPO was completely eliminated, suggesting that the low molecular compounds were responsible of the pear PPO inhibitory effects.

Moreover, when onion extracts were heated the pear PPO inhibition was more efficient. Lee (2007) also found that heat treated onion extracts inhibited banana PPO. Furthermore, it was shown that Maillard reaction products (MRP) significantly inhibited banana PPO as well as the addition of various antibrowning agents.

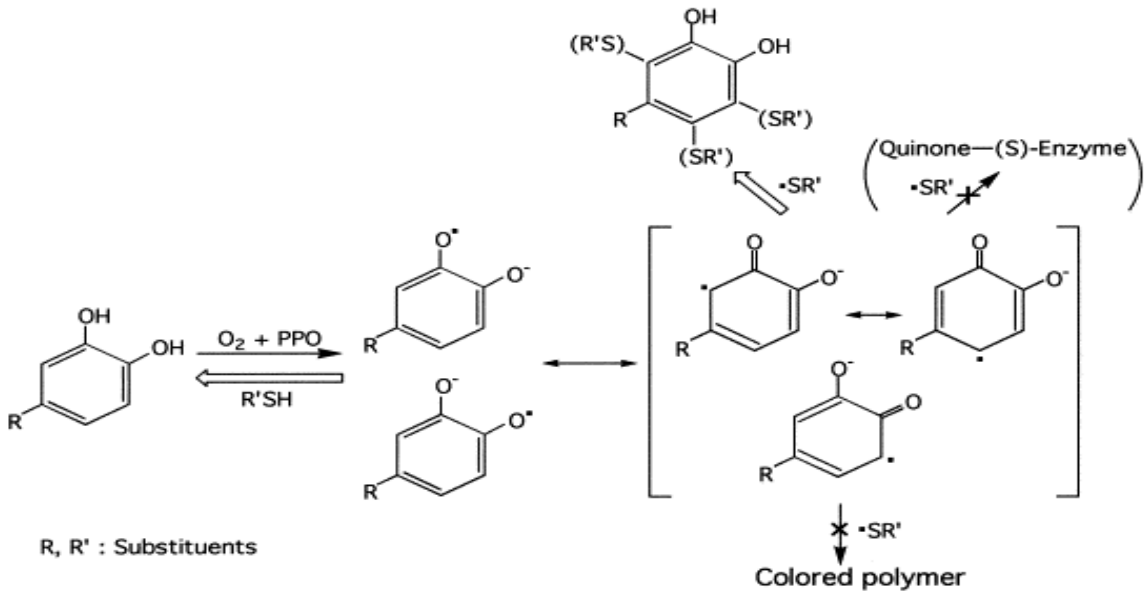


Figure 7. Mechanism of inhibition of enzymatic browning by thiol compounds and protection of active sites of sulfhydryl enzymes (Negishi & Ozawa, 2000)

1.3.2. Onion antioxidant properties

Onion antioxidant properties together with antibrowning and antimicrobial properties give an added value to this vegetable that would represent a new challenge for the food industry for the development of onion products that would preserve different food items of being oxidized.

From a nutritional point of view, it has been shown that *Allium* species may help to prevent tumor promotion, cardiovascular diseases and aging; all processes that are associated with free radicals (Stajner *et al*., 2006).

In terms of onion *in vitro* antioxidant activity, the assessment of the antioxidant activities and comparison with previously reported findings show that the antioxidant activity of 5 portions of onion is equivalent to 1 glass (150 mL) red wine, 12 glasses white wine, 2 cups of tea, 4 apples, 5.5 portions egg plant, 3.5 glasses of blackcurrant juice, 3.5 (500 mL) glasses of beer, 7 glasses of orange juice, and 20 glasses of apple juice (long life) (Paganga *et al*., 1999).

Onions had clearly higher radical scavenging activities than garlic, red onion being more active than yellow onion. Quercetin and its derivates together with the anthocyans, have been widely reported to have antioxidant properties. Flavonoid compounds and particularly those having o-dihydroxy substituent in the B-ring were shown to be effective antioxidants (Ly *et al*., 2005). It was demonstrated that the skin extracts of onion possessed the highest antioxidant activities. Levels of quercetin and $DPPH^{\bullet}$ radical scavenging activity increase from onion core to skin (Nutila *et al*., 2002; Nutila *et al*. 2003; Ly *et al*., 2005; Kim, & Kim, 2006). Onion phenolic and sulfur compounds are among the onion bioactive compounds involved in the assessment of onion antioxidant properties (Benkeblia, 2005).

Moreover, onion *in vitro* antioxidant activity depends not only on the onion varieties or cultivars (Yang *et al.*, 2004, Santas *et al*., 2008) but also on the processing or on the different heat treatment applied to onions (Yang, 2004; Moreno *et al*., 2006; Woo *et al*., 2007).

In terms of onion *in vivo* antioxidant activity, different studies carried out in rats or in humans assessed that onion has positive health effects acting as an antioxidant vegetable helpful in the prevention of some nowadays prevalent diseases such as CVD. Early results by Helen *et al*. (2000) indicate that onion oil is an effective antioxidant against the oxidative damage caused by nicotine in rats as compared to vitamin E. Later on, several onion fed rat studies corroborated those onion antioxidant effects (Azuma *et al*., 2007; Park *et al.* 2007; Slobodianik *et al*., 2007). The same antioxidants effects have been shown in human studies including onion in the diet (McAnlis *et al*., 1999; Boyle *et al*., 2000).

1.3.3. Onion antimicrobial properties

Allium vegetables have long been known for their antimicrobial activity against various microorganisms, including Gram-positive and Gram-negative bacteria (Zohri *et al*., 1995) (*Staphilococcus aureus* and *Salmonella enteriditis)* and fungi *(Aspergillus niger*, *Penicillium cyclopium* and *Fusarium oxysporum*) (Benkeblia & Varoquaux, 2003). There have been few applications of *Allium* vegetables as natural food preservatives, in spite of numerous studies on antimicrobial activity of these vegetables. Relative instability of the antimicrobial compounds and the strong odor of their mother plants seem to limit the use of them as practical food preservatives (Kyung & Lee, 2001). A recent study showed that the addition of garlic and onion powders in enhanced

meats had an antioxidant activity as effective as that of sodium ascorbate and also an antimicrobial effect to inhibit the growth of total bacteria and bacteria from the *Enterobacteriaceae* family (Park *et al*., 2008).

Different onion extracts and oils have proven antimicrobial and antioxidant properties which are interesting from a technological and nutritional point of view (Irkin & Korukluoglu, 2007; Choi *et al*., 2008). Organosulfur compounds (OSCs) and phenolic compounds have been reported to be involved in the onion antimicrobial activity (Takahama & Hirota, 2000; Griffiths *et al*., 2002; Kim *et al*., 2004a; Kim *et al*., 2004b). Other compounds such as the peptide Allicepin have antifungal properties and have been isolated from onion bulbs (Wang & Ng, 2004).

2. Onion biological and nutritional properties

2.1. Onion nutritional content

Onion (*Allium cepa* L.) has an approximately 90% content of water. Onion is low in calories and have a moderately high content of dietary fibre and sugars (Table 1). In terms of vitamins and minerals content (Tables 2 and 3) onion has low sodium content and a high content of vitamin B_6, folic acid, calcium, magnesium, phosphorus and potassium. By contrast, onion has low content of lipids (Table 4) and among the amino acid content only arginine and glutamic acid are remarkable (Table 5). Raw onion nutritional content is shown in Tables 1 to 5 (Annex I).

2.2. Onion bioactive compounds

Onion added into different food products as a food ingredient made these products richer in bioactive compounds such as flavonoids, dietary fibre, and organosulfur compounds (OSCs). These onion bioactive compounds have potential beneficial health effects. Moreover, some of the onion bioactive compounds are also interesting from a technological point of view due to their properties as preservatives agents, OSCs due to their antioxidant and antibrowning properties or dietary fibre due to possibly texture enhancer properties.

2.2.1. Flavonoids. Quercetin and quercetin glucosides

Onion is among the richest source of dietary flavonoids and contributes to a large extent to the overall intake of flavonoids. Two flavonoid subgroups are found in onion, the anthocyanins, which impart a red/purple colour to some varieties and the flavonols such as quercetin (Figure 8) and its derivatives responsible for the yellow and brown skins of many other varieties (Griffiths *et al*., 2002). Flavonols are the predominant pigments of onions. Only compounds belonging to the flavonols, the anthocyanins, and the dihydroflavonols have been reported to occur in onion bulbs.

At least 25 different flavonols have been characterized, and quercetin derivatives are the most important ones in all onion cultivars. Their glycosyl moieties are almost exclusively glucose, which is mainly attached to the 4', 3, and/or 7-positions of the aglycones. Analogous derivatives of kaempferol and isorhamnetin have been identified as minor onion pigments (Slimestad *et al*., 2007).

Quercetin 3,4'-diglucoside (Q_{DG}) and quercetin 4'-glucoside (Q_{MG}) are in most cases reported as the main onion flavonols in recent literature (Caridi *et al*., 2007). These glucosides of quercetin represent about the 90% of the overall contents in different *Allium* species (Bonacorsi *et al*., 2008). Significant differences in the levels and ratios of the two compounds were seen between red (‘Redwing’; Q_{DG} 191 mg/100 g dry weight-dw and Q_{MG} 85 mg/100 g dw), brown (‘Cream Gold’, Q_{DG} 153 mg/100 g dw, Q_{MG} 58 mg/100 g dw), and white onion varieties (‘Spanish white’; $Q_{DG} < 1$ mg/100 g dw, $Q_{MG} < 1$ mg/100 g dw) (Caridi *et al*., 2007).

Distribution of quercetin and its glycosides within the onion bulb changes during onion processing, different `cooking´ methods, and exposure to fluorescent light (Ewald *et al*., 1999; Mogren *et al*., 2007; Nemeth & Piskula, 2007; Lee *et al.*, 2008; Mogren *et al*., 2008). After ingestion quercetin and its glycosides undergoes extensive metabolism and microbial action resulting in its altered or degraded structure; therefore, most of the effects shown in *in vitro* experiments with the pure compound cannot be directly extrapolated to *in vivo* systems (Nemeth & Piskula, 2007).

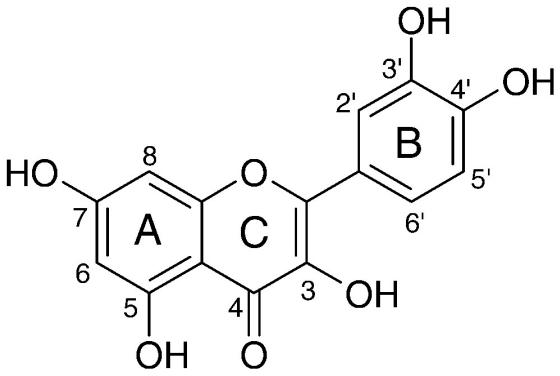


Figure 8. Quercetin chemical structure

Absorption and metabolism of the flavonol quercetin and its glycosides have been described by Aherne & O'Brien (2002). Quercetin glycosides, the mostly present form in onions, are converted to the respective aglycones in the large intestine by the glycosidase activity of intestinal bacteria and absorbed. Several studies have demonstrated that quercetin glycosides are absorbed more efficiently than quercetin aglycone (Hollman *et al*., 1995; Moon *et al*., 2000) irrespective of the position of their glucose moiety (Olthof *et al*., 1998). After being absorbed, quercetin is metabolized an excreted (Day *et al*., 2001). Mullen *et al.* (2006) found five metabolites in quantifiable amounts in human plasma after onion ingestion (quercetin-3-glucuronide, quercetin-3'-sulfate, isorhamnetin-3-glucuronide, a quercetin diglucuronide and a quercetin glucuronide sulfate). They also reported that total urinary excretion of quercetin metabolites was 12.9 mmol, corresponding to 4.7% of intake (Figure 9).

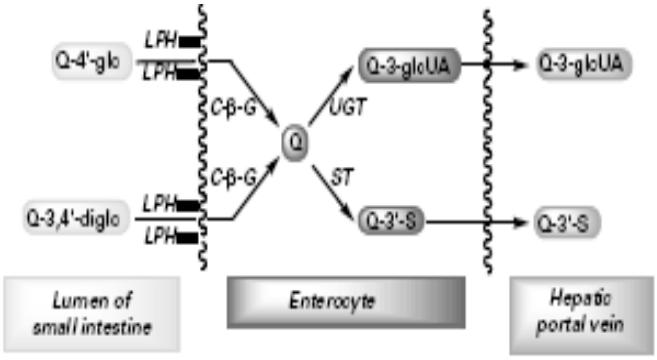


Figure 9. Absorption, excretion and metabolite profiling of quercetin conjugates. Schematic of the proposed metabolic fate of quercetin-4'-glucoside and quercetin-3,4'-diglucoside as they pass from the lumen of the small intestine into the hepatic portal vein. C-β-G, cytosolic β-glucosidase; diglc, diglucoside; glc, glucoside; glcUA, glucuronide; Q, quercetin; LPH, lactase phlorizen hydrolase; S, sulfate; ST, sulphotransferase; UGT, glucuronyltransferase (Mullen *et al*., 2006)

In general, quercetin has been investigated for their widespread health benefits which have generally been ascribed to its combination of antioxidant and antiinflammatory activities (Davis *et al*., 2009). Quercetin beneficial health effects include protection against various diseases such as osteoporosis, certain forms of cancer, pulmonary, and CVD but also against aging. Especially the ability of quercetin to scavenge highly reactive species such as peroxynitrite and the hydroxyl radical is suggested to be involved in these possible beneficial health effects (Boots *et al*., 2008).

The flavonol quercetin has antiproliferative effects in many cancer cell lines. Antioxidant or pro-oxidant activities and kinase inhibition have been proposed as molecular mechanisms for these effects. In addition, an estrogenic activity has been observed. Findings by Galluzo *et al.* (2009) suggest that quercetin results in HeLa cell death through a transfected estrogen receptor-dependent mechanism involving caspase and p38 kinase activation. These findings indicate new potential chemopreventive actions of flavonoids on cancer growth.

2.2.2. Organosulfur compounds (OSCs)

Allium plants contain high concentrations of alk(en)yl cysteine sulfoxides (ACSOs). Among them, onions, shallots, and leeks contain the methyl, propyl, and prop-1-enyl cysteine sulfoxides.

When the onion tissue is disrupted by cutting, crushing or chewing, the ACSOs are enzymatically degraded by the enzyme alliinase to iminopropionic acid and alk(en)yl cysteine sulfenic acids.

The iminopropionic acid spontaneously hydrolyses to form ammonia and pyruvic acid. The sulfenic acids decompose spontaneously. Methyl and propyl sulfenic acids yield mainly thiosulfinates, while prop-1-enyl sulfenic acid forms both, the corresponding thiosulfinate and thiopropanal S-oxide, the onion lachrymatory factor (Figure 10) (Block *et al*., 1993).

Onion pungency is caused by a range of sulfur compounds that cause a pungent, burning sensation in the back of the mouth and the throat. Others produce the milder, more pleasant and typical onion flavour. All these flavours are produced when the onion is cut and alliinase is released and instantly breaks down the flavour precursors previously described. A convenient method to measure onion pungency is to analyze pyruvic acid, which is formed in stoichiometric amount to the thiosulfinates. Pyruvic acid has been shown to correlate well with flavour perception. The balance between

level of pungency and level of sugars determines the perception of sweetness in an onion. High pungency can mask a high level of sugars so that the onion is not perceived as sweet. Also, onions with low pungency and low sugar content can be perceived as bland. Ideally, a sweet onion would have a high level of sugars and low pungency (Vagen & Slimestad, 2008).

Thiosulfinates themselves are unstable, particularly on heating, and break down to a complex mixture of compounds, in which mono-, di-, tri- and tetra-sulfides predominate (Munday & Munday, 2004; Rose *et al*., 2005) (Figure 11).

Dipropyl disulfide, dipropyl trisulfide, and propenil disulfides are the major constituents of onion volatiles, although many others compounds have been identified, including dipropyl sulfide and dipropenyl sulfide (Munday & Munday, 2001).

Defining the mechanism by which organosulfur compounds (OSCs) derived from *Allium* vegetables inhibit cancer cell growth has been the topic of intense research in the last two decades. Some *Allium* vegetable constituents have also entered clinical trials to assess their safety and anticancer efficacy (Powolny & Singh, 2008). OSCs have been shown to exert diverse biological effects such as: induction of carcinogen detoxification, inhibition of tumor cell proliferation, antimicrobial effect, free radical scavenging, inhibition of DNA adduct formation, induction of cell cycle arrest, and induction of apoptosis. It has been suggested that these compounds act as chemopreventive agents through a combination of above mechanisms (Moriarty *et al*., 2007).

It has also been proposed that the chemoprotective effect of onion sulfides is due, at least in part, to the ability of these sulfides to increase tissue activities of phase II detoxification enzymes (Guyonnet, 2001; Teyssier *et al*., 2001,). These enzymes, which include glutathione S-transferase (GST), epoxide hydrolase (EH), quinone reductase (QR, DT-diaphorase, NAD[P]H:quinone-acceptor oxidoreductase) and UDP-glucuronosyl transferase (UDPGT), inactivate many electrophilic substances, including certain carcinogens, and facilitate their elimination from the body.

Taking into account the relationship between structure and enzyme inducing ability by OSCs it can be drawn the importance of unsaturation in the alkyl chain, showing prop-1-enyl derivatives a higher level of induction than the propyl in many tissues. The importance of the number of sulfur atoms in the molecule was also important; the general rule could be that the higher activity is found with increasing number of sulfur atoms, although the direction of the effect was different in the prop-1-enyl sulfide which was stronger inducer than the disulfide (Munday & Munday, 2004).

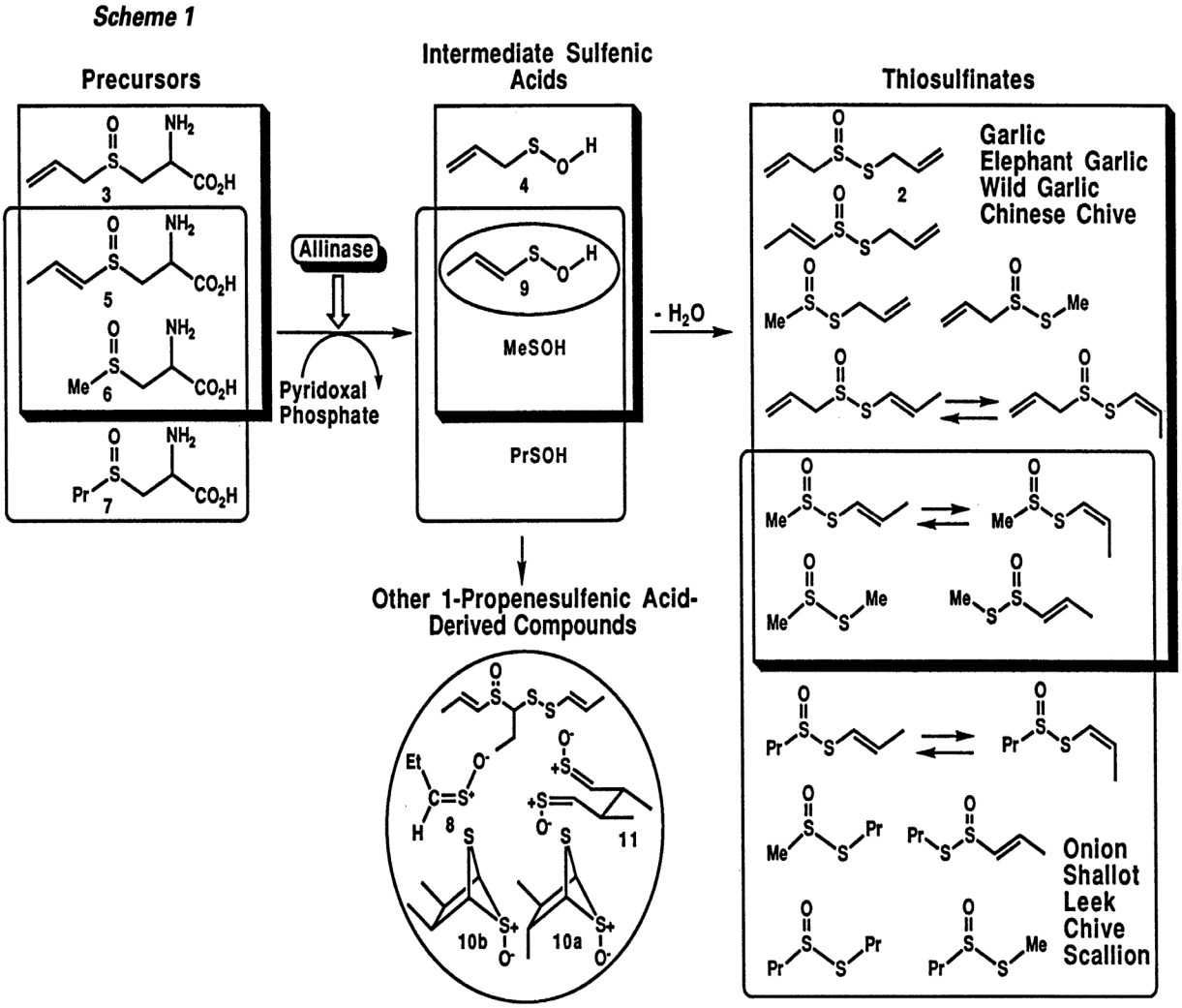


Figure 10. Biosynthetic pathway of thiosulfinates (Block *et al.*, 1993)

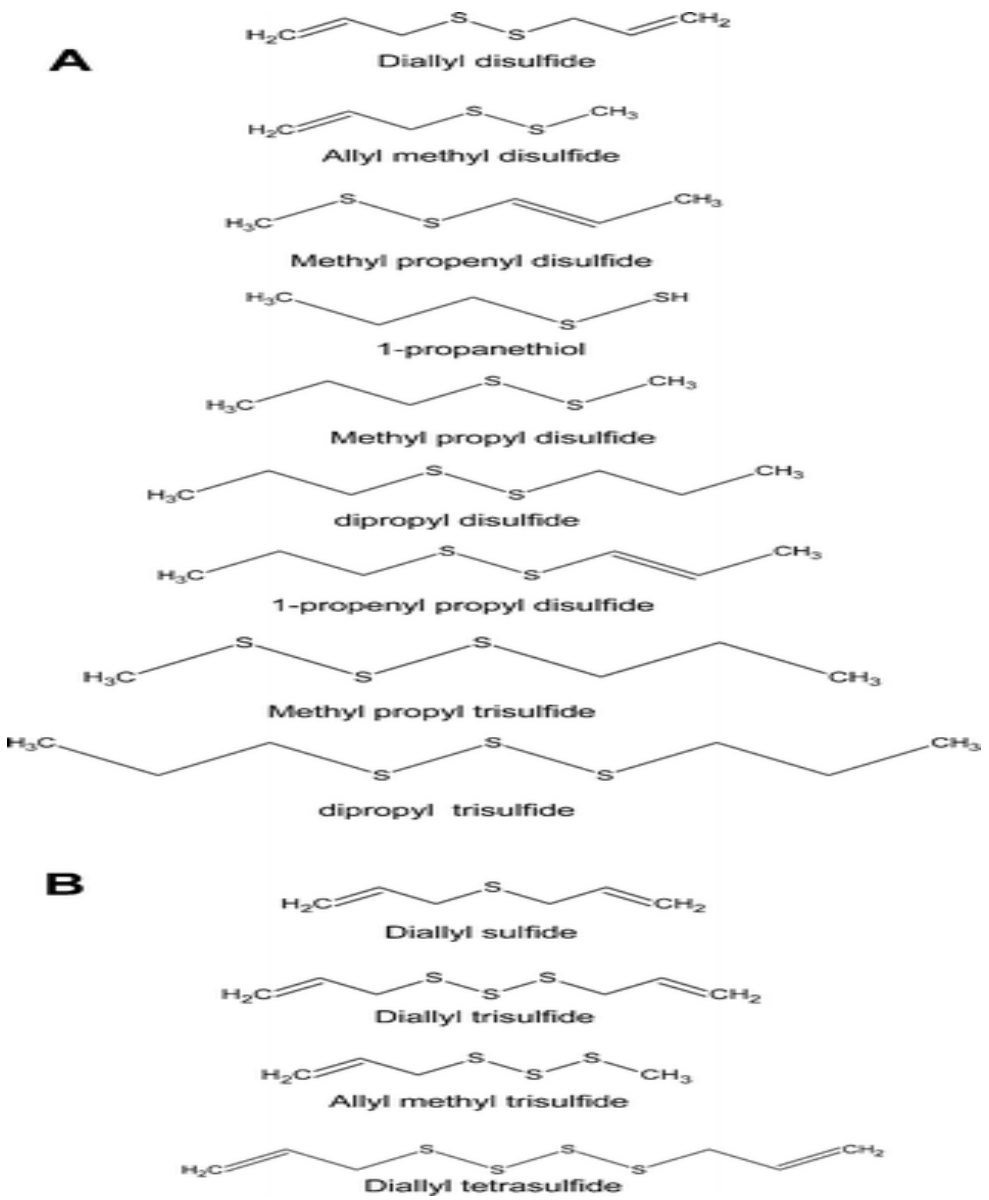


Figure 11. Sulfides identified in the headspace volatiles of crushed *Allium* tissues: (A) onion volatiles, and (B) garlic volatiles (Rose *et al*., 2005)

2.2.3. Fructans and fructooligosaccharides (FOS)

Bulb dry matter content is an important quality parameter of onion, also important in the onion dehydration industry because it directly relates to the energy needed for drying. About 65 to 80% of bulb dry matter consists of non-structural carbohydrates (Kahane *et al*., 2001; Benkeblia *et al*., 2005).

The onion predominant non-structural carbohydrates are glucose, fructose, sucrose and low-molecular-weight fructans, while starch and raffinose are absent. Fructans, also

known as fructooligosaccharides (FOS), are polyfructosyl sucroses of varying molecular size that constitute the main carbohydrate reserve of onion. Fructans accumulate during bulbing and are then catabolized during regrowth and sprout development of the bulbs (Benkeblia *et al*., 2005).

It is generally accepted that FOS is a common name only for fructose oligomers that are mainly composed of kestose (GF_2), nystose (GF_3) and fructofuranosylnystose (GF_4), in which fructosyl units (F) are bound by ß-linkage at the position of sucrose (glucose+fructose-GF) respectively. There is a clear predominance of kestose (GF_2) in every onion tissue and no occurrence of highly polymerized fructans. The tissues richest in fructans are the fleshy layers, so that the outer two fleshy layers turn out to be the best onion by-product as a possible fructan source (Jaime *et al*., 2001; Jaime *et al*., 2002).

The fructan degree of polymerization level in onion is mostly in between 3 and 15. Short chain fructans, with a degree of polymerization less than 5, are potentially used as natural low-calorie sweeteners. Onion bulbs with fructans of a high DP may be used for lipid replacement with consequential health benefits (Kahane *et al*., 2001).

Onion showed a better soluble/insoluble dietary fibre (SDF:IDF) ratio than other vegetables that will be connected with different metabolic and physiological effects SDF increases the viscosity of the stomach contents, thereby allowing down-mixing and absorption of nutrients, whereas IDF reduces intestinal transit time and increases the bulk of the food mass (Jaime *et al*., 2002). Fructans could act as osmoregulators due to their solubility in water inside the vacuole.

Fructans act stimulating the growth of specific microorganisms in the colon (*Bifidobacteria* and *Lactobacilli*) with a general positive health effect, including on colonic inflammation (Ernst & Feldheim, 2000; Lara-Villoslada, 2006; Roberfroid, 2007). Administration of FOS significantly lowered fasting glycemia and total cholesterol, increasing the intestinal absorption and bone density of calcium and magnesium.

2.2.4. Other onion bioactive compounds

Recent literature reported that several interesting novel compounds have been isolated from onion. Among them, saponins and peptides have been isolated and studied for their potentially beneficial health effects.

5-hydroxy-3-methyl-4-propylsulfanyl-5H-furan-2-one, and four others compounds, were isolated and confirmed to be quinone reductase and glutathione *S*-transferase inducers *in vitro* (Xiao & Parkin, 2007), therefore they could act as chemopreventive agents. This warrants further research to isolate and identify more agents for their potential for phase II enzyme induction *in vitro* and *in vivo*.

Several research reports have demonstrated antifungal, antitumor, cytotoxicity, blood coagulability, antispasmodic and cholesterol-lowering effects of saponins isolated from onion and garlic (Lanzotti, 2006).

Four furostanol saponins, two of which were new compounds, named ceparoside A and ceparoside B were isolated from the seeds of *Allium cepa* L. (Yuan *et al*., 2008). Other new saponins were found years earlier by Corea *et al*. (2005), they were reported to possess antispasmodic activity in the guinea pig isolated ileum; such an effect might contribute to explaining the traditional use of onion in the treatment of disturbances of the gastrointestinal tract.

Recently, it was also reported that an onion gamma-glutamyl peptide from onion (Welti *et al*., 2004) inhibits the development and activity of osteoclasts *in vitro* (Langos *et al*., 2007).

2.3. Onion health effects

Allium vegetables health properties have been supported by numerous *in vitro*, *in vivo*, and *ex-vivo* studies. Particularly, onion has been described to have several health benefits related to its antioxidant, anticarcinogenic, hypolipidemic, hypoglycaemic, or antiaggreagatory effects.

From a medical and nutritionally point of view, it has to be taken into account that the onion used as a food or a food ingredient in the elaboration of many dishes also exerts a wide variety of medicinal effects which are very interesting for its human health potential benefits. Traditionally, in the folk medicine, it has been described the use of onion as an antimicrobial, cardiovascular-supportive, hypoglycemic, antioxidant/anticancer, and asthma-protective agent.

It has been described that a diet rich in *Allium* vegetables, including onion, would lead to several and different health benefits that could be helpful in the prevention of two of the more relevant and prevalent diseases nowadays such as cancer or CVD.

2.3.1. Onion and cancer

The association between consumption of *Allium* vegetables and risk for cancer has been assessed in several epidemiologic studies, mainly case-control (Bianchini & Vainio, 2001; Galeone *et al*., 2006).

In general, these studies are more consistent in reporting a protective effect of onion in gastric cancer. However, onion consumption has been also consistently related with a decreased colorectal cancer risk.

In addition, onion consumption was reported to decrease the risk for the cancer of the lung (Sankaranarayanan *et al*., 1994) and of the brain (Hu *et al*., 1999) in case-control studies.

Onion consumption was significantly inversely correlated with the risk of the stomach cancer. Recently, Gónzalez *et al.* (2006) observed in the European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST) a probable protective effect of total vegetables and *Allium* vegetables intake on the intestinal type of gastric cancer (González *et al*., 2006). Most of the case-control studies concerning onion were conducted in China (You *et al*., 1989; Gao *et al*., 1999) and several of them in Asia and Europe (Boeing *et al*., 1991, González *et al*., 1991; Tuyns *et al*., 1992, Hansson *et al*., 1993). A cohort study was conducted in The Netherlands (Dorant *et al*., 1996). The chemopreventive effects of onion against stomach and esophageal cancers may be related to their antibacterial properties. Inhibition of bacterial growth in the gastric cavity may result in less conversion of nitrate to nitrite in the stomach, a decreased probability of endogenous formation of carcinogenic N-nitroso compounds, and reduction in *Helicobacter pylori* infection specifically.

Diets rich in fruit and deep-yellow vegetables, dark-green vegetables, and onions and garlic are modestly associated with reduced risk of colorectal adenoma, a precursor of colorectal cancer (Millen *et al*., 2007). Decreased risk for colorectal cancer with the consumption of onion was generally found in case-control studies. The effect was particularly significant for consumption of cooked onions and leeks in Belgium (Tuyns *et al*., 1988), for a combination of garlic, onions, and pepper in Argentina (Iscovich *et al*., 1992). In a case-control study in Australia (Steinmetz *et al*., 1993) it was reported a lower risk for both sexes, with a more pronounced decrease for women and for cancer of the proximal compared with the distal colon. However, a cohort study in The Netherlands showed no significant effect of consumption of onions, leeks, and garlic supplements (Dorant *et al*., 1996).

2.3.2. Onion and cardiovascular disease (CVD) prevention. Antiaggregatory and hypolipidemic effects

CVD include coronary heart disease (heart attacks), cerebrovascular disease, raised blood pressure (hypertension), peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure. If current trends are allowed to continue, by 2015 an estimated 20 million people will die from CVD (mainly from heart attacks and strokes) (WHO. Cardiovascular disease). Therefore, CVD have a major impact on the mortality and quality of life of human populations across the world, despite improvements in lifestyle and innovations in the prevention and treatment of CVD in previous decades (Wensing *et al*., 2009).

CVD risk factors are mainly determined by uncontrollable causes (heredity, gender and age) and lifestyle-related causes (smoking, physical inactivity, stress and unhealthy diet), which are possible to be modified. Established CVD risk factors include dyslipidemia, obesity, hypertension, and diabetes mellitus (WHO. Prevention of cardiovascular disease: Guidelines for assessment and management cardiovascular risk).

The study by Galeone *et al.* (2009), the first from Mediterranean countries, suggests that a diet rich in onions may have a favorable effect on the risk of acute myocardial infarction; therefore these vegetables could be useful in a CVD preventive diet.

Several biomarkers are measured to predict CVD events including blood lipids levels (LDL-cholesterol and triglycerides), fibrinogen (a marker of thrombosis and inflammation), D-dimer (a marker of thrombosis), plasminogen-activator inhibitor type 1 (a marker of fibrinolytic potential and endothelial function), high-sensitivity C-reactive protein (CRP) (inflammation marker), homocysteine (a marker of endothelial function and oxidant stress), B-type and N-terminal pro–atrial natriuretic peptides, serum aldosterone, plasma renin (markers of neurohormonal activity), and urinary albumin-to-creatinine ratio (a marker of glomerular endothelial function) (Kannel, 2005; Wang *et al*., 2006).

Alterations in lipid profiles, diabetes, hypertension, and obesity are risk factors conventionally associated to the early appearance of CVD. Onion has been described to have hypolipidemic, hypoglycaemic, and antithrombotic effects and therefore could be useful in the CVD prevention.

Focusing on onion lipid lowering effects, this vegetable has been reported to exert moderately hypolipidemic effects in experimental animal such as healthy pigs fed a high

fat diet and consequently potentially reduce risk indices of CVD and obesity (Otrowska *et al*., 2003; Gabler *et al*., 2006).

Among bioactive compounds involved in onion hypolipidemic effects, quercetin has shown to have the ability to reduce serum cholesterol levels and arteriosclerosis severity (Glasser *et al*., 2002). A recent study by Kumari & Augusti, 2007 also proclaimed for the lipid lowering action of the S-methyl cysteine sulfoxide (SMCS) isolated from *Allium cepa* L.

Onion has also been reported to have hypoglycaemic effects (Srinivasan, 2005). It was inferred that this beneficial ameliorating influence of dietary onion on diabetic nephropathy may be mediated through onion's ability to lower blood cholesterol levels and to reduce lipid peroxidation, dietary onion caused significant beneficial modulation of the progression of renal lesions in diabetic rats (Babu & Srinivasan, 1999). Other rat studies have assessed onion hypoglycaemic effects (El-Demerdash *et al*., 2005). Recently, a study by Lee *et al.* (2008) showed that onion skin was effective in controlling hyperglycemia in animal models of type 2 diabetes mellitus, at least in part by inhibiting alpha-glucosidase activity (Lee *et al*., 2008).

Thrombosis complications play a major role in CVD. Blood clot formation depends on an intricate series of events involving platelets, other cells, and the activation of specific blood proteins, known as coagulation factors. A thrombus is a blood clot formed when there is an imbalance in the blood coagulation system that can block the flow of blood through a vein or artery, and can detach from the vessel wall to become a life-threatening embolus when it lodges in the lungs or other vital organs. Blood clots in coronary arteries cause acute coronary syndrome and blood clots that form in the heart are the major cause of stroke in people with atrial fibrillation. Onion inhibits platelet aggregation *in vitro* and *in vivo* (Ali *et al*., 1999; Ali *et al*., 2000; Briggs *et al*., 2001; Jung *et al*., 2002; Hubbard *et al*., 2006). The mechanism by which onion exerts its antithrombotic effect has been shown to involve the inhibition of thromboxane A2 formation, potent inducer of platelet aggregation (Moon *et al*., 2000).

The antiplatelet activity observed in onion is influenced by genotype, environmental factors and genotypically determined sulfur content of the bulb (Goldman *et al*., 1996; Sance *et al*., 2008) having onion α-sulfinil-disulfides (cepaenes) a demonstrated antithrombotic activity (Block *et al*., 1997).

2.3.3. Other onion health-promoting health effects

Onion consumption has also been reported to be involved in the bone metabolism and in the behaviour as a possible antidepressant agent.

A recent study by Matheson *et al*. (2009) reported that onion consumption seems to have a beneficial effect on bone density in perimenopausal and postmenopausal women. Furthermore, older women who consume onions most frequently may decrease their risk of hip fracture by more than 20% *versus* those who never consume onions. Prevention of low bone mass is important to reduce the incidence of osteoporotic fractures. Onion retains its bone resorption inhibitory activity in the rat when added to a vegetarian diet (Muhlbauer *et al*., 2002).

Another recent study by Sakakibara *et al*. (2008) suggests that onion exerted antidepressant-like activity in a behavioural model that acted independently of the hypothalamic-pituitary-adrenal axis.

2.4. Annex I

Table 1. Raw onion (*Allium cepa* L.) proximates

PROXIMATES	Unit	Mean ± SD
Water	g	89.11 ± 0.248
Energy	kcal	40 ± 0
Energy.	kJ	166 ± 0
Protein (N x 6.25)	g	1.10 ± 0.036
Total lipid (fat)	g	0.10 ± 0.005
Ash	g	0.35 ± 0.003
Carbohydrate, by difference	g	9.34 ± 0
Fibre, total dietary	g	1.7 ± 0.048
Sugars, total	g	4.24 ± 0
Sucrose	g	0.99 ± 0.050
Glucose (dextrose)	g	1.97 ± 0.054
Fructose	g	1.29 ± 0.052

Amount in 100 grams of edible portion

Table 2. Raw onion (*Allium cepa* L.) vitamins

VITAMINS	Unit	Mean ± SD
Vitamin C, total ascorbic acid	mg	7.4 ± 0.053
Thiamin	mg	0.046 ± 0.001
Riboflavin	mg	0.027 ± 0.002
Niacin	mg	0.116 ± 0.003
Pantothenic acid	mg	0.123 ± 0.002
Vitamin B-6	mg	0.120 ± 0.004
Folate, total	mcg	19 ± 0.059
Choline, total	mg	6.1 ± 0
Betaine	mg	0.1 ± 1
β-Carotene	µg	1 ± 0
Vitamin A, IU	IU	2 ± 0
Lutein + zeaxanthin	µg	4 ± 0
Vitamin E (α-tocopherol)	mg	0.02 ± 0
Vitamin K (phylloquinone)	µg	0.4 ± 0.011

Amount in 100 grams of edible portion

Table 3. Raw onion (*Allium cepa* L.) minerals

MINERALS	Unit	Mean ± SD
Calcium, Ca	mg	23 ± 0.568
Iron, Fe	mg	0.21 ± 0.08
Magnesium, Mg	mg	10 ± 0.152
Phosphorus, P	mg	29 ± 0.584
Potassium, K	mg	146 ± 2.951
Sodium, Na	mg	4 ± 0.158
Zinc, Zn	mg	0.17 ± 0.004
Copper, Cu.	mg	0.039 ± 0.002
Manganese, Mn	mg	0.129 ± 0.004
Selenium, Se	μg	0.5 ± 0.149

Amount in 100 grams of edible portion

Table 4. Raw onion (*Allium cepa* L.) lipids

LIPIDS	Unit	Mean ± SD
Fatty acids, total saturated	g	0.042
14:0	g	0.004
16:0	g	0.034 ± 0.003
18:0	g	0.004
Fatty acids, total monounsaturated	g	0.013 ± 0.02
18:1 undifferentiated	g	0.013 ± 0.02
Fatty acids, total polyunsaturated	g	0.017
18:2 undifferentiated	g	0.013 ± 0.02
18:3 undifferentiated	g	0.004
Phytosterols	mg	15 ± 1

Amount in 100 grams of edible portion

Table 5. Raw onion (*Allium cepa* L.) amino acids

AMINO ACIDS	Unit	Mean
Tryptophan	g	0.014
Threonine	g	0.021
Isoleucine	g	0.014
Leucine	g	0.025
Lysine	g	0.039
Methionine.	g	0.002
Cystine.	g	0.004
Phenylalanine	g	0.025
Tyrosine	g	0.014
Valine	g	0.021
Arginine	g	0.104
Histidine	g	0.014
Alanine.	g	0.021
Aspartic acid	g	0.091
Glutamic acid	g	0.258
Glycine	g	0.025
Proline	g	0.012
Serine	g	0.021

Amount in 100 grams of edible portion

USDA National Nutrient Database for Standard Reference, Release 21 (2008). Food Group: 11: Vegetables and Vegetable Products. Raw onion (*Allium cepa* L.) Refuse: 10% Stem ends, sprouts and defects.

Chapter 2

Research questions and hypotheses

Objectives

Chapter 2. Research questions and hypotheses. Objectives

The main aim of the present PhD Thesis was to investigate the biological activity and nutritional properties of processed onion products. Several research questions and hypotheses were postulated triggering to different scientific reports.

1. Research questions and hypotheses

Hypotheses I and II - How do food preservation and processing technologies (freezing, pasteurization, sterilization, and high hydrostatic pressure) affect onion nutritional and technological quality (onion by-products and fresh onion)?

Which processing technology and onion by-product would be the most adequate for the potential technological development of an antioxidant and antibrowning functional food ingredient?

Hypothesis I: In the *first hypothesis* we postulate that the processing technologies (freezing, pasteurization, and sterilization) which stabilize onion by-products (juice, paste, and bagasse) would also affect onion composition and properties as total phenols, quercetin content, antioxidant and antibrowning properties. As a consequence, it would be possible to select an onion by-product which shows the best bioactive compound content and technological properties for its potential food ingredient development ***(Paper I)***.

Which would be the effects of processing fresh onion by high hydrostatic pressure combined with temperature on onion bioactive compounds extractability and antioxidant activity?

Hypothesis II: In the *second hypothesis* we postulate that processing onion with high hydrostatic pressure combined with temperature at a constant time would increase total phenols, quercetin, quercetin glucosides extractability, and antioxidant activity compared with nonprocessed fresh onion ***(Paper II)***.

Hypotheses III, IV, V, and VI - Which are the 'in vivo' biological activity and nutritional properties of onion products?

Which would be the main biological responses in healthy rats fed onion by-products?

Hypothesis III: In the *third hypothesis* we postulate that onion by-products might have antioxidant and prebiotic properties and therefore could exert health beneficial

effects. In order to better understand the biological behaviour of the different onion bioactive compounds, we extracted the soluble dietary fibre from the onion by-product chosen in our previous study (pasteurised onion paste). Thus, we aimed to study the biological effects in healthy rats fed onion as a whole onion by-product and as two derived onion fractions, an onion extract rich in fructooligosaccharides (FOS) and an onion residue **(*Paper III*)**.

Would it be possible to elucidate which organosulfur compounds (OSCs) are present in the urine from rats fed onion by-products?

Hypothesis IV: In the *fourth hypothesis* we postulate that novel metabolites with an organosulfur structure would be found in urine from healthy rats fed onion by-products. Thus, using nutri-metabonomic study that combines ^{1}H NMR spectroscopy and chemometrics we could be able to elucidate the structure of novel biomarkers for onion intake **(*Paper IV*)**.

How would onion by-products intake affect plasma lipids and platelet aggregation in healthy rats?

Hypothesis V: In the *fifth hypothesis* we postulate that onion by-products might exert hypolipidemic and antithrombotic effects and the intake of food ingredients vegetables from the *Allium* genus could be useful in the cardiovascular disease (CVD) prevention **(*Paper V*)**.

Which would be the main biological and nutritional responses in overweight humans eating precooked dishes with an added onion food ingredient?

Hypothesis VI: In the *sixth hypothesis* we postulate that an onion product elaborated from fresh onion could be added to different precooked dishes as a food ingredient and it would possibly enhance their antioxidant capacity and exert antiaggregatory, hypolipidemic and antiinflammatory effects in overweight subjects **(*Paper VI-in preparation*)**.

2. Objectives

Overall, in the present PhD Thesis two main objectives with several secondary objectives were formulated.

Objective 1

The first aim was to evaluate *in vitro* the effects of food processing and preservation technologies on nutritional and technological properties of onion.

Objective 1.1.

Evaluation of the effects caused by processing technologies (sterilization, pasteurization, and freezing) on quercetin, total phenols, antioxidant activity and antibrowning properties of onion by-products (juice, paste, and bagasse) from two different onion cultivars (*Allium cepa* L. var. *cepa*, 'Recas' and *Allium cepa* L. var. *cepa*, 'Figueres').

Objective 1.2.

Study of the effects caused by the high-pressure processing technology combined with temperature at a constant time on the content of quercetin, quercetin glucosides, total phenols content, and antioxidant activity of fresh onion (*Allium cepa* L. var *cepa,* 'Grano de Oro').

Objective 2

Our second aim was to evaluate *in vivo* the biological activity and nutritional properties of onion products.

Objective 2.1.

Assessment of oxidative stress biomarkers, genotoxicity, and gut environment in healthy rats fed onion by-products: For that purpose a soluble dietary fibre rich fraction was extracted from a freeze-dried pasteurized onion paste (*Allium cepa* L. var *cepa*, 'Recas') and a four-week study was conducted with rats fed the onion by-product, a fibre rich extract, and a residue.

Objective 2.2.

Searching for novel dietary biomarkers for onion intake with an organosulfur compound (OSC) structure in urine of rats fed onion by-products ('Recas' pasteurized onion paste) by a nutri-metabonomic study combining ^{1}H NMR spectroscopy and chemometrics.

Objective 2.3.

Evaluation of plasma lipids and platelet aggregation in healthy rats fed onion by-products ('Recas' pasteurized onion paste) and study of the possible usefulness of onion by-products as food ingredients with hypolipidemic and antithrombotic properties included in a cardiovascular disease (CVD) preventive diet.

Objective 2.4.

We aimed to evaluate the possible antioxidant, hypolipidemic, antiaggregatory, and antiinflamatory responses in overweight humans eating four precooked dishes (two soups and two dishes with meat) with an added onion food ingredient ('Recas' freeze-dried fresh onion).

Capítulo 2

Preguntas científicas e hipótesis

Objetivos

Capítulo 2. *Preguntas científicas e hipótesis. Objetivos*

El objetivo principal de la presente Tesis Doctoral fue la investigación de la actividad biológica y las propiedades nutricionales de productos de cebolla procesados. Varias preguntas e hipótesis fueron postuladas.

1. Preguntas científicas e hipótesis

Hipótesis I y II -.¿Cómo influyen tecnologías de conservación y procesado de alimentos (congelación, pasteurización y esterilización, y alta presión hidrostática) en la calidad nutricional y tecnológica de productos de cebolla (subproductos de cebolla y cebolla fresca)?

¿Qué tecnología de conservación y qué subproducto de cebolla serían los más adecuados para el desarrollo tecnológico de un ingrediente alimentario funcional antioxidante y con propiedades de antipardeamiento enzimático?

Hipótesis I: En la *primera hipótesis* postulamos que las tecnologías de conservación (congelación, pasteurización, y esterilización) llevadas a cabo para estabilizar los subproductos de cebolla (zumo, pasta y bagazo) afectarían a su contenido en fenoles totales, quercetina, actividad antioxidante y capacidad de prevención del pardeamiento enzimático. En consecuencia, la caracterización de subproductos de cebolla haría posible la elección de aquel que presente un buen contenido en compuestos bioactivos y unas buenas características tecnológicas para su desarrollo como ingrediente alimentario **(*Paper I*).**

¿Cuáles son los principales efectos del procesado de la cebolla fresca con alta presión hidrostática combinada con temperatura en la extractabilidad de compuestos bioactivos y actividad antioxidante de productos de cebolla?

Hipótesis II: En la *segunda hipótesis* postulamos que el procesado de cebolla con alta presión hidrostática combinado con temperatura a tiempo constante incrementaría la extractabilidad de fenoles totales, quercetina y glucósidos de quercetina así como su actividad antioxidante respecto a la cebolla fresca no procesada **(*Paper II*)**.

Hipótesis III, IV, V y VI - ¿Cuáles son las actividades biológicas y propiedades nutricionales 'in vivo' de productos de cebolla?

¿Cuales serían las principales respuestas biológicas de ratas sanas cuando son alimentadas con subproductos de cebolla?

Hipótesis III: En la *tercera hipótesis* postulamos que subproductos de cebolla podrían tener propiedades antioxidantes y prebióticas. Para comprender el comportamiento biológico de los diferentes compuestos bioactivos de la cebolla se realizó una extracción previa de la fibra dietética soluble de cebolla a partir del subproducto de cebolla escogido en el estudio anterior (pasta pasteurizada). Así, queríamos estudiar los efectos biológicos de la cebolla en ratas sanas, a través de un subproducto de cebolla entero y dos subproductos de cebolla derivados de él, que contuvieran diferentes compuestos bioactivos de cebolla, un extracto rico en fructoligosacáridos (FOS) y un residuo **(*Paper III*)**.

¿Sería posible discernir qué compuestos organosulfurados estarían presentes en la orina de ratas alimentadas con subproductos de cebolla?

Hipótesis IV: En la *cuarta hipótesis* postulamos qué nuevos metabolitos derivados de compuestos organosulfurados se encontrarían en orina de ratas sanas alimentadas con subproductos de cebolla. Por tanto, por medio del estudio nutri-metabonómico en la orina de estos tres grupos de ratas y haciendo uso de técnicas como la espectroscopía ^{1}H NMR y la quimiometría se podrían elucidar la estructura de nuevos biomarcadores de la ingesta de cebolla **(*Paper IV*)**.

¿Cómo afectarían la ingesta de subproductos de cebolla a los lípidos plasmáticos y agregación plaquetaria en ratas sanas?

Hipótesis V: En la *quinta hipótesis* postulamos que los subproductos de cebolla podrían tener efectos hipolipidémicos y antitrombóticos y que la ingesta de ingredientes alimentarios de vegetales del género *Allium* podría ser útil en la prevención de enfermedades cardiovasculares **(*Paper V*)**.

¿Cuáles serían las principales respuestas biológicas y nutricionales en humanos con sobrepeso alimentados con platos precocinados con un ingrediente de cebolla añadido?

Hipótesis VI

En la *sexta hipótesis* postulamos que un producto elaborado partir de cebolla fresca podría ser adicionado a platos precocinados como un ingrediente alimentario y posiblemente aumentaría la capacidad antioxidante, anticoagulante y antiinflamatoria en sujetos sanos con sobrepeso **(*Paper VI-en preparación*)**.

2. Objetivos

En la presente Tesis Doctoral se formularon dos objetivos principales con varios objetivos secundarios.

Objetivo 1

El primer objetivo fue la evaluación *in vitro* de los efectos de tecnologías de procesado y conservación en las propiedades nutricionales y tecnológicas de la cebolla.

Objetivo 1.1.

Estudio del efecto de tecnologías de procesado y conservación (esterilización, pasteurización, y congelación) en el contenido de quercetina, fenoles totales, actividad antioxidante y propiedades de antipardeamiento enzimático de subproductos de cebolla (zumo, pasta y bagazo) de dos variedades de cebolla (*Allium cepa* L. var. *cepa*, 'Recas' y *Allium cepa* L. var. *cepa*, 'Figueres').

Objetivo 1.2.

Evaluar los efectos de la alta presión hidrostática combinada con temperatura a tiempo constante en la extractablidad de quercetina, glucósidos de quercetina, fenoles totales y actividad antioxidante de cebolla fresca (*Allium cepa* L. var *cepa,* 'Grano de Oro').

Objetivo 2

El segundo objetivo fue la evaluación *in vivo* de la actividad biológica y las propiedades nutricionales de productos de cebolla.

Objetivo 2.1.

Estudio de biomarcadores de estrés oxidativo, genotoxicidad y ambiente intestinal de ratas alimentadas con subproductos de cebolla. Para dicho propósito se extrajo una fracción de fibra dietética soluble de pasta de cebolla pasteurizada (*Allium cepa* L. var *cepa*, 'Recas') y se llevó a cabo un estudio durante cuatro semanas en ratas alimentadas con un subproducto de cebolla, una fracción rica en fibra y un residuo.

Objetivo 2.2.

Búsqueda de nuevos bioamrcadores dietéticos con estructuras de compuestos organosulfurados en orina de ratas alimentadas con subproductos de cebolla (pasta pasteurizada 'Recas'). Estudio nurti-metabonómico mediante espectroscopía ^{1}H NMR y quimiometría.

Objetivo 2.3.

Evaluación de lípidos plasmáticos y agregación plaquetaria en ratas sanas alimentadas con subproductos de cebolla (pasta pasteurizada 'Recas') y estudio de la posible utilidad de subproductos de cebolla como ingredientes alimentarios con propiedades hipolipidémicas y antitrombóticas incluidos en una dieta preventiva de enfermedades cardiovasculares.

Objetivo 2.4.

Evaluación de las posibles respuestas antioxidantes, hipolipidémicas, antiagregante plaquetarias y antiinflamatorias en sujetos con sobrepeso alimentados con cuatro platos precocinados (dos sopas y dos platos con carne) con un ingrediente de cebolla añadido (cebolla fresca liofilizada 'Recas').

Chapter 3

Overview of the experimental work

Chapter 3. Overview of the experimental work

This PhD Thesis based on the biological activity and nutritional properties of processed onion products has been conducted at the Department of Plant Food Science and Technology, Instituto del Frío, Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain; the Department of Toxicology and Risk Assessment, National Food Institute, Technical University of Denmark, Søborg, Denmark; and the Department of Human Nutrition, University of Copenhagen, Copenhagen, Denmark.

The experimental part can be divided into two principal sections that compile the onion *in vitro* and *in vivo* studies carried out. The onion samples used and the main techniques and analysis performed are briefly explained below for each of the study performed. Moreover, the methodologies used together with the techniques and the analysis performed are also detailed in each paper included in the PhD.

1. Onion *in vitro* studies

Two *in vitro* studies including onion from different onion cultivars were carried out. Our main aim was to study the effects of processing technologies on onion bioactive content, antioxidant activity, and antibrowning properties.

1.1. Onion by-products

Onion juices, pastes and bagasses of two onion cultivars: *Allium cepa* L. var. *cepa*, 'Recas' and *Allium cepa* L. var. *cepa*, 'Figueres' were processed and stabilized by sterilization, pasteurization, and freezing.

The onion by-products ('Figueres' and 'Recas' sterilized, pasteurized, and frozen onion juices, pastes, and bagasses) were freeze-dried before measuring the bioactive compound content, antibrowning, and antioxidant activities. Each onion sample was analyzed in triplicate.

As a preparatory step for the next *in vivo* study (rat study) an approximately amount of 1500 g of pasteurized onion paste (*Allium cepa* L. var. *cepa*, 'Recas') was freeze-dried (Figure 1).

The soluble dietary fibre (fructans and fructooligosaccharides) was extracted from this onion by-product freeze-dried powder following the modified Shiomi method with minor modifications described by Jaime *et al*. (2001). As a result, an onion extract, fibre rich, and an onion residue were obtained. Therefore, three onion by-products were used for the onion diets in the rat study.

Figure 1. Pasteurized onion paste (*Allium cepa* L. var. *cepa*, 'Recas')

1.2. Onion high hydrostatic pressure processing

Fresh onion (*Allium cepa* L. var *cepa*, 'Grano de Oro') was peeled, washed, cut it into pieces, vacuum packaged and sealed with a Multivac sealer (Wolferchweden, Germany). Plastic bags used were BB4L, CRYOVAC Europe, Grace S. A., Sant Boi de Llobregat, Barcelona, Spain. Oxygen permeability was 30 cm^3/ (m^2 24 h bar) at 23 ºC and 0% relative humidity (RH). BB4L is a heat shrinkable co-extruded material, containing polyethylene-vinyl acetate (EVA) co-polymer (Figure 2).

Response surface methodology (RSM) was employed to study the effect of combined treatments of high-pressure and temperature on onion bioactive compound content and antioxidant activity. The experiment was carried out according to a central composite face-centered design. Three levels of each independent variable (pressure and temperature) were chosen (Table 1).

Following the design, ten selected processes of two variables were performed. Fresh onion was pressurized following the treatments from 1 to 10 (Figure 3).

T1 = 100 MPa/5 ºC

T2 = 100 MPa/27.5 ºC

T3 = 100 MPa/50 ºC

T4 = 250 MPa/5ºC

T5 = 250 MPa/27.5 ºC

T6 = 250 MPa/27.5 ºC

T7 = 250 MPa/50 ºC

T8 = 400 MPa/5 ºC

T9 = 400 MPa/27.5 ºC

T10 = 400 MPa/50 ºC

Table 1. Independent variables and their levels used for central composite design

Independent variables	**Symbol**	**Coded variable levels**		
		-1	0	1
Pressure (MPa)	X_1	100	250	400
Temperature (ºC)	X_2	5	27.5	50

1.3. Methodologies, techniques, and analysis (onion *in vitro* studies)

-High performance liquid chromatography (HPLC) for the characterization and quantification of quercetin and glucosides of quercetin (Hertog *et al*., 1992).

-Spectrophotometry for the measurement of onion total phenols (Vinson *et al*., 1998), onion antibrowning capacity (inhibition of avocado polyphenol oxidase (PPO) activity) (Kim *et al*., 2005) and antioxidant activity (DPPH˙ assay) (Sánchez-Moreno *et al*., 1998).

Figure 2. Onion processing. Peeling, washing, cutting, and vacuum into bags

Figure 3. High-pressure processing (HP- Equipment at Instituto del Frío (CSIC) and samples packaged subjected to HP treatments)

2. Onion *in vivo* studies

2.1. Rat study

Rat Study Design: Thirty-two rats were allocated into four groups of eight rats and fed during four weeks with a control diet added 10% of onion by-product, a control diet added 7% of onion extract or a control diet added 3% onion residue. The composition of the rat feed is shown in Table 2.

Table 2. Composition of animal diets

Ingredient (g/kg feed)	Control	Onion by-product	Onion extract	Onion residue
Onion by-product	0	100 g	0	0
Onion extract	0	0	70 g	0
Onion residue	0	0	0	30 g
Control feed	1000 g	900 g	930g	970 g
Total feed	1000 g	1000 g	1000 g	1000 g

The samples taken from the rats fed onion by-products one day before the first sacrifice day were urine and faeces.

The samples taken from the rats fed onion by-products during the sacrifice days were blood (plasma, red blood cells (RBC), white blood cells (WBC)), caecal, colon, and liver samples.

2.2. Human intervention study

Four precooked dishes, two soups (Figures 2 and 3) and two dishes with meat (Figures 4 and 5) were prepared. Freeze-dried powder elaborated from fresh onion (*Allium cepa* L. var. *cepa*, 'Recas') were added or not to each meal (Table 3).

Figure 2. Potato soup

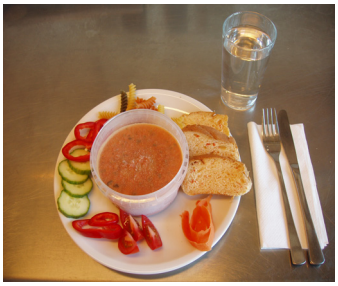


Figure 3. Tomato soup

Figure 4. Minced beef stew

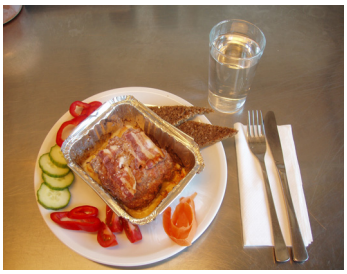


Figure 5. Meat loaf

Table 3. Total energy and energy distribution per serving of onion meals and no onion meals. Source: Dankost

	Total Energy [KJ]	Energy Distribution [%]
Potato soup (Onion)	770.4	Protein: 6.2 Fat: 56.2 Carbohydrate: 35.7
Potato soup (No onion)	675	Protein: 5 Fat: 63.3 Carbohydrate: 30.6
Tomato soup (Onion)	1199	Protein: 7.3 Fat: 68.4 Carbohydrate: 22.1
Tomato soup (No onion)	1103.5	Protein: 6.7 Fat: 73.8 Carbohydrate: 17.8
Meat loaf (Onion)	1505.4	Protein: 36.8 Fat: 48.4 Carbohydrate: 14
Meat loaf (No onion)	1410	Protein: 38,3 Fat: 51,3 Carbohydrate: 10,1
Minced beef stew (Onion)	1056.1	Protein: 38.4 Fat: 47.1 Carbohydrate: 14.4
Minced beef stew (No onion)	951.9	Protein: 41.2 Fat: 51.7 Carbohydrate: 7.1

Human Study Design

The study was a randomized double-blinded crossover design study composed of two 14-day dietary intervention periods including onion or not onion diets with a 25-day wash-out period between dietary intervention periods. A restriction diet was given to the overweight participants to be followed for eight days before each intervention period start and continuing during each period (Figure 6).

Measurement of blood pressure (Figure 7), collection of blood samples (Figure 8), urine and faeces delivery were taken during each intervention period according Table 4.

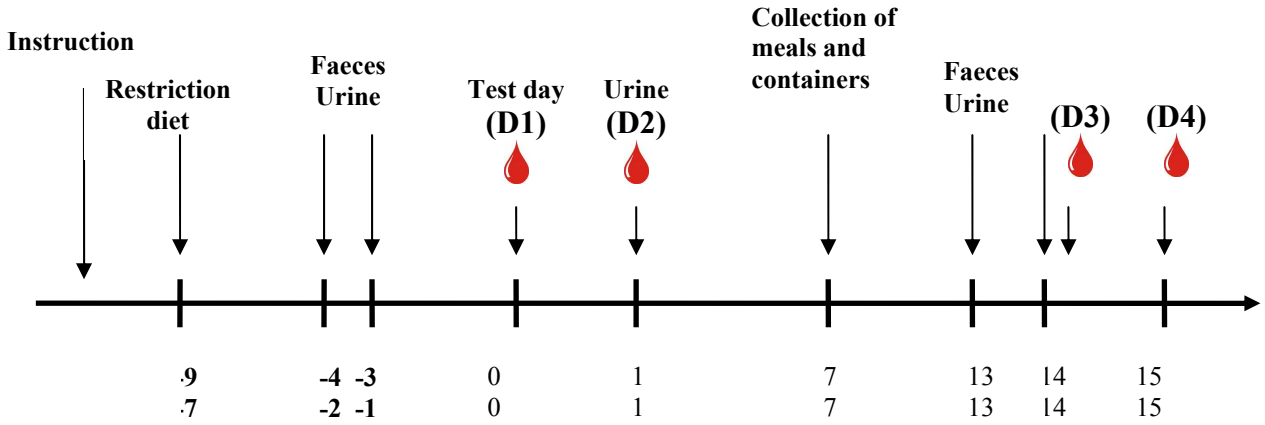


Figure 6. Timeline (days) of the study

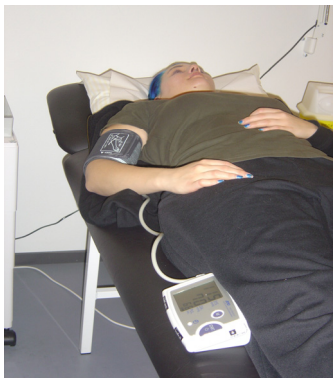


Figure 7. Blood Pressure

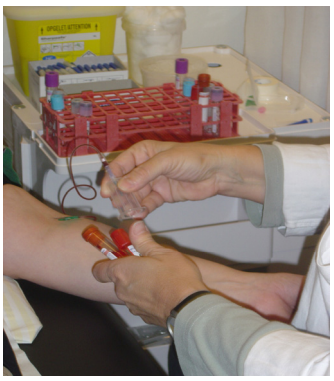


Figure 8. Blood collection

Table 4. Blood, urine sample collection, and blood pressure days during each onion dietary intervention period

Day-hour	0 h	2 h	4 h	24 h
0	Blood pressure Blood samples* Urine sample	Blood samples* Urine sample	Blood pressure Blood samples Urine sample	
1				Blood pressure Blood samples* Urine sample
14	Blood pressure Blood samples*			
15	Blood pressure Blood samples			

* Blood sample for coagulation time and blood smears analysis

2.3. Methodologies, techniques, and analysis (onion *in vivo* studies)

-Comet Assay or Single Cell Gel Electrophoresis -SCGE- Assay. Determination of DNA damage in rat liver and white blood cells (WBC) samples (Tice *et al*., 2000).

-Automated Roche/Hitachi 912 Analyzer (Roche Diagnostic A/S, Hvidovre, Denmark) (spectophotometry). Antioxidant enzymes in rat red blood cells (RBC) and liver samples. Bacterial enzymes in rat caecal samples (Paglia & Valentine, 1967; Goldberg & Spooner, 1983; Johansson & Borg HLA, 1988).

-Autoanalizer Cobas Mira (spectophotometry). Antioxidant enzymes and antioxidant capacity in human RBC and plasma, respectively.

-Quantitative real time PCR. Quantitative Real-time PCR, ABI 7900HT FAST System. For determining the gene expression of antioxidant enzymes, phase II enzymes in rat RBC and liver samples, enzymes involved in lipid and platelet metabolism (rat liver and WBC samples).

-Capillary electrophoresis with indirect UV detection for the short chain fatty acids (SCFA) in rat caecal samples (Hansen *et al*., 2008).

-The pH in the rat caecum content near the colon outlet was determined using a microelectrode (Knick, Portamess 751 calimatic pH meter equipped with a Hamilton biotrode)-pH.

-LC/MS/MS methodology for bile acid quantification. Faeces samples and bile acids standards were analysed on an Acquity UPLC with a TQ detector (Waters operated in MRM mode). The individual compounds were quantified using QuanLynx version 4.1 (Waters) based on internal standards and external calibrants

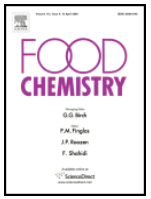
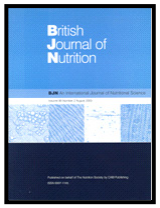
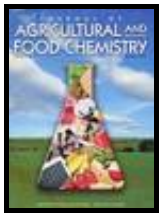
-^{1}H NMR methodology for biomarkers and lipid quantification. Spectra were recorded for rat and human urine and plasma samples. The spectra for urine samples were acquired on a Bruker Avance Ultra Shield 400 spectrometer (Bruker Biospin Gmbh, Rheinstetten, Germany) operating at 400.13 MHz using a broad band inverse detection probe head. For plasma samples NMR spectra were acquired on a Bruker Avance 400 MHz spectrometer (9.4 T) (Bruker Biospin Gmbh, Rheinstetten, Germany) at 311K, which corresponds to the body temperature of rats for quantification.

-Chemometric analysis and software. Multivariate data analysis in the form of principal component analysis (PCA) (Hotelling *et al*., 1933) and PLS (Wold *et al*., 1983) was applied to obtain optimal quantitative and qualitative biomarkers information from the measured spectra in urine samples. Plasma lipids (total cholesterol and cholesterol content in high density (HDL), low density (LDL) and very low density (VLDL) lipoproteins) were then predicted by a previously developed chemometric models based on NMR data and interval partial least square (PLS) models.

All the 5 papers included in this PhD Thesis contain the results from these *in vitro* (Paper I and II) and *in vivo* studies (Paper III, IV and V). The sixth paper which includes the human study is in preparation.

An overview with the focus, aims, and main outcomes of each paper is shown in Table 5.

Table 5. Overview of the five papers included in this PhD Thesis

	Paper I	**Paper II**	**Paper III**	**Paper IV**	**Paper V**
Title	Characterisation of onion (*Allium cepa* L.) by-products as food ingredients with antioxidant and antibrowning properties	Onion high-pressure processing: Flavonol content and antioxidant activity	Effect of an onion by-product on bioactivity and safety markers in healthy rats	An exploratory NMR nutri-metabonomic investigation reveals dimethyl sulfone as a dietary biomarker for onion intake	Effects of an onion by-products on plasma lipids and platelet aggregation in healthy rats
Journal	Food Chem (2008) 108, 907-916 	LWT Food Sci Technol (2009) 42, 835-841 	Br J Nutr (2009) In press 	Analyst (2009) 134, 2344-2351 	J Agric Food Chem (2009) Under review 
Studies	Onion by-products *Allium cepa* L. var. *cepa,* 'Recas' and *Allium cepa* L. var. *cepa,* 'Figueres'	Fresh onion *Allium cepa* L. var *cepa* 'Grano de Oro'	Rat study Onion by-products *Allium cepa* L. var. *cepa,* 'Recas'	Rat study Onion by-products *Allium cepa* L. var. *cepa*, 'Recas'	Rat study Onion by-products *Allium cepa* L. var. *cepa,* 'Recas'
Focus	Effects of processing and preservation technologies on onion total phenols, quercetin, antioxidant, and antibrowning properties	Effects of high-pressure processing on fresh onion total phenols, quercetin, quercetin glucosides, and antioxidant activity	How onion by-products affect DNA, antioxidant enzymes, gene expression (antioxidant and phase II enzymes), and gut environment in healthy rats	Combine NMR with chemometrics as a tool for searching for novel biomarkers for onion intake	Effects of an onion by-product on CVD risk factors (TC, LDL-C; VLDL-C, and HDL-C, *Hmcgr* expression) and platelet aggregation (*Txas* expression) in healthy rats
Primary outcomes	Pasteurized onion pastes offer good technological and nutritional characteristics for being develop as onion food ingredients	400 MPa/5 ºC-processed onion showed higher quercetin-4´ glucoside content and maintained the antioxidant activity compared with the untreated onion	The onion by-product and the two derived onion fractions (extract and residue) are not genotoxic and exert *in vivo* antioxidant and prebiotic properties	Dimethyl sulfone is identified as a dietary biomarker for onion intake. Quantify dietary intake could be beneficial as control in diet intervention studies	The onion by-product and the onion extract decrease *Txas* expression. The onion extract might find use for isolation of inhibitors of platelet aggregation

Chapter 4

Characterisation of onion (*Allium cepa* L.) by-products as food ingredients with antioxidant and antibrowning properties

Eduvigis Roldán, Concepción Sánchez-Moreno, Begoña de Ancos, M. Pilar Cano

Food Chemistry **(2008) 108, 907-916**

Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 108 (2008) 907–916
Food Chemistry
www.elsevier.com/locate/foodchem

Characterisation of onion (*Allium cepa* L.) by-products as food ingredients with antioxidant and antibrowning properties

Eduvigis Roldán, Concepción Sánchez-Moreno *, Begoña de Ancos, M. Pilar Cano

Department of Plant Foods Science and Technology, Instituto del Frío, Consejo Superior de Investigaciones Científicas (CSIC), Cl José Antonio Novais 10, Ciudad Universitaria, E-28040 Madrid, Spain

Received 19 July 2007; received in revised form 22 November 2007; accepted 23 November 2007

Abstract

Processing and stabilising onion wastes (residues and surpluses of onion) could solve the environmental problem derived from a great onion wastes disposal. Moreover, obtaining stabilised onion by-products as natural antioxidant food ingredients could be advantageous to food industry, not only to improve the use of onion wastes but also to obtain new natural and functional ingredients. The aim of this study was to characterise onion by-products – juice, paste and bagasse – from two Spanish onion cultivars – 'Figueres' and 'Recas' – that have been stabilised by thermal treatments – freezing, pasteurisation and sterilisation – in order to evaluate the effect of the processing and stabilisation treatment on the bioactive composition, antioxidant activity and polyphenol oxidase (PPO) enzyme inhibition capacity. The results obtained triggered to choose one onion by-product offering better characteristics for its potential development as a food ingredient: source of antioxidant and antibrowning bioactive compounds. In this study it was shown that processing of 'Recas' onion wastes to obtain a paste (mixture content) and applying a mild pasteurisation were the best alternatives to obtain an interesting stabilised onion by-product with good antioxidant properties that made useful its use as functional food ingredient.

Keywords: Onion by-products; Stabilisation treatments; Freezing; Pasteurisation; Sterilisation; Bioactive compounds; Antioxidant; Enzymatic browning; Functional food ingredient

1. Introduction

Onion (*Allium cepa* L.) is one of the major vegetable crops grown in Europe which production and cultivated area has increased constantly since 1998. More than 450,000 tonnes of onion wastes is produced annually in the European Union, mainly in UK, Holland and Spain. Nowadays, the food and agricultural products processing industries generate substantial quantities of phenolic-rich by-products, which could be valuable natural sources of antioxidants to be employed as ingredients. Some of these by-products have been the subject of investigations and have proven to be effective sources of phenolic antioxidants (Balasundram, Sundram, & Samman, 2006; Peschel et al, 2006).

There is a concern over the production of large quantities of industrial onion waste or by-products and its disposal. Onion wastes are not suitable for fodder, or landfill disposal due to the rapid growth of phytopathogens, e.g. *Sclerotium cepivorum* (white rot). Valorisation of by-products, particularly exploitation of them for profitable production of food-grade products will benefit the onion producers and processors (Lecain, Ng, Parker, Smith, & Waldron, 1999).

Processing and stabilising onion wastes (residues and surpluses of onion) could represent both advantages: a solution of the environmental problem derived from the great onion wastes disposal and the obtaining of stabilised onion by-products as natural antioxidant food ingredients. Spain is one of the major Mundial onion-producing coun-

* Corresponding author. Tel.: +34 915492300; fax: +34 915493627.
E-mail address: csanchezm@if.csic.es (C. Sánchez-Moreno).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.11.058

tries. It produced 936,827 tonnes of onion in a cultivated area of 21,324 hectares in 2003. Different varieties and cultivars of onion are spread out among all the regions of Spain, being Castilla-La Mancha, Levant and Andalusia the main producing areas. Catalonia produced 55,368 tonnes of onion in 2004. Onion industry produces wastes that yield an approximated 15% of the total production that is annually changeable. Therefore, this variability among harvests every year leads the industry to have an onion overproduction those years with a high volume of onion production. The 90% of onion produced in Catalonia is cultivated in Lleida. In Catalonia the production in 2004 was 18,250 tonnes of 'Recas' onion cultivar, 13,600 tonnes of 'Figueres' onion cultivar and 3000 tonnes of the rest of onion cultivars and varieties.

Onion nutritional composition is very complex. It has been shown that it is one of the major sources of dietary flavonoids in many countries. Specifically, onion has been characterised for its flavonol quercetin and quercetin derivates. Moreover, it is rich in other bioactive compounds such as frutooligosaccharides and sulfur compounds.

Epidemiological studies have indicated that the consumption of fruits and vegetables is associated with a reduced risk for the development of chronic diseases, such as cardiovascular disease and cancer. Phytochemicals, including phenolics and flavonoids, are suggested to be the major bioactive compounds contributing to the health benefits of fruits and vegetables (Yang, Meyers, Van der Heide, & Liu, 2004). Quercetin is one of the abundant flavonol-type flavonoids commonly found in vegetables and fruits (Moon, Nakata, Oshima, Inakuma, & Terao, 2000). Onion ranked highest in quercetin content in a survey of 28 vegetables and 9 fruits (Hertog, Hollman, & Venema, 1992). It shows a variety of pharmacological effects such as growth inhibition of tumour and microbial cells, reduction of cancer risk, scavenging of free radicals, and protection against cardiovascular disease, which are attributed to specific sulfur-containing compounds and flavonoids (Ly et al., 2005). In addition, onions have been found to have antioxidant properties in different *in vitro* models (Kim & Kim, 2006; Nuutila, Puupponen-Pimiä, Aarni, & Oksman-Caldentey, 2003).

A number of by-products have been previously studied as potential sources of antioxidants. In fact, an interesting approach to utilise by-products is their potential use as sources of natural compounds with high antioxidant activity (Larrosa, Llorach, Espín, & Tomás-Barberán, 2002). Onion wastes adequately processed and stabilised could be useful in the food industry as functional ingredients to be added to processed foods due to the increasing demand by consumers for substituting synthetic compounds by natural substances as food ingredients. Compounds of inherently natural origin would be widely accepted by consumers in the market (Jang, Sanada, Ushio, Tanaka, & Ohshima, 2002).

Nowadays, one of the major concern for the food industry is to prevent the development of enzymatic browning prior to or during the processing of fruits and vegetables because of the alteration in the organoleptic and visual properties of the product. A quality loss is also a fact to take into account due to the phenolic compounds content decrease that occurs during the enzymatic browning (Tomás-Barberán & Espín, 2001). Recent studies have shown that sulfhydryl (SH or thiol) groups are good inhibitors of the enzyme PPO (Ding, Chachin, Ueda, & Wang, 2002), Therefore, it is assumed that the thiol compounds contained in onion might be the active components responsible for the PPO inhibitory effect of onion. Onion extracts could be used as natural food ingredients for the prevention of browning caused by PPO (Kim, Kim, & Park, 2005).

In this work, we attempt to evaluate onion by-products stabilised by different treatments in order to show their bioactive, antioxidant, and antibrowning properties. This would trigger to choose the onion by-product showing better characteristics for its potential use as antioxidant and antibrowning food ingredient.

2. Materials and methods

2.1. Chemicals

Acetonitrile and methanol were obtained from Labscan Ltd. (Dublin, Ireland). Di-sodium hydrogen phosphate anhydrous, sodium dihydrogen phosphate monohydrate, and sodium carbonate anhydrous were purchased from Merck KGaA (Darmstadt, Germany). Hydrochloric acid and *ortho*-phosphoric acid were purchased from Panreac Química, S. A. (Barcelona, Spain). Catechol, chlorogenic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), Folin–Ciocalteu's phenol reagent, polyvinylpolypyrrolidone, and quercetin were obtained from Sigma–Aldrich, Inc. (St. Louis, MO, USA).

2.2. Samples

2.2.1. Onion by-products. Processing and stabilisation treatments

'Figueres' and 'Recas' onion wastes from the harvesting period of 2005 (*Allium cepa* L. var. *cepa*) were supplied by a producing onion industry, CEBACAT (Asociación Catalana de Productores y Comercializadores de Cebolla) in Lleida (Catalonia, Spain). Their processing and stabilisation was held in The National Center for Food Technology and Safety (CNTA) in San Adrián (Navarra, Spain). Stabilised onion by-products analyses were performed in Instituto del Frío, Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain.

Previously, onions wastes roots were removed and sheered with a 10×10 mm rack. Then, these onions were processed with a friction screw press to obtain the following three onion by-products: onion juice (the liquid fraction), onion paste (a mixture between the solid and the liquid fractions) and onion bagasse (the solid fraction).

Juice, paste, and bagasse by-products from 'Figueres' and 'Recas' onion cultivars wastes were packed into sterilisable bags (PET/ALU/OPA/PP, Amcor Flexibles Hispania S. A., Granollers, Barcelona, Spain) for the sterilisation and pasteurisation treatments; and into trays (PP/EVOH/PP, EDV, Llinars del Vallés, Barcelona, Spain) for the freezing treatment. Sterilisation (at 115 °C, 17–31 min) and pasteurisation (at 100 °C, 11–17 min) took place in a conventional autoclave. Sterilised and pasteurised onion by-products were stored at −4 °C until analysis. Freezing treatment (at −70 °C) was carried out in a liquid nitrogen cabinet (Frigothermic, model L.S.1, Martorell, Barcelona, Spain) until the product reached −18 °C. Frozen onion by-products were stored at −18 °C until analysis.

2.3. Analysis

Stabilised onion by-products were analysed for their bioactive composition, and their antioxidant and antibrowning properties.

2.3.1. Bioactive composition

2.3.1.1. Total phenols. Total Phenols were determined spectrophotometrically (Vinson, Hao, Su, & Zubik, 1998). Analyses were performed by visible spectrophotometry at 760 nm after reaction with Folin–Ciocalteu's reagent.

Juice (50 mL), paste or bagasse (10 g) plus 25 mL methanol/water (80:20, v/v) were homogenised in duplicate in an ultrahomogeniser (Omni mixer, model ES-270, Omni International Inc., Gainesville, VA, USA). Extracts were made up to 100 mL with methanol for juice and up to 50 mL for paste and bagasse. Next, they were introduced into test tubes and then 1.0 mL Folin–Ciocalteu's reagent and 0.8 mL sodium carbonate (7.5%) were added. The absorbance of all samples was measured at 760 nm after incubating at room temperature for 1 h. Results were calculated by a calibration curve obtained from chlorogenic acid and expressed as milligrams of chlorogenic acid equivalents (CAE) per 100 g of dry weight (dw).

2.3.1.2. Extraction, separation, identification and quantification of quercetin. Total quercetin was determined by high performance liquid chromatography (HPLC). The extraction was carried out according to the method by Hertog et al. (1992) with minor modifications.

2.3.1.2.1. Hydrolysis mixture. Juice (50 mL), paste or bagasse (10 g) plus 25 mL methanol/water (80:20, v/v) were mixed with 5 mL of a 6 M HCl solution. No antioxidants were added to the hydrolysis mixture. The hydrolysis was performed in duplicate. After refluxing at 90 °C for 4 h, the extract was allowed to cool, vacuum filtered, made up to 100 mL with methanol for juice and up to 50 mL for paste and bagasse, next sonicated. The extracts were filtered through a 0.45 μm membrane filter for organic solvents prior to injection. Duplicates of 20 μL for each extract were analysed by HPLC.

2.3.1.2.2. HPLC procedure. The analytical HPLC system employed consisted of a Hewlett-Packard (Palo Alto, CA, USA) Model 1050 coupled with a quaternary solvent delivery pump and equipped with an autosampler (G1329A ALS) with a 20 μL sample loop and a Hewlett-Packard 1040A rapid scanning UV–vis photodiode array detector. Separation of flavonoids was performed on a reverse-phase Zorbax Eclipse XDB C_{18} Hypersil ODS (5 μm) stainless steel column (250x4.6 mm i.d., 5 μm particle size) (Agilent, Spain). The mobile phase was deionised Milli-Q water adjusted to a pH 2.5 with *ortho*-phosphoric acid (solution A) and acetonitrile (solution B). The program began with a gradient elution from 90% to 65% A, and from 10% to 35% B for 20 min, followed by a gradient from 65% to 90% A, and from 35% to 10% B for the next 5 min. The flow rate was fixed at 1 mL/min and runs were monitored with the UV–vis photodiode array detector which was set at 370 nm. The data were stored and processed using a Hewlett-Packard (Palo Alto, CA, USA) ChemStation and related software. Identification of the quercetin was carried out by HPLC by comparing the retention time and UV–vis absorption spectrum with those of the quercetin standards. The quantification was achieved by the absorbance recorded in the chromatograms relative to the external standards of flavonoids previously referred to. Total quercetin content was expressed as milligrams of total quercetin per 100 g of dry weight (dw).

2.3.2. Scavenging effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH·) radical

Antioxidant activity was determined by the measurement of the DPPH· radical scavenging (Sánchez-Moreno, Larrauri, & Saura-Calixto, 1998).

2.3.2.1. Extraction. Juice (50 mL), paste or bagasse (10 g) plus 25 mL methanol/water (80:20, v/v) were mixed with 5 mL of a 6 M HCl solution. No antioxidants were added to the hydrolysis mixture. The hydrolysis was performed in duplicate. The refluxing period in this case was 2 h.

2.3.2.2. DPPH· radical scavenging capacity. The determination of the radical scavenging capacity was evaluated with the stable radical DPPH·. The method is described extensively elsewhere (Sánchez-Moreno, Plaza, de Ancos, & Cano, 2003). The parameters EC_{50}, which reflects 50% depletion of initial DPPH· radical and the time needed to reach the steady state at EC_{50} concentration (T_{EC50}) were calculated. The antiradical efficiency ($AE = 1/EC_{50} \times T_{EC_{50}}$), a parameter that combines both factors, was also calculated.

2.3.3. Polyphenol oxidase (PPO) inhibition assay

Minor modifications of the Kim et al. (2005) research were carried out in order to evaluate the inhibitory effect of onion stabilised by-products extracts on avocado polyphenol oxidase.

2.3.3.1. Avocado PPO extraction. Avocados (*Persea americana* Miller var. *americana* 'Fuerte') were purchased from a Spanish local supermarket. They were peeled, cut into small pieces and frozen with liquid nitrogen. Afterwards, the frozen pieces were grinded and homogenised into a blender (Osterizer, NC, USA) and stored at −20 °C until their analysis.

Avocado frozen powder (2 g) was mixed with polyvinylpolypyrrolidone (PVPP) (0.8 g) and homogenised in an ultrahomogeniser with 20 mL of a sodium phosphate buffer solution (0.1 M, pH 6.5) for 3 min. The homogenate was centrifuged at 9500*g* for 15 min at 4 °C. The supernatant was collected and filtered through a six coat cheese cloth. The filtered was used as avocado PPO enzyme extract throughout this experiment. All steps were carried out at 4 °C.

2.3.3.2. Onion extracts preparation. The previous step was to freeze-dry the onion by-products in a lyophilizer (model Lyoalfa, Telstar, S. A., Barcelona, Spain). Freeze dried onion by-products (1.2 g) were homogenised with distilled water (20 mL) for 3 min in the ultrahomogeniser. The homogenate was centrifuged at 17,500*g* for 20 min at 4 °C. The supernatants were vacuum filtered through a 0.45 μm membrane filter. Each extraction was prepared in duplicate.

2.3.3.3. PPO inhibition assay. The PPO activity was assayed with 0.07 M catechol as a substrate by a spectophotometric procedure (Kim et al., 2005).

Polyphenol oxidase activity was assayed using the stabilised onion by-products extracts (1 mL), the PPO avocado extract (0.1 mL) and a solution of 0.07 M catechol (1 mL) in a sodium phosphate buffer (0.05 M, pH 6.5) (0.9 mL). The total volume of the PPO inhibition assay was 3 mL. Firstly, the inhibition reaction mixture (stabilised onion by-product extracts and PPO extract) was incubated for 5 min at 25 °C. Immediately after, the rest of the reactants were added. Absorbance at 420 nm was monitored at 25 °C for 30 s.

The results were expressed as relative enzymatic activity (REA): the percentage of PPO activity were measured and extrapolated to 100% REA (in percentage, %). Thus, REA represents the residual PPO activity reached after adding different onion by-products as natural inhibitors to the model solution.

2.3.4. Statistical analysis

Results were given as mean ± standard deviation of six independent determinations. One-way analysis of variance (ANOVA) was used to compare the means. Differences were considered significant at $P < 0.05$. All statistical analyses were performed with Statgraphics Plus 5.1 (Statistical Graphics Corporation, Inc., Rockville, MD, USA).

3. Results and discussion

The following results are exposed regarding different onion by-products within the same stabilisation treatment. Thus, discussion will compare the bioactive composition (total phenols and total quercetin), the antioxidant activity, and the inhibition PPO capacity parameters in 'Recas' and 'Figueres' frozen, pasteurised and sterilised onion by-products (juice, paste and bagasse).

3.1. Bioactive composition (total phenol and total quercetin content)

From a nutritional point of view, it is desirable to minimise the loss of the biological activity of onion by-products throughout processing by controlling all the technological and stabilisation parameters involved in all operations of the process. Therefore, to obtain a representative onion by-product offering better characteristics as a food ingredient it is crucial to focus on the type of onion by-product and on the stabilisation treatment applied.

In our work, total phenols and total quercetin content were measured in onion by-products in order to evaluate their bioactive composition.

Regarding different onion by-products within the same stabilisation treatment our results were the following:

Frozen 'Recas' paste showed the highest total phenol content among all the frozen 'Recas' by-products analysed. Frozen 'Figueres' onion by-products showed significantly different ($P < 0.05$) total phenol content among them (Table 1). Frozen 'Recas' paste was also the onion by-product which reached the highest total quercetin content (4431.21 ± 415.23 mg/100 g dw) among all the stabilised

Table 1
Bioactive compounds and antioxidant activity of frozen onion by-products[a]

By-product	Cultivar	Total phenols (mg CAE/100 g dw)	Total quercetin (mg/100 g dw)	EC_{50} (g dw/g DPPH•)	$T_{EC_{50}}$ (min)
Juice	'Figueres'	118.56 ± 4.01Aa	57.90 ± 13.13Aa	10.75 ± 0.17Ca	31.12 ± 3.55Aa
	'Recas'	183.96 ± 23.74Ab	214.64 ± 18.22Ab	12.35 ± 0.38Bb	45.33 ± 1.34Cb
Paste	'Figueres'	238.95 ± 43.62Ba	671.48 ± 51.54Ca	1.76 ± 0.007Ba	22.23 ± 4.07Aa
	'Recas'	441.31 ± 50.93Cb	4431.21 ± 415.23Cb	4.05 ± 0.04Ab	28.15 ± 0.16Aa
Bagasse	'Figueres'	407.64 ± 32.02Cb	600.72 ± 7.24Ba	1.47 ± 0.39Aa	63.35 ± 7.98Bb
	'Recas'	330.40 ± 10.81Ba	2230.89 ± 277.25Bb	4.12 ± 0.34Ab	36.27 ± 4.64Ba

[a] Values are means ± SD, $n = 6$. Means within a column with different capital letters in different by-products for the same cultivar are significantly different at $P < 0.05$. Means within a column with different small letters in the same by-product for different cultivars are significantly different at $P < 0.05$.

Table 2
Bioactive compounds and antioxidant activity of pasteurised onion by-products[a]

By-product	Cultivar	Total phenols (mg CAE/100 g dw)	Total quercetin (mg/100 g dw)	EC_{50} (g dw/g DPPH·)	$T_{EC_{50}}$ (min)
Juice	'Figueres'	128.23 ± 33.7Aa	23.13 ± 4.10Aa	3.37 ± 0.03Ba	58.75 ± 3.29Bb
	'Recas'	151.03 ± 10.71Aa	31.44 ± 2.02Ab	3.96 ± 0.04Cb	52.47 ± 0.37Aa
Paste	'Figueres'	143.01 ± 7.55Aa	131.98 ± 13.68Ba	1.33 ± 0.32Aa	52.86 ± 2.17Aa
	'Recas'	329.77 ± 83.49Bb	195.17 ± 7.27Bb	2.30 ± 0.04Ab	52.98 ± 1.04Aa
Bagasse	'Figueres'	143.55 ± 11.13Aa	212.19 ± 29.19Ca	3.21 ± 0.011Bb	54.99 ± 1.94ABa
	'Recas'	453.29 ± 29.36Cb	721.37 ± 4.94Cb	2.65 ± 0.020Ba	61.18 ± 0.17Bb

[a] Values are means ± SD, $n = 6$. Means within a column with different capital letters in different by-products for the same cultivar are significantly different at $P < 0.05$. Means within a column with different small letters in the same by-product for different cultivars are significantly different at $P < 0.05$.

Table 3
Bioactive compounds and antioxidant activity of sterilised onion by-products[a]

By-product	Cultivar	Total phenols (mg CAE/100 g dw)	Total quercetin (mg/100 g dw)	EC_{50} (g dw/g DPPH·)	$T_{EC_{50}}$ (min)
Juice	'Figueres'	153.15 ± 39.28Aa	11.83 ± 0.11Aa	32.48 ± 2.83Bb	43.89 ± 3.96Ba
	'Recas'	213.79 ± 31.08Aa	79.08 ± 7.81Ab	14.86 ± 0.41Ca	57.25 ± 3.23Cb
Paste	'Figueres'	416.21 ± 38.53Ba	260.17 ± 3.30Ba	4.07 ± 0.09Ab	27.05 ± 2.58Aa
	'Recas'	591.25 ± 21.01Cb	489.78 ± 9.48Bb	3.34 ± 0.02Ba	43.01 ± 0.18Ab
Bagasse	'Figueres'	220.51 ± 37.30Aa	310.92 ± 36.38Ca	6.13 ± 0.40Ab	29.78 ± 0.53Aa
	'Recas'	398.79 ± 26.61Bb	724.72 ± 5.78Cb	2.61 ± 0.05Aa	51.23 ± 0.54Bb

[a] Values are means ± SD, $n = 6$. Means within a column with different capital letters in different by-products for the same cultivar are significantly different at $P < 0.05$. Means within a column with different small letters in the same by-product for different cultivars are significantly different at $P < 0.05$.

by-products analysed. Frozen 'Figueres' onion by-products did not reached such accused total quercetin content (Table 1).

Pasteurised 'Recas' bagasse showed higher total phenol content than those shown by pasteurised 'Recas' paste or juice. Pasteurised 'Figueres' by-products did not show significantly difference ($P > 0.05$) in their total phenol content among them (Table 2). Likewise, pasteurised 'Recas' bagasse was the onion pasteurised by-product which showed the highest total quercetin content (721.37 ± 4.94 mg/100 g dw) followed by 'Recas' paste or juice, significantly different ($P < 0.05$) among them. Pasteurised 'Figueres' by-products were also significantly different ($P < 0.05$) among them regarding total quercetin content. Pasteurised 'Figueres' bagasse showed the highest total quercetin content (212.19 ± 29.19 mg/100 g dw) followed by pasteurised 'Figueres' paste and juice (Table 2).

Sterilised 'Recas' paste showed higher total phenol content than sterilised 'Recas' bagasse and juice. In the same way, sterilised 'Figueres' paste showed higher total phenol content than sterilised 'Figueres' bagasse or juice (Table 3). Sterilised 'Recas' bagasse showed significantly higher ($P < 0.05$) total quercetin content (724.72 ± 5.78 mg/100 g dw) than sterilised 'Recas' paste or juice. Sterilised 'Figueres' by-products had the same behaviour than pasteurised 'Recas' by-products, being sterilised 'Figueres' bagasse the by-product which reached the highest total quercetin content (310.92 ± 36.38 mg/100 g dw) followed by paste or juice (Table 3). Sterilised onion by-products did not show significant differences ($P < 0.05$) compared to pasteurised ones, being 'Recas' and 'Figueres' bagasses the by-products showing the highest total quercetin content followed by 'Recas' and 'Figueres' pastes or juices.

Generally, stabilised by-products from 'Recas' onion cultivar had a significantly higher ($P < 0.05$) bioactive composition (total phenols and quercetin) than those by-products from 'Figueres' cultivar (Tables 1–3).

Concerning total phenol content among 'Recas' onion by-products analysed, sterilised and frozen pastes showed the highest values followed by pasteurised 'Recas' bagasse. In addition, freezing and pasteurisation stabilisation treatments did not rend significantly differences ($P > 0.05$) in total phenol content when 'Recas' paste was analysed.

Referring to total quercetin content in 'Recas' onion by-products, it was shown that frozen 'Recas' paste had the highest content among all the stabilised pastes analysed, followed by pasteurised and sterilised 'Recas' bagasses which did not show significant differences ($P > 0.05$). Pasteurised and sterilised 'Recas' paste were significantly different ($P < 0.05$) to frozen 'Recas' paste.

Our results showed that 'Recas' onion cultivar and paste by-product were one of the best choices due to their higher bioactive content. In this work, it has been shown that bioactive compounds are highly concentrated in those onion by-products containing more wall cells like paste or bagasse than in juice which loss a great proportion of wall cells during its process. Moreover, the by-products from the colourful cultivar 'Recas' showed moderately high bioactive compounds values. Previous works stated that onion unutilised outer layers of a red variety had the higher contents of total phenols followed by a continuous decrease towards the inner part of the bulb. They were a rich source of quercetin with high antioxidant activity and showed significant protection of DNA damage caused by free radicals (Prakash, Singh, & Upadhyay, 2007).

Stabilisation treatments applied would have to be carefully chosen. These treatments not only would have to maintain as higher bioactive composition as possible but also ensure the safety and stability of these onion by-products during its whole self-life. In our work, sterilisation was a thermal treatment (115 °C) which provoked a higher phenol release compared with the pasteurisation (100 °C), and freezing (−18 °C). This fact is in agreement with previous works that attribute to the thermal treatment an increase in the release of bioactive compounds from the cell walls of the onion skin or the onion outer tissues (Lombard, Peffley, Geoffriau, Thompson, & Herring, 2005). In addition, Kim et al (2006) showed that the total phenol content of grape seed extracts was significantly increased by heat treatments, indicating that phenolic compounds in these extracts were liberated by heat treatments. Onions contain large amounts of quercetin glycosides and they are often subject to thermal processes in food production. The thermal treatment led to a degradation of the quercetin glycosides. The main product is the aglycone quercetin, which remained stable during further roasting (180 °C) (Rohn, Buchner, Driemel, Rauser, & Kroh, 2007). Thus, this flavonol may be stable at the 115 °C temperature applied in the sterilisation and it would be stable at lower temperatures applied in pasteurisation or freezing treatments.

Analysing the applied stabilisation treatments is crucial to choose one treatment that does not involve microbiological risk in order to develop a safe food ingredient. In our study, freezing was a treatment which may not be chosen as a stabilisation treatment due to the microbiological risk it could involve (data not shown). In addition, sterilisation may produce caramelised compounds in the onion by-products stabilised by this treatment. This fact could influence on their nutritional composition by causing a great loss in the bioactive composition measured, total quercetin content indeed. By contrast, pasteurisation as a mild thermal treatment would represent the best choice to stabilise onion by-products maintaining mainly intact their bioactive composition. Our results showed that this stabilisation treatment caused a low decrease in the total phenols and quercetin content measured in the onion by-products analysed (compared to freezing or sterilisation).

3.2. Antioxidant activity (DPPH· stable radical scavenging)

Several radical scavenging parameters were measured: EC_{50}, $T_{EC_{50}}$, and antiradical efficiency (AE). The AE was calculate in order to evaluate the total antioxidant activity, this parameter combines both factors (EC_{50} and $T_{EC_{50}}$) (Sánchez-Moreno et al., 1998).

Regarding AE as antioxidant parameter and comparing the effect of the onion processing within the same stabilisation treatment the results were the following:

Frozen 'Recas' paste ($8.7 \pm 0.003 \times 10^{-3}$) showed a significantly higher ($P < 0.05$) AE value than frozen 'Recas' bagasse ($3.4 \pm 0.58 \times 10^{-3}$) or juice ($1.7 \pm 0.12 \times 10^{-3}$). When analysing 'Figueres' onion cultivar, frozen paste ($25.8 \pm 4.85 \times 10^{-3}$) showed significantly higher ($P < 0.05$) AE value than frozen 'Figueres' bagasse ($9.5 \pm 6.36 \times 10^{-3}$) or juice ($3.0 \pm 0.14 \times 10^{-3}$) (Fig. 1a).

Pasteurised and frozen 'Recas' paste had similar AE values ($P > 0.05$). In addition, pasteurised 'Recas' paste was more efficient scavenging radicals than pasteurised 'Recas' bagasse or juice ($8.0 \pm 0.3 \times 10^{-3}$ *vs.* $6.1 \pm 0.06 \times 10^{-3}$ and $4.8 \pm 0.1 \times 10^{-3}$, respectively). Pasteurised 'Figueres' paste ($15.0 \pm 4.2 \times 10^{-3}$) showed significantly higher AE value than pasteurised 'Figueres' bagasse ($5.6 \pm 0.2 \times 10^{-3}$) or juice ($5.0 \pm 0.3 \times 10^{-3}$). Thus, pasteurised 'Figueres' paste ($15.0 \pm 4.2 \times 10^{-3}$) showed the highest value

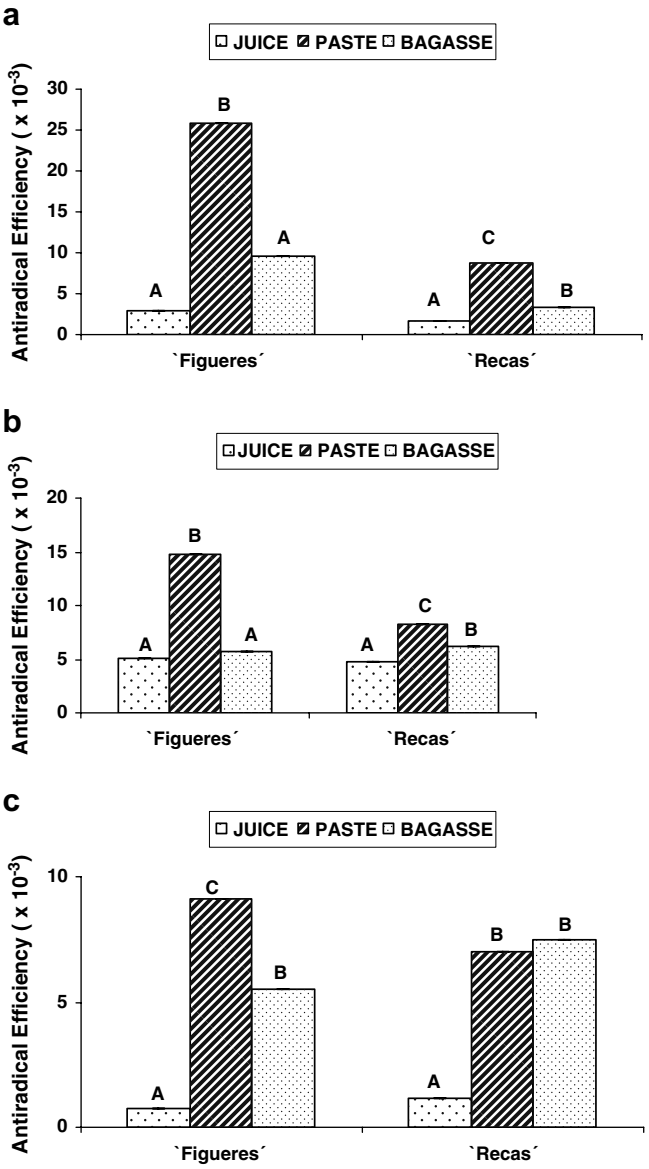


Fig. 1. Antiradical efficiency (AE) of frozen onion by-products (a), pasteurised onion by-products (b), and sterilised onion by-products (c). Bars with different capital letters in different by-products for the same cultivar are significantly different at $P < 0.05$. AE expressed as $1/[EC_{50}$ (g dw/g DPPH·) $\times T_{EC_{50}}$ (min)].

among all the pasteurised onion by-products analysed ('Recas' and 'Figueres') (Fig. 1b).

There were not significantly differences ($P > 0.05$) between sterilised 'Recas' paste ($7.0 \pm 0.6 \times 10^{-3}$) and bagasse ($7.5 \pm 0.007 \times 10^{-3}$) AE values. The AE value shown by sterilised 'Recas' juice ($1.17 \pm 0.03 \times 10^{-3}$) was significantly lower ($P < 0.05$) than sterilised 'Recas' paste ($7.0 \pm 0.6 \times 10^{-3}$) and bagasse ($7.5 \pm 0.007 \times 10^{-3}$). Sterilised 'Figueres' paste ($9.1 \pm 0.6 \times 10^{-3}$) AE value was significantly higher than sterilised 'Figueres' bagasse and juice ($5.5 \pm 0.5 \times 10^{-3}$, and $0.72 \pm 0.04 \times 10^{-3}$, respectively) (Fig. 1c).

In general, pastes from the two onion cultivars assayed showed higher antiradical efficiency values. Therefore, they showed better characteristics as potential antioxidant food ingredients. Pasteurised and frozen 'Recas' pastes reached the following AE values: $8.0 \pm 0.3 \times 10^{-3}$ and $8.7 \pm 0.003 \times 10^{-3}$, respectively. These values were significantly higher than that found in sterilised 'Recas' paste ($7.0 \pm 0.6 \times 10^{-3}$).

The correlation between antioxidant capacity and bioactive composition (total phenols and flavonoids) has been widely studied (Aviram & Aviram, 2002; Pyo, Lee, Logendra, & Rosen, 2004; Sánchez-Moreno et al., 2003; Sellappan & Akoh, 2002). Nuutila et al. (2003) found an observable correlation between high radical scavenging/antioxidant activity and high amounts of total phenolics and flavonoids of the onion extracts, resulting the phenolic compounds of *Allium* plants contribute to their antioxidative properties. Moreover, these authors showed that the skin extracts of onion possessed the highest activities (Nuutila et al., 2003). Thus, in our work the correlation between antioxidant capacity and bioactive composition was also studied in the two onion cultivars by-products assayed.

Concerning 'Recas' paste, our results showed that the EC_{50} parameter and total phenols were inversely correlated in the frozen and sterilised 'Recas' paste ($r = -0.9552$; $P = 0.0001$ *vs.* $r = -0.8196$; $P = 0.0068$, respectively). EC_{50} parameter and total quercetin showed a significant inverse correlation in frozen and sterilised 'Recas' paste ($r = -0.9832$; $P = 0.0001$ *vs.* $r = -0.9495$; $P = 0.0001$, respectively). A significant inverse correlation between the EC_{50} parameter and total phenols ($r = -0.7566$; $P = 0.0183$) was shown in pasteurised 'Recas' paste. Probably other bioactive compounds that have not been analysed in this research would be responsible of the antioxidant capacity found in pasteurised paste.

Antioxidant capacity of onion has been widely studied. There have been shown different antioxidant capacities among different cultivars or varieties (Aoyama & Yamamoto, 2007; Benkeblia, 2005; Nuutila et al., 2003; Yang et al., 2004). Moreover, it has been elucidated an increasing antioxidant activity from the inner to the outer part of the onion (Kim et al., 2006; Ly et al., 2005; Suh, Lee, Cho, & Chung, 1999). In concordance, our results showed that there was a difference between the two cultivars analysed 'Figueres' and 'Recas'. Generally, 'Recas' onion by-products assayed offered better radical scavenger properties than 'Figueres' onion by-products. In addition, by-products with a higher content of outer parts of onion (paste and bagasse) showed higher antioxidant activity than juices.

Processing and stabilising onion wastes may have an impact on the antioxidant activity measured. As different thermal treatments applied to onion caused a lost in the free radical scavenging properties found in this fresh vegetable, the temperature used to stabilise onion by-products must be carefully controlled in order not to lose the potential antioxidant properties of these by-products (Agostini, Jimenez, Ramón, & Gómez, 2004; Fu, 2004; Kawamoto, Sakai, Okamura, & Yamamoto, 2004; Yin & Cheng, 1998).

In our study, pasteurised 'Recas' paste offered better characteristics than pasteurised 'Recas' bagasse or juice as antioxidant food ingredient due to the lower concentration (EC_{50}) needed to scavenge the stable radical DPPH$^{\cdot}$ (Table 2). In this context, it is important to take into account that onion by-products have been used to increase antioxidant characteristics in tomato juice (Larrosa et al., 2002).

Pasteurisation was a mild treatment that did not reach the high temperatures found when sterilisation was applied, maintaining better the antioxidant properties of the by-products analysed.

3.3. Antibrowning activity (polyphenol oxidase inhibition assay)

The use of natural inhibitors of PPO is still stimulated by the need to replace sulfating agents in order to prevent or minimize the loss of fresh or processed foodstuffs (Billaud, Brun-Mérimée, Louarme, & Nicolas, 2004). From a technological point of view, it would be conceivable to use natural antibrowning agents in processed fruits provided that their safety is assessed and their commercial feasibility is demonstrated. Among the numerous compounds capable of reducing enzymatic browning and/or oxidoreductase activity, onion has been found to have bioactive compounds with such properties (Eissa, Fadel, Ibrahim, Hassan, & Abd Elrashid, 2006).

In this work, PPO activities of avocado fruit were significantly reduced by the different onion by-products analysed. In order to measure their antibrowning capacity, we compared the onion by-products within the same stabilisation treatment and the results showed the following behaviour:

Frozen 'Recas' paste reduced significantly ($P < 0.05$) the avocado PPO activity (57.08%) followed by frozen 'Recas' juice and bagasse, averaging 39.69%. By contrast, when 'Figueres' by-products were analysed, frozen bagasse was the by-product with a significantly higher ($P < 0.05$) inhibitory enzymatic effect (55.82%), followed by 'Figueres' frozen paste (34.51%) and juice (29.25%) (Table 4).

Pasteurised 'Recas' paste and juice reduced PPO activity 53.49% and 65.52%, respectively meanwhile pasteurised

Table 4
Inhibitory enzymatic effect of onion by-products[a]

Antibrowning agent		Relative enzymatic activity (REA, %)	
Onion by-product	Stabilisation treatments	Cultivar	
		'Figueres'	'Recas'
Juice	Freezing	70.75 ± 3.25Bb	60.38 ± 3.00Ca
	Pasteurisation	51.81 ± 4.91Ab	34.48 ± 1.81Aa
	Sterilisation	63.51 ± 4.84Bb	40.36 ± 2.50Ba
Paste	Freezing	65.49 ± 3.27Bb	42.92 ± 3.40Ba
	Pasteurisation	67.18 ± 4.57Bb	46.50 ± 2.78Ba
	Sterilisation	31.86 ± 1.84Ab	10.29 ± 0.81Aa
Bagasse	Freezing	44.18 ± 9.70Aa	60.23 ± 1.96Bb
	Pasteurisation	72.53 ± 7.04Ba	86.08 ± 9.24Ca
	Sterilisation	61.22 ± 2.68Bb	27.32 ± 3.41Aa

[a] Values are means ± SD, $n = 6$. Means within a column with different capital letters in different by-products for the same cultivar and stabilisation treatment are significantly different at $P < 0.05$. Means within a column with different small letters in the same by-product and stabilisation treatment for different cultivars are significantly different at $P < 0.05$.

'Recas' bagasse reduced it 13.92%. Pasteurised 'Figueres' juice reduced PPO activity 48.19%, and pasteurised 'Figueres' bagasse and paste did not show significantly difference ($P > 0.05$) among them. Pasteurised 'Recas' paste inhibitory capacity towards avocado PPO was higher (53.49%) than the capacity shown by pasteurised 'Figueres' paste (32.82%) (Table 4).

Sterilised 'Recas' paste reduced PPO activity 89.71% meanwhile sterilised 'Recas' bagasse and juice did it 72.68% and 59.64%, respectively. Sterilised 'Figueres' paste reduced PPO activity 68.14%. Sterilised 'Figueres'bagasse and juice reduced it 38.78% and 36.49%, respectively.

Interestingly, in our work it was shown the same behaviour by paste onion by-products when pasteurisation or sterilisation were applied. However, sterilised by-products showed more accused inhibitory effect than pasteurised ones. Thus, sterilised 'Recas' paste inhibitory capacity towards avocado PPO was higher (89.71%) than the capacity shown by pasteurised 'Recas' paste (53.49%) (Table 4).

The percentage of relative enzymatic activity found when pasteurised (110 °C, 11–17 min) 'Recas' paste (46.50%) was used as an antibrowning agent was similar to that found (45.9%) by Kim et al. (2005) when using heated onion extracts (100 °C, 10 min).

Technological and stabilisation processes applied to onion may influence significantly on their PPO inhibition capacity. Higher antibrowning activity was found in sterilised by-products followed by pasteurised and frozen ones. Sterilised 'Recas' and 'Figueres' pastes showed a high antibrowning effect reducing the PPO activity in 89.71% and 68.14%, respectively.

Recent studies have shown that sulfhydryl (SH or thiol) compounds are good inhibitors of the enzyme PPO (Ding et al., 2002; Jang et al., 2002; Martínez & Whitaker, 1995; Negishi & Ozawa, 2000). Onions are rich in two chemical compounds flavonoids and alk(en)yl cysteine sulfoxides (ACSO) (Griffiths, Trueman, Crowther, Thomas, & Smith, 2002). Therefore, it is generally assumed that sulfur compounds of low molecular weight contained in onions are responsible of the PPO inhibition.

It has been shown that heated onion extracts were more effective in prevention of pear and banana browning than fresh onion extracts (Kim et al., 2005; Lee, 2007). In our work, we have studied the effect caused by the temperature used to stabilise onion by-products on avocado PPO inhibition. The onion processing used to obtain the different onion by-products was also studied.

The positive effect of a temperature rise in onion extracts towards different fruits or vegetables PPO inhibition has been widely studied (Ding et al., 2002; Hosoda & Iwahashi, 2002; Kim et al., 2005; Lee et al., 2002). Moreover, a synergic effect among sulfur compounds (contained in onions), Maillard compounds and caramelisation products formed at high temperatures had also been postulated and studied in several researches (Billaud et al., 2004; Cheriot, Billaud, Maillard, & Nicolas, 2007; Gruber, Vieths, Wangorsch, Nerkamp, & Hofmann, 2004; Kim et al., 2005; Wagner, Reichhold, Koschutnig, Chériot, & Billaud, 2007).

Results of our research were in concordance with the researches previously cited. Generally, 'Recas' cultivar displayed PPO inhibiting properties more potent than that found in 'Figueres' cultivar in all the stabilisation treatments and onion by-products assayed. A temperature rise offered better antibrowning properties in all onion by-products assayed standing out paste. Data suggested that thermal treatments (pasteurisation and sterilisation) were mainly responsible of the avocado polyphenol oxidase inhibition, whereas non-thermal treatments (freezing) did not show such accused effect. Interestingly, it was shown that sterilised 'Recas' paste was the by-product with the strongest PPO inhibitory effect among all the onion by-products analysed.

Therefore, stabilising onion by-products by sterilisation would offer better antibrowning properties to these potential food ingredients than pasteurisation or freezing. By contrast, applying sterilisation as a stabilisation treatment would have the added problem of caramelisation and it might show the disadvantages exposed above. Thus, pasteurisation could represent a better choice in order to develop a food ingredient with an interesting added antibrowning property. Moreover this thermal treatment would maintain the safety of the food ingredient.

4. Conclusions

After analysing bioactive composition, antioxidant activity, and polyphenol oxidase inhibition capacity in the stabilised onion by-products from both cultivars ('Figueres' and 'Recas'), it was concluded that those by-products obtained from the 'Recas' onion cultivar showed better characteristics. Pasteurisation (100 °C, 11–17 min) applied as stabilisation treatment kept bioactive and technological characteristics of onion by-products. This treatment did not trigger the adverse effects caused by thermal

sterilisation such as caramelisation. Thus, pasteurised 'Recas' paste was chosen to be the most appropriate onion by-product for developing an antioxidant food ingredient among all the onion by-products analysed. It showed several advantages: a remarkable antioxidant activity (AE), a moderate high bioactive composition (total phenols and quercetin), and an excellent antibrowning effect from a technological point of view.

By-products derived from the manipulation and preparation of onion for its marketing involves a great economic loss for that sector food industry. From this study, it could be concluded that there is a real possibility of using those onion by-products for developing natural food ingredients with functional properties.

Acknowledgement

This research was supported by funding from the Ministry of Science and Technology (AGL2003-09138-C04-01). E. Roldán wishes to thank the Ministry of Education and Science for a Predoctoral Fellowship.

References

Agostini, L. R., Jimenez, M. J. M., Ramón, A. N., & Gómez, A. A. (2004). Determination of the antioxidant capacity of flavonoids in fruits and fresh and thermally treated vegetables. *Archivos Latinoamericanos de Nutrición, 54*, 89–92.

Aoyama, S., & Yamamoto, Y. (2007). Antioxidant activity and flavonoid content of Welsh onion (*Allium fistulosum*) and the effect of thermal treatment. *Food Science and Technology Research, 13*, 67–72.

Aviram, R., & Aviram, M. (2002). Onion juice polyphenols inhibits LDL oxidation: Stimulatory effect of juice storage and of the onion outer peel juice. *Free Radical Research, 36*, 69–70.

Balasundram, N., Sundram, K., & Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry, 99*, 191–203.

Benkeblia, N. (2005). Free-radical scavenging capacity and antioxidant properties of some selected onions (*Allium cepa* L.) and garlic (*Allium sativum* L.) extracts. *Brazilian Archives of Biology and Technology, 48*, 753–759.

Billaud, C., Brun-Mérimée, S., Louarme, L., & Nicolas, J. (2004). Effect of glutathione and Maillard reaction products prepared from glucose or fructose with glutathione on polyphenoloxidase from apple - I: Enzymatic browning and enzyme activity inhibition. *Food Chemistry, 84*, 223–233.

Cheriot, S., Billaud, C., Maillard, M.-N., & Nicolas, J. (2007). Inhibition of polyphenoloxidase activity by mixtures of heated cysteine derivatives with carbonyl compounds. *Molecular Nutrition & Food Research, 51*, 395–403.

Ding, C. K., Chachin, K., Ueda, Y., & Wang, C. Y. (2002). Inhibition of loquat enzymatic browning by sulfhydryl compounds. *Food Chemistry, 76*, 213–218.

Eissa, H. A., Fadel, H. H. M., Ibrahim, G. E., Hassan, I. M., & Abd Elrashid, A. (2006). Thiol containing compounds as controlling agents of enzymatic browning in some apple products. *Food Research International, 39*, 855–863.

Fu, H. Y. (2004). Free radical scavenging and leukemia cell growth inhibitory properties of onion powders treated by different heating processes. *Journal of Food Science, 69*, 50–54.

Griffiths, G., Trueman, L., Crowther, T., Thomas, B., & Smith, B. (2002). Onions – A global benefit to health. *Phytotherapy Research, 16*, 603–615.

Gruber, P., Vieths, S., Wangorsch, A., Nerkamp, J., & Hofmann, T. (2004). Maillard reaction and enzymatic browning affect the allergenicity of pru av 1, the major allergen from cherry (*Prunus avium*). *Journal of Agricultural and Food Chemistry, 52*, 4002–4007.

Hertog, M. G. L., Hollman, P. C. H., & Venema, D. P. (1992). Optimization of a quantitative HPLC determination of potentially anticarcinogenic flavonoids in vegetables and fruits. *Journal of Agricultural and Food Chemistry, 40*, 1591–1598.

Hosoda, H., & Iwahashi, Y. (2002). Inhibition of browning of apple slice and juice by onion juice. *Journal of the Japanese Society for Horticultural Science, 71*, 452–454.

Jang, M. S., Sanada, A., Ushio, H., Tanaka, M., & Ohshima, T. (2002). Inhibitory effects of 'enokitake' mushroom extracts on polyphenol oxidase and prevention of apple browning. *LWT-Food Science and Technology, 35*, 697–702.

Kawamoto, E., Sakai, Y., Okamura, Y., & Yamamoto, Y. (2004). Effects of boiling on the antihypertensive and antioxidant activities of onion. *Journal of Nutritional Science and Vitaminology, 50*, 171–176.

Kim, S. Y., Jeong, S. M., Park, W. P., Nam, K. C., Ahn, D. U., & Lee, S. C. (2006). Effect of heating conditions of grape seeds on the antioxidant activity of grape seed extracts. *Food Chemistry, 97*, 472–479.

Kim, S. J., & Kim, G. H. (2006). Quantification of quercetin in different parts of onion and its DPPH radical scavenging and antibacterial activity. *Food Science and Biotechnology, 15*, 39–43.

Kim, M., Kim, C. Y., & Park, I. (2005). Prevention of enzymatic browning of pear by onion extract. *Food Chemistry, 89*, 181–184.

Larrosa, M., Llorach, R., Espín, J. C., & Tomás-Barberán, F. A. (2002). Increase of antioxidant activity of tomato juice upon functionalisation with vegetable byproduct extracts. *LWT-Food Science and Technology, 35*, 532–542.

Lecain, S., Ng, A., Parker, M. L., Smith, A. C., & Waldron, K. W. (1999). Modification of cell-wall polymers of onion waste – Part I. Effect of pressure-cooking. *Carbohydrate Polymers, 38*, 59–67.

Lee, K. M. (2007). Inhibitory effect of banana polyphenol oxidase during ripening of banana by onion extract and Maillard reaction products. *Food Chemistry, 102*, 146–149.

Lee, M. K., Kim, Y. M., Kim, N. Y., Kim, G. N., Kim, S. H., Bang, K. S., et al. (2002). Prevention of browning in potato with a heat-treated onion extract. *Bioscience Biotechnology and Biochemistry, 66*, 856–858.

Lombard, K., Peffley, E., Geoffriau, E., Thompson, L., & Herring, A. (2005). Quercetin in onion (*Allium cepa* L.) after heat-treatment simulating home preparation. *Journal of Food Composition and Analysis, 18*, 571–581.

Ly, T. N., Hazama, C., Shimoyamada, M., Ando, H., Kato, K., & Yamauchi, R. (2005). Antioxidative compounds from the outer scales of onion. *Journal of Agricultural and Food Chemistry, 53*, 8183–8189.

Martínez, M. V, & Whitaker, J. R. (1995). The biochemistry and control of enzymatic browning. *Trends in Food Science and Technology, 6*, 195–200.

Moon, J. H, Nakata, R., Oshima, S., Inakuma, T., & Terao, J. (2000). Accumulation of quercetin conjugates in blood plasma after the short-term ingestion of onion by women. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology, 279*, R461–R467.

Negishi, O., & Ozawa, T. (2000). Inhibition of enzymatic browning and protection of sulfhydryl enzymes by thiol compounds. *Phytochemistry, 54*, 481–487.

Nuutila, A. M., Puupponen-Pimiä, R., Aarni, M., & Oksman-Caldentey, K.-M. (2003). Comparison of antioxidant activities of onion and garlic extracts by inhibition of lipid peroxidation and radical scavenging activity. *Food Chemistry, 81*, 485–493.

Peschel, W., Sánchez-Rabaneda, F., Diekmann, W., Plescher, A., Gartzia, I., Jiménez, D., et al. (2006). An industrial approach in the search of natural antioxidants from vegetable and fruit wastes. *Food Chemistry, 97*, 137–150.

Prakash, D., Singh, B. N., & Upadhyay, G. (2007). Antioxidant and free radical scavenging activities of phenols from onion (*Allium cepa*). *Food Chemistry, 102*, 1389–1393.

Pyo, Y. H., Lee, T. C., Logendra, L., & Rosen, R. T. (2004). Antioxidant activity and phenolic compounds of Swiss chard (*Beta vulgaris* subspecies *cycla*) extracts. *Food Chemistry, 85*, 19–26.

Rohn, S., Buchner, N., Driemel, G., Rauser, M., & Kroh, L. W. (2007). Thermal degradation of onion quercetin glucosides under roasting conditions. *Journal of Agricultural and Food Chemistry, 55*, 1568–1573.

Sánchez-Moreno, C., Larrauri, J. A., & Saura-Calixto, F. (1998). A procedure to measure the antiradical efficiency of polyphenols. *Journal of the Science of Food and Agriculture, 76*, 270–276.

Sánchez-Moreno, C., Plaza, L., de Ancos, B., & Cano, M. P. (2003). Effect of high-pressure processing on health-promoting attributes of freshly squeezed orange juice (*Citrus sinensis* L.) during chilled storage. *European Food Research and Technology, 216*, 18–22.

Sellappan, S., & Akoh, C. C. (2002). Flavonoids and antioxidant capacity of Georgia-grown Vidalia onions. *Journal of Agricultural and Food Chemistry, 50*, 5338–5342.

Suh, H. J., Lee, J. M., Cho, J. S., & Chung, S. H. (1999). Radical scavenging compounds in onion skin. *Food Research International, 32*, 659–664.

Tomás-Barberán, F. A., & Espín, J. C. (2001). Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *Journal of the Science of Food and Agriculture, 81*, 853–876.

Vinson, J. A., Hao, Y., Su, X. H., & Zubik, L. (1998). Phenol antioxidant quantity and quality in foods: vegetables. *Journal of Agricultural and Food Chemistry, 46*, 3630–3634.

Wagner, K. H., Reichhold, S., Koschutnig, K., Chériot, S., & Billaud, C. (2007). The potential antimutagenic and antioxidant effects of Maillard reaction products used as "natural antibrowning" agents. *Molecular Nutrition & Food Research, 51*, 496–504.

Yang, J., Meyers, K. J., Van der Heide, J., & Liu, R. H. (2004). Varietal differences in phenolic content and antioxidant and antiproliferative activities of onions. *Journal of Agricultural and Food Chemistry, 52*, 6787–6793.

Yin, M. C., & Cheng, W. S. (1998). Antioxidant activity of several Allium members. *Journal of Agricultural and Food Chemistry, 46*, 4097–4101.

Chapter 5

Onion high-pressure processing: Flavonol content and antioxidant activity

Eduvigis Roldán-Marín, Concepción Sánchez-Moreno, Rosana Lloría, Begoña de Ancos, M. Pilar Cano

LWT-Food Science and Technolology **(2009) 42, 835-841**

LWT - Food Science and Technology 42 (2009) 835–841

Contents lists available at ScienceDirect

LWT - Food Science and Technology

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

Onion high-pressure processing: Flavonol content and antioxidant activity

Eduvigis Roldán-Marín, Concepción Sánchez-Moreno*, Rosana Lloría, Begoña de Ancos, M. Pilar Cano

Department of Plant Foods Science and Technology, Instituto del Frío-CSIC, Madrid, Spain

ARTICLE INFO

Article history:
Received 2 June 2008
Received in revised form
8 October 2008
Accepted 13 November 2008

Keywords:
Onion
High-pressure processing
Flavonols
Quercetin glucosides
Antioxidant activity

ABSTRACT

Onion flavonol content and antioxidant activity have been related to human health promoting effects. Quercetin and quercetin glucosides (quercetin-4′-glucoside and quercetin-3,4′-diglucoside) have been reported the main onion flavonols in recent literature. Impact of combined treatments of high-pressure processing (HPP) and temperature on onion nutritional attributes has been scarcely studied.

Our study aimed to investigate the impact of HPP technology combined with temperature on onion (*Allium cepa* L. var. *cepa*, 'Grano de Oro') total phenol content, flavonol content, and antioxidant capacity. The experimental design comprised a response surface methodology according to a central composite face-centred design. The variable ranges were 100–400 MPa (pressure) and 5–50 °C (temperature), time was set up constant to 5 min.

Response surfaces of onion total quercetin, quercetin-4′-glucoside, and quercetin-3,4′-diglucoside content showed a similar pattern. The application of low temperature (5 °C) combined with pressures of 100 and 400 MPa triggered to a better extraction of these flavonols among the treatments analysed. Response surface of the EC_{50} antioxidant parameter as a function of pressure and temperature showed a clear trend towards an increase in onion antioxidant activity when applying pressures from 100 to 400 MPa. Four hundred megapascals/5 °C-processed onion showed an approximately 33% higher quercetin-4′-glucoside content compared with the untreated onion, and maintained the antioxidant activity of the untreated onion.

1. Introduction

Onions are a natural part of the daily diet for most of the world's population. Common yellow onion (*Allium cepa* L.) is a crop of great economic importance grown all over the world (Mogren, Olssen, & Gertsson, 2007). Onion phenol compounds, particularly flavonols, are known to be potent free radical scavengers and antioxidants; they are considered to be protective against cardiovascular diseases and to contribute in the prevention of colorectal cancers in humans (Caridi, Trenerry, Rochfort, Duong, Laugher, & Jones, 2007; Moskaug, Carlsen, Myhrstad, & Blomhoff, 2004; Prakash, Singh, & Upadhyay, 2007; Wenzel, Herzog, Kuntz, & Daniel, 2004). At least 25 different flavonols have been characterised in onions being quercetin and quercetin derivates the most predominant pigments in all onion cultivars. Quercetin-4′-glucoside and quercetin-3,4′-diglucoside are in most cases reported as the main onion flavonols in recent literature (Bonaccorsi, Caristi, Gargiulli, & Leuzzi, 2005; Bonaccorsi, Caristi, Gargiulli, & Leuzzi, 2008; Slimestad, Fossen, & Vågen, 2007).

Examining the manner in which onion is processed and consumed when considering its usefulness in preventing cardiovascular disease and obtaining the maximum health effects has to be taken into account. It was shown that onions have to be eaten raw or moderately cooked for obtaining those beneficial effects (Cavagnaro, Sance, & Galmarini, 2007). High-pressure processing (HPP) is a novel technology that has enormous potential in the food industry, controlling food spoilage, improving food safety, and extending product shelf life while retaining the characteristics of fresh, preservative-free, minimally processed food (Considine, Kelly, Fitzgerald, Hill, & Sleator, 2008). HPP foods have the distinct advantage of having the potential to be marketed as value-added foods due to the retention of organoleptic and nutritional qualities similar to those of 'fresh' unprocessed products (Rastogi, Raghavarao, Balasubramaniam, Niranjan, & Knorr, 2007). Thus, from a nutritional perspective, HPP is an attractive food preservation technology that clearly offers opportunities for horticultural and food processing industries to meet the growing demand from consumers for healthier food products and that has reached them

* Corresponding author. Department of Plant Foods Science and Technology, Instituto del Frío, Consejo Superior de Investigaciones Científicas (CSIC), C/ José Antonio Novais 10, Ciudad Universitaria, E-28040 Madrid, Spain, Tel.: +34 9 1549 2300; fax: +34 9 1549 3627.
E-mail address: csanchezm@if.csic.es (C. Sánchez-Moreno).

0023-6438/$34.00
doi:10.1016/j.lwt.2008.11.013

with a variety of new products (McInerney, Seccafien, Stewart, & Bird, 2007; Torres & Velazquez, 2005).

Onion processing may decrease the flavonoid content as a consequence of preparation and/or leakage from the vegetable (Lee et al., 2008). However, onion processing could increase the flavonoid extractability from the matrix resulting in a higher apparent content. Deglycosylation can occur due to enzymes released from disrupted plant tissue, and after consumption, due to β-glucosidases of the consumer's body, and those of microbial origin (Nemeth & Piskula, 2007). Onion processing can also lead to the deterioration of onion quality through colour changes and the formation of pink or green–blue pigments (Kubec, Hrbacova, Musah, & Velisek, 2004; Toivonen & Brummell, 2008).

In addition, when processing onion it is important to consider not only the consumer's perception and onion safety and quality but also onion nutritional attributes. Therefore, it was of our interest to analyse how a processing technology affects onion nutritional properties. High-pressure onion processing treatments could offer safe new onion products with similar organoleptic properties of fresh onion that additionally could offer potential human health benefits.

In our study, we decided to choose the Spanish onion variety (*Allium cepa* L. var. *cepa*, 'Grano de Oro') with the highest production and cultivated area in the year 2005, 581,074 and 10,485 tonnes and hectares, respectively (MAPA, 2006). Thus, we also took into account the potential economic importance of the raw material selected. Possible interactions between genetics and HPP have been recently reported for other vegetables varieties (Wolbang, Fitos, & Treeby, 2008), therefore potential interactions between the cultivar and the onion variety selected along with the HPP technology could be expected.

Our study aimed to investigate the effects of high-pressure processing (HPP) combined with temperature at constant time (5 min) applied to onion, focussing on onion nutritional attributes in terms of total phenol content, flavonol content (total quercetin (TQ), quercetin-4′-glucoside (Q_{MG}), quercetin-3,4′-diglucoside (Q_{DG})), and antioxidant capacity.

2. Materials and methods

2.1. Chemicals

Acetonitrile and methanol were obtained from Labscan Ltd. (Dublin, Ireland). Sodium carbonate anhydrous was purchased from Merck KGaA (Darmstadt, Germany). Hydrochloric acid and *ortho*-phosphoric acid were purchased from Panreac Química, S. A. (Barcelona, Spain). Chlorogenic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH•), Folin–Ciocalteu's phenol reagent and quercetin was obtained from Sigma–Aldrich Inc. (St. Louis, MO). Quercetin 4′-*O*-glucoside (spiraeoside) and quercetin-3,4′-*O*-glucoside were purchased from Extrasynthèse (France) and Polyphenols (Sandnes, Norway), respectively.

2.2. Raw material

Raw onions (*Allium cepa* L. var *cepa*, 'Grano de Oro') were supplied by a local supermarket in Madrid (Spain). Onions were harvested in October 2006 in Toledo (Spain). Their bulbs were free of external damages and exhibit a diameter of 70–90 mm. Initial characteristics of raw onion are shown in Table 1. Onions were hand peeled for their following analysis.

Table 1
Initial physical, physicochemical, and chemical characteristics of onion (*Allium cepa* L. var. *cepa*, 'Grano de Oro').

Characteristic	Value[a]
pH	5.02 ± 0.06
Titratable acidity (g citric acid/100 g fw)	2.00 ± 0.01
Soluble solids (°Brix at 20 °C)	6.51 ± 0.12
Total solids (g/100 g fw[b])	10.52 ± 0.02
L^*	56.22 ± 1.08
a^*	−5.95 ± 0.62
b^*	7.81 ± 0.84
h [$\tan^{-1}(b^*/a^*)$]	−0.91 ± 0.03
C [$(a^{*2} + b^{*2})^{1/2}$]	9.82 ± 0.84
Total phenols (mg CAE[c]/100 g dw[d])	438.88 ± 15.05
Total quercetin (mg/100 g dw)	237.03 ± 21.04
Quercetin-4′-glucoside (mg/100 g dw)	33.08 ± 0.73
Quercetin-3,4′-diglucoside (mg/100 g dw)	241.04 ± 7.24
EC_{50} (g dw/g DPPH•)	49.04 ± 1.23

[a] Values are the mean of three independent determinations ± standard deviation.
[b] fw: fresh weight.
[c] CAE: chlorogenic acid equivalents.
[d] dw: dry weight.

2.3. Analysis of raw onion

2.3.1. pH and titratable acidity

Ten grams of onion was blended with 20 mL of deionised water in an ultrahomogeniser (Omni mixer, model ES-207, Omni International Inc., Gainesville, VA). The mixture was heated to 100 °C, then 20 mL of deionised water was added and the resulting mixture was cooled to 20 °C. The pH was measured at this temperature using a pH metre (Microph 2000, Crison, Barcelona, Spain). After the determination of pH, the solution was titrated with 0.1 N NaOH to pH 8.1, and the results were expressed as percentage of citric acid (g of citric acid per 100 g of fresh weight (fw)).

2.3.2. Soluble solids

Soluble solids of onion were determined using a digital refractometer (ATAGO, Tokyo, Japan) at 20 °C, and results were reported as degrees Brix.

2.3.3. Total solids

The AOAC method was minimally modified (AOAC, 2000). Total solids were measured with a microwave oven operating at 200 W for 20 min until constant weight, and results were expressed as g of total solids per 100 g fw.

2.3.4. Colour

The colour of onion pulp was measured using a tristimulus reflectance colorimeter (HunterLab, model D25 A9, Hunter Associates Laboratory, Inc., Reston, VA) calibrated with a white standard tile ($X = 82.51$; $Y = 84.53$; $Z = 101.23$). Samples were placed in petri dishes and filled to the top, and colour was recorded using the CIELab uniform colour space. L^* (lightness), a^* (green–red tonality), and b^* (blue–yellow tonality) values were recorded, and the results were expressed as: hue angle, h [$\tan^{-1}$ (b^*/a^*)] and saturation (or chroma), C [$(a^{*2} + b^{*2})^{1/2}$].

2.4. Experimental design

Response surface methodology (RSM) was employed to study the effect of combined treatments of high-pressure and temperature on onion total phenol content, flavonol content, and antioxidant activity. The experiment was carried out according to a central composite face-centred design. Three levels of each independent variable (pressure and temperature) were chosen (Table 2).

Table 2
Independent variables and their levels used for central composite design.

Independent variables	Symbol	Coded variable levels		
		−1	0	1
Pressure (MPa)	X_1	100	250	400
Temperature (°C)	X_2	5	27.5	50

Following the design, 10 selected processes of two variables were performed. Holding time of each combination high-pressure and temperature was set constant to 5 min. A second order polynomial equation was used to express the responses as a function of independent variables, which is given as:

$$Y = b_0 + Sb_1X_1 + Sb_2X_2 + Sb_{12}X_1X_2$$

Y represents the dependent variable (estimated response); b_0, b_1, b_2, b_{12} represent the equation coefficients; X_1 and X_2 represent the independent variables studied, pressure and temperature, respectively.

Analysis of variance was performed for each response variable using the full models where P-values (partitioned into linear, quadratic and interaction factors) indicated if the terms were significant. Lack of fit determined whether the model selected was adequate to describe the significance of the observed data. None of the predicted models had a significant lack of fit. Analysis of variance and the coefficients of the second polynomial equation (b_0, b_1, b_2, b_{12}) are shown in Table 3.

2.5. Onion high-pressure processing

2.5.1. Onions

Raw onions were hand peeled, washed with tap water at 4 °C for 1 min, rinsed with distilled water 30 s and dried. Immediately after, onions were cut into approximately 10-mm pieces with a kitchen knife. Samples of 120 g chopped onion were vacuum packaged and sealed with a Multivac sealer (Wolferchweden, Germany). Plastic bags used were BB4L, CRYOVAC Europe, Grace S. A., Sant Boi de Llobregat, Barcelona, Spain. Oxygen permeability was 30 $cm^3/(m^2$ 24 h bar) at 23 °C and 0% relative humidity (RH). BB4L is a heat shrinkable co-extruded material, containing polyethylene-vinyl acetate (EVA) co-polymer.

2.5.2. High-pressure processing

Three onion plastic bags per process were introduced into the pressure unit filled with pressure medium (water). Duplicates of each process were performed. High-pressure and temperature treatments were performed in a hydrostatic pressure unit with a 2350 mL capacity, a maximum pressure of 500 MPa, and a potential maximum temperature of 95 °C (GEC Alsthom ACB 900 HP, type ACIP 665, Nantes, France). Pressure was increased and released at 2.5 MPa/s.

Pressurised onion samples were freeze-dried into a lyophiliser (model Lyoalfa, Telstar, S. A., Barcelona, Spain), immediately after they were finely grinded with a mortar and stored at −20 ± 0.5 °C until their analysis. Afterwards, freeze-dried onion samples were analysed for total phenol content, flavonol content (total quercetin (TQ), quercetin-4′-glucoside (Q_{MG}), quercetin-3,4′-diglucoside (Q_{DG})), and antioxidant capacity.

2.6. Analysis of high-pressure processed onion

2.6.1. Extraction and quantification of total phenols

Total phenols were determined spectrophotometrically (Vinson, Hao, Su, & Zubik, 1998). Analyses were performed by visible spectrophotometry at 760 nm after reaction with Folin–Ciocalteu's reagent. Each onion sample (1 g) plus 25 mL methanol/water (80:20, v/v) was homogenised in duplicate in an ultrahomogeniser (Omni mixer, model ES-270, Omni International Inc., Gainesville, VA) at 8000 rpm for 4.5 min. Extracts were made up to 50 mL with methanol. Next, onion extracts were introduced into test tubes adding 1.0 mL Folin–Ciocalteu's reagent and 0.8 mL sodium carbonate (7.5%). The absorbance of all samples was measured at 760 nm after incubating at room temperature for 1 h. Results were calculated by a calibration curve obtained from chlorogenic acid and expressed as milligrams of chlorogenic acid equivalents (CAE) per 100 g of dry weight (dw).

2.6.2. Extraction, separation, identification, and quantification of flavonols

Flavonols quantified in our study were total quercetin (TQ), quercetin-4′-glucoside (Q_{MG}), and quercetin-3,4′-diglucoside (Q_{DG}). They were determined by high performance liquid chromatography (HPLC). The extraction was carried out in duplicate according to the method by Hertog, Hollman, and Venema (1992) with minor modifications.

For TQ determination the extraction included a hydrolysis. Each onion sample (1 g) was homogenised with 25 mL methanol/water (80:20, v/v) in an ultrahomogeniser (Omni mixer, model ES-270, Omni International Inc., Gainesville, VA) at 8000 rpm for 4.5 min. Immediately after, 5 mL of a 6 M HCl solution was added to the mixture. No antioxidants were added to the hydrolysis mixture. The hydrolysis was performed in duplicate. Afterwards, the hydrolysed extracts were refluxed at 90 °C for 4 h and then allowed to cool, vacuum filtered, made up to 50 mL with methanol and sonicated. Onion hydrolysed extracts were filtered through a 0.45 μm membrane filter for organic solvents prior to injection. Triplicates of 20 μL for each onion extract were analysed by HPLC. The concentration of total quercetin was expressed as mg of TQ per 100 g dw.

Table 3
Analysis of variance and regression coefficients of the second order polynomial equation for each dependent response variable: total phenol content (TP), flavonol content, total quercetin (TQ), quercetin-4′-glucoside (Q_{MG}), quercetin-3,4′-diglucoside (Q_{DG}), and antioxidant activity (EC_{50}).

Regression coefficients		TP	TQ	Q_{MG}	Q_{DG}	EC_{50}
Intercept	b_0	444.153	389.266	58.7538	370.855	67.9059
Linear	b_1	−0.528467	−1.05509	−0.178756****	−1.0048	−0.0423873**
	b_2	3.58153	−5.37309***	−0.57278***	−5.10511***	−0.332081
Quadratic	b_{11}	0.00158134	0.00224381**	0.00037746***	0.00211946**	0.0000058412
	b_{22}	−0.0362485	0.105286**	0.012134**	0.0994922**	0.00518801
Interaction	b_{12}	−0.00628089	−0.00539993	−0.000979259****	−0.00493778	0.000191111
	r^2	0.45	0.90	0.92	0.90	0.82

*$P \leq 0.01$; **$P \leq 0.05$; ***$P \leq 0.10$; ****$P \leq 0.15$. Independent variables: pressure (X_1) and temperature (X_2).

For Q_{MG} and Q_{DG} determination no hydrolysis was performed. Duplicates of each onion sample (1 g) were mixed with 25 mL methanol/water (80:20, v/v) and homogenised in an ultra-homogeniser (Omni mixer, model ES-270, Omni International Inc., Gainesville, VA) at 8000 rpm for 4.5 min. These onion extracts were vacuum filtered, made up to 50 mL with methanol and sonicated. Next, the onion extracts were filtered through a 0.45 μm membrane filter for organic solvents prior to injection. Triplicate of 20 μL for each onion extract were analysed by HPLC. Q_{MG} and Q_{DG} concentrations were expressed as mg of Q equivalents per 100 g dw.

2.6.2.1. HPLC procedure. The analytical HPLC system employed consisted of a Hewlett-Packard (Palo Alto, CA) Model 1050 coupled with a quaternary solvent delivery pump and equipped with an autosampler (G1329A ALS) with a 20 μL sample loop and a Hewlett-Packard 1040A rapid scanning UV-vis photodiode array detector. Separation of flavonoids was performed on a reverse-phase Eclipse XDB-C18 stainless steel column (4.6 × 250 mm, 5 μm) (Agilent, Spain). The mobile phase was deionised Milli-Q water adjusted to a pH 2.5 with *ortho*-phosphoric acid (solution A) and acetonitrile (solution B). The program began with a gradient elution from 90 to 65% A, and from 10 to 35% B for 20 min, followed by a gradient from 65 to 90% A, and from 35 to 10% B for the next 5 min. The flow rate was fixed at 1 mL/min and runs were monitored with the UV-vis photodiode array detector which was set at 370 nm.

The data were stored and processed using a Hewlett-Packard (Palo Alto, CA) ChemStation and related software. Identification of the quercetin was carried out by HPLC by comparing the retention time and UV-vis absorption spectrum with those of the standards. The quantification was achieved by the absorbance and retention times recorded in the chromatograms relative to the external standards of the flavonols previously referred to.

2.6.3. DPPH• radical scavenging capacity

Antioxidant activity was determined by the measurement of the DPPH• radical scavenging (Sánchez-Moreno, Larrauri, & Saura-Calixto, 1998). The extraction followed included a hydrolysis with a refluxing period in this case of 2 h. Triplicates of each onion extract were analysed. The method is described extensively elsewhere (Sánchez-Moreno, Plaza, De Ancos, & Cano, 2003). EC_{50} reflects the 50% depletion of initial DPPH•.

2.7. Statistical analysis

Results were given as mean ± standard deviation of six independent determinations. One-way analysis of variance (ANOVA) was used to compare the means. Differences were considered significant at $P < 0.05$. All statistical analyses were performed with Statgraphics Plus 5.1 (Statistical Graphics Corporation, Inc., Rockville, MD).

3. Results and discussion

The effect of high-pressure processing (HPP) combined with other technologies on bioactive compound extractability and antioxidant activity of different fruits, vegetables, juices, and purees have been widely studied by our group (Plaza, Sánchez-Moreno, Elez-Martínez, De Ancos, Martín-Belloso, & Cano, 2006b; Sánchez-Moreno, Plaza, De Ancos, & Cano, 2004; Sánchez-Moreno, Plaza, Elez-Martínez, De Ancos, Martín-Belloso, & Cano, 2005). In the current study, response surface methodology (RSM) was performed in order to establish different high-pressure and temperature processing treatments subjected to study. As a result, chopped onion (*Allium cepa* L. var. *cepa*, 'Grano de Oro') was processed under ten different treatments that combine different pressures (100, 250, and 400 MPa) and temperatures (5, 27.5, and 50 °C). We aimed to investigate the impact of ten combined treatments of HPP and temperature at a constant time applied to onion (*Allium cepa* L. var. *cepa*, 'Grano de Oro') on onion bioactive content and antioxidant activity compared with untreated onion.

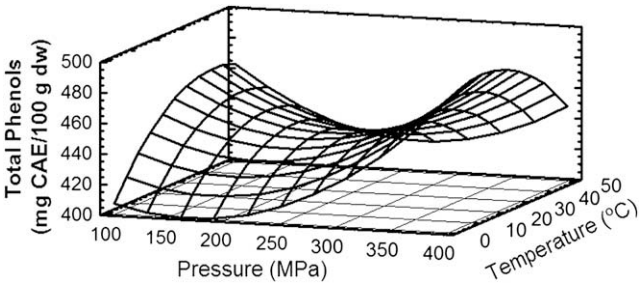


Fig. 1. Response surface for onion total phenol content.

3.1. Onion bioactive compound content

3.1.1. Total phenol content

Response surface for onion total phenol (TP) content as a function of pressure and temperature is showed in Fig. 1. None of the terms analysed (regression coefficients) showed significant effects on the onion TP content (Table 3). Initial onion TP concentration was 438.88 ± 15.05 mg CAE/100 g dw (Table 1). A rise of approximately 12% on TP content was found when treatments applied to onion included pressures of 100 and 400 MPa combined with high (50 °C) and low (5 °C) temperatures, respectively.

3.1.2. Flavonol content

3.1.2.1. Total quercetin. Onion total quercetin (TQ) response surface is shown in Fig. 2. Lineal term of temperature ($P < 0.10$), quadratic term of pressure ($P < 0.05$) and temperature ($P < 0.05$) had significant effects on onion TQ.

Untreated onion showed a TQ concentration of 237.03 ± 21.04 mg/100 g dw (Table 2). Our results showed that onion treated at low (5 °C) temperature combined with 100 and 400 MPa had a 26% higher TQ content compared with untreated onion. One hundred megapascals/50 °C-treated onion showed 18% higher TQ content than untreated onion.

3.1.2.2. Quercetin-4′-glucoside. Fig. 3 showed response surface of onion quercetin-4′-glucoside (Q_{MG}). Lineal term of pressure ($P < 0.15$), lineal term of temperature ($P < 0.10$), quadratic term of pressure ($P < 0.10$), quadratic term of temperature ($P < 0.05$) and interaction term of pressure and temperature ($P < 0.15$) had significant effects on onion Q_{MG}.

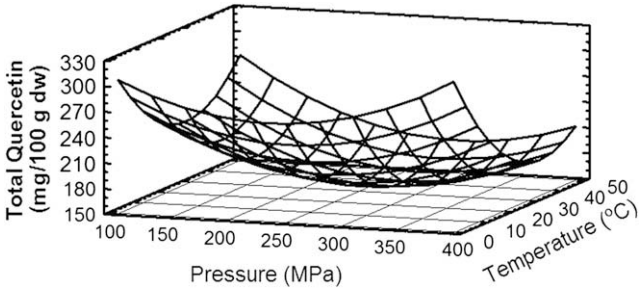


Fig. 2. Response surface for onion total quercetin.

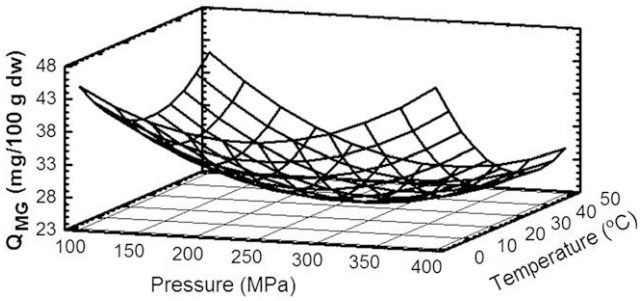


Fig. 3. Response surface for onion quercetin-4′-glucoside.

Untreated onion showed an initial Q_{MG} concentration of 33.08 ± 0.73 mg/100 g dw. When onion is processed at low temperature (5 °C) in combination with 100 or 400 MPa, the highest Q_{MG} content was found. Onion treated at 100 MPa/5 °C and 400 MPa/5 °C had approximately 33% higher Q_{MG} content than untreated onion. Onion treated at 100 MPa/50 °C showed approximately 28% higher Q_{MG} content than untreated onion.

3.1.2.3. Quercetin-3,4′-diglucoside. Fig. 4 showed response surface of quercetin-3,4′-diglucoside (Q_{DG}). Lineal term of temperature ($P < 0.10$), quadratic term of pressure ($P < 0.05$), quadratic term of temperature ($P < 0.05$) had significant effects on Q_{DG}.

Untreated onion showed an initial Q_{DG} concentration of 241.04 ± 7.24 mg/100 g dw. Q_{DG} content found in pressurised onion showed similar behaviour than those found when TQ and Q_{MG} content were analysed. Thus, onion treated at 100 MPa/5 °C and 400 MPa/5 °C had approximately 17% higher Q_{DG} content than untreated onion. One hundred megapascals/50 °C-processed onion had approximately 10% higher Q_{DG} content than untreated onion.

Free quercetin was also quantified in non-hydrolysed pressurised onion samples but its content showed a low percentage compared with Q_{DG} and Q_{MG}. The profile of the target flavonols in our study (Q_{DG} and Q_{MG}) and the free quercetin (data not shown) was the following: Q_{DG} (82.75%), Q_{MG} (12.90%) and free quercetin (4.35%). Therefore, we found higher Q_{DG} content in all high-pressure processed onion samples followed by Q_{MG} and free quercetin. If we compare untreated onion and processed onion at low temperature (5 °C) and HPP (100 and 400 MPa), we found a higher increase in onion Q_{MG} content compared with the increase found in Q_{DG} and free quercetin content. Therefore, processing onion at low temperature (5 °C) and high-pressure triggered to a higher Q_{MG} extractability compared with Q_{DG} and free quercetin extractability. It could be also inferred that processing onion with high-pressure did not affect β-glycosidic linkage of Q_{MG} and Q_{DG}.

Response surface of onion TP content showed that the highest TP concentration (493.10 ± 28.95 mg CAE/100 g dw) was found when low temperature (5 °C) was combined with 400 MPa (Fig. 1).

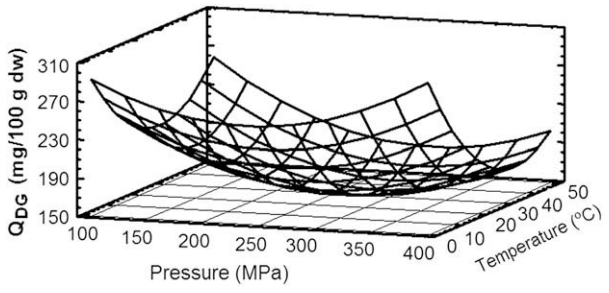


Fig. 4. Response surface for onion quercetin-3,4′-diglucoside.

Response surfaces when analysing onion flavonol content, TQ, Q_{MG}, and Q_{DG} (Figs. 2–4), showed a similar pattern. It was shown that their extractions were strongly affected by the independent variables of pressure and temperature applied in the different treatments. Onion processed at low temperature (5 °C) and pressures of 100 and 400 MPa had higher flavonol content than those found when medium or high temperatures (27.5 or 50 °C) were combined with 250 MPa. Thus, it could be expected that not only onion flavonol compounds but also other unidentified phenol compounds were mainly extracted at low temperature (5 °C) and 400 MPa.

The highest onion Q_{MG} concentration was found when onion was processed at 100 MPa/5 °C and 400 MPa/5 °C, showing Q_{MG} concentrations of 44.18 ± 0.13 mg/100 g dw and 44.02 ± 0.55 mg/100 g dw, respectively (Fig. 3). Moreover, at those treatments the Q_{MG} extraction yield was the highest found followed by those for TQ and Q_{DG}. The highest TQ and Q_{DG} concentrations values were found when processing onion at 100 MPa/5 °C and 400 MPa/5 °C among the treatments applied, 296.46 ± 7.10 mg/100 g dw and 300.23 ± 5.80 mg/100 g dw for TQ respectively; and 282.34 ± 1.48 mg/100 g dw and 283. 17 ± 5.75 mg/100 g dw, for Q_{DG} respectively (Figs. 2 and 4).

Early microscopic studies revealed severe damage to the vacuoles of onion epidermis cells treated by 300 MPa at 25 °C (Butz, Koller, Tauscher, & Wolf, 1994). We found that the combination of low temperature (5 °C) and pressure of 400 MPa led to higher onion total phenol content, particularly flavonol content, than those found at medium or high temperatures (27.5 or 50 °C). Thus, we found an increased flavonol extractability at 400 MPa/5 °C. The use of high temperatures is commonly known to cause detrimental changes on processed products. These undesirable changes affect nutritional as well as organoleptic attributes. In addition, other studies reported that thermal treatments triggered to an onion flavonol content loss (Makris & Rossiter, 2001; Price, Bacon, & Rhodes, 1997; Rohn, Buchner, Driemel, Rauser, & Kroh, 2007).

It could be inferred that the increase in the total phenol and flavonol content found in 400 MPa/5 °C-processed onion could be due to the disruption of the onion vegetative vacuoles where these phenolic compounds are confined. In addition, the low temperature applied did not cause an important bioactive content loss. Other hypothesis could be that high-pressure treatments could help the extraction of cell wall membrane fixed polyphenols. Several authors have been described a similar effect with carotenoids in pressurised orange juice, persimmon puree and tomato-based products (De Ancos, González, & Cano, 2000; De Ancos, Sgroppo, Plaza, & Cano, 2002; Krebbers, Matser, Hoogerwerf, Moezelaar, Tomassen, & van den Berg, 2003; Plaza, Sánchez-Moreno, De Ancos, & Cano, 2006a; Sánchez-Moreno et al., 2004; Sánchez-Moreno, Plaza, De Ancos, & Cano, 2006). However, it has to be taken into account that although in most cases high-pressure processing does not lead to significant loss of potential beneficial substances as flavonol compounds it could cause structural changes in food matrices which may affect their bioavailability (Butz, Edenharder, Fernandez-Garcia, Fister, Merkel, & Tauscher, 2002). Further human intervention studies would have to be done in order to investigate if these potential structural changes in onion matrix could affect onion bioactive compound bioavailability.

Regarding onion sensory characteristics, Arroyo, Sanz, and Prestamo (1999) showed that these characteristics were not affected by high-pressure treatments. At pressures of 350 and 400 MPa/5 °C for 30 min onion texture was firm and its flavour was good. At 350 MPa/5 °C for 30 min the onion colour was unchanged. Onion is highly susceptible to weakening of the tissue matrix during heat treatment. It was reported that the firmness of onion decreased gradually with increasing heating time at a relatively high temperature of 90 and 100 °C (Kim, 2006). In addition, outer layer

tissue of pre-peeled onions exposed to heat treatment at 80 °C had been shown to have irreversible membrane damage (Hyun-Hee, Seok-In, Young-Sook, & Dongman, 2003). Thus, no changes on onion sensory properties are expected in the processed onion of our study at low temperature (5 °C) and high pressure (400 MPa). Moreover, other study concluded that the missing ability of industrially processed onions to develop a brown colour may be overcome by storage of the peeled onions for a few hours or even overnight at 5 °C (Kaack, Christensen, Hansen, & Grevsen, 2004). Antibrowning properties of onion by-products at different temperatures (115, 100, and −70 °C) were reported in our previous study (Roldán, Sánchez-Moreno, De Ancos, & Cano, 2008). Thus, further preservation studies of 400 MPa/5 °C-processed onion would be of interest in order to investigate deeply the implication of antibrowning compounds during the storage at 5 °C.

3.2. Antioxidant activity

Fig. 5 showed response surface of the antioxidant parameter EC_{50} as a function of pressure and temperature. Lineal term of pressure ($P < 0.05$) had significant effects on EC_{50} parameter analysed. It was shown a clear trend towards an increase in onion antioxidant activity when applying pressures from 100 to 400 MPa. Therefore, the higher the pressure values applied to onion, the lower the EC_{50} values found. Treatments at high pressure (400 MPa) and low (5 °C) or medium (27.5 °C) temperatures showed the lowest EC_{50} value (averaging 49.71 g dw/g DPPH•) among the pressurised onion analysed. Moreover, it was shown no significance difference ($P \leq 0.05$) between untreated onion (Table 2) and 400 MPa/5 °C-processed onion.

There was a significant inverse correlation between onion total phenol content and the EC_{50} antioxidant parameter ($P = 0.0318$, $r^2 = 0.71$) when comparing the different pressures applied (100, 250, and 400 MPa) at 5 °C. Therefore, total phenol content played an important role exerting influence on onion antioxidant activity at 5 °C.

The *o*-dihydroxyl group in the B-ring structure of quercetin is responsible for a greater proportion of its free radical scavenging activity (Rice-Evans, Miller, & Paganga, 1996). The decreased inhibition of azo radical-induced lipid peroxidation by Q_{MG} is because of the loss of the *o*-dihydroxyl group in the B-ring due to the introduction of a sugar group in the 4′ position of the quercetin structure. Q_{MG} yielded higher amounts of quercetin aglycone that can act as powerful antioxidant in the intestinal mucosa (Murota, Mitsukuni, Ichikawa, Tsushida, Miyamoto, & Terao, 2004).

Our results are in agreement with those of McInerney et al. (2007) who reported that antioxidant activity and levels of carotenoids of some vegetables (carrots, green beans and broccoli) before and after exposure to high pressures (up to 600 MPa for 2 min) were essentially no different. Their data suggested that micronutrients and phytochemicals in certain vegetables may be more bioavailable by high-pressure treatments.

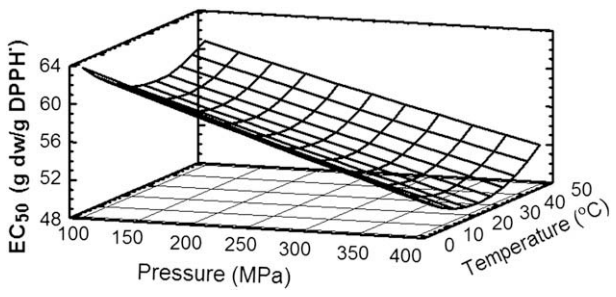


Fig. 5. Response surface for onion antioxidant activity (EC_{50}).

4. Conclusions

Onion total phenols, flavonol content, and antioxidant activity were affected by pressure and temperature variables of the 10 high-pressure processing treatments selected in our study.

Our results showed that processing onion (*Allium cepa* L. var. *cepa*, 'Grano de Oro') with treatments that combine low temperature (5 °C) with pressures of 100 and 400 MPa at a constant time (5 min) significantly increased the extractability of quercetin-4′-glucoside, total quercetin, and quercetin-3,4′-diglucoside, yielding an increase in their contents of 33, 26, and 17%, respectively, compared with untreated onion. Low temperature (5 °C) and high pressure (400 MPa) treatments increased the extractability of total phenol from onion. Moreover, processing onion at low (5 °C) and medium (27.5 °C) temperatures combined with a high pressure of 400 MPa maintained the antioxidant activity of untreated onion. It was shown a clear trend towards an increase in antioxidant activity in pressurised onion from 100 to 400 MPa.

Concluding, in our study it was shown that processing onion at 400 MPa/5 °C could represent a good high-pressure treatment that would increase total phenol and flavonol extractability compared with untreated onion. Processing onion at 400 MPa/5 °C would maintain the antioxidant capacity of the untreated onion. Thus, 400 MPa/5 °C-processed onion would improve nutritional functionality of fresh onion and would retain fresh onion antioxidant activity.

Further studies would have to be done in order to establish the optima range of pressures and temperatures that maintain onion antioxidant activity and achieve higher bioactive compound extractability compared with untreated onion. Bioavailability of onion bioactive compounds that might be extracted during high-pressure processing would have to be studied in depth.

Acknowledgements

This research was supported by funding from the Ministry of Science and Technology (AGL2003-09138-C04-01). In addition, the authors acknowledge the financial support of the Ministry of Education and Science (200670I081 and Program Consolider-Ingenio 2010, FUN-C-FOOD, CSD2007-00063). E. Roldán-Marín wishes to thank the Ministry of Education and Science for a Predoctoral Fellowship.

References

AOAC. (2000). *Official Methods of Analysis no 985.26* (17th edn).. Washington, DC: Association of Official Analytical Chemists.

Arroyo, G., Sanz, P. D., & Prestamo, G. (1999). Response to high-pressure, low-temperature treatment in vegetables: determination of survival rates of microbial populations using flow cytometry and detection of peroxidase activity using confocal microscopy. *Journal of Applied Microbiology, 86*, 544–556.

Bonaccorsi, P., Caristi, C., Gargiulli, C., & Leuzzi, U. (2005). Flavonol glucoside profile of southern Italian red onion (*Allium cepa* L.). *Journal of Agricultural and Food Chemistry, 53*, 2733–2740.

Bonaccorsi, P., Caristi, C., Gargiulli, C., & Leuzzi, U. (2008). Flavonol glucosides in *Allium* species: a comparative study by means of HPLC-DAD-ESI-MS-MS. *Food Chemistry, 107*, 1668–1673.

Butz, P., Edenharder, R., Fernandez-Garcia, A., Fister, H., Merkel, C., & Tauscher, B. (2002). Changes in functional properties of vegetables induced by high pressure treatment. *Food Research International, 35*, 295–300.

Butz, P., Koller, W. D., Tauscher, B., & Wolf, S. (1994). Ultra-high pressure processing of onions: chemical and sensory changes. *Lebensmittel-Wissenschaft und -Technologie, 27*, 463–467.

Caridi, D., Trenerry, V. C., Rochfort, S., Duong, S., Laugher, D., & Jones, R. (2007). Profiling and quantifying quercetin glucosides in onion (*Allium cepa* L.) varieties using capillary zone electrophoresis and high performance liquid chromatography. *Food Chemistry, 105*, 691–699.

Cavagnaro, P. F., Sance, M. M., & Galmarini, C. R. (2007). Effect of heating on onion (*Allium cepa* L.) antiplatelet activity and pungency sensory perception. *Food Science and Technology International, 13*, 447–453.

Considine, K. M., Kelly, A. L., Fitzgerald, G. F., Hill, C., & Sleator, R. D. (2008). High-pressure processing – effects on microbial food safety and food quality. *FEMS Microbiology Letters, 281*, 1–9.

De Ancos, B., González, E., & Cano, M. P. (2000). Effect of high-pressure treatment on the carotenoid composition and the radical scavenging activity of persimmon fruit purees. *Journal of Agricultural and Food Chemistry, 48*, 3542–3548.

De Ancos, B., Sgroppo, S., Plaza, L., & Cano, M. P. (2002). Possible nutritional and health-related value promotion in orange juice preserved by high-pressure treatment. *Journal of the Science of Food and Agriculture, 82*, 790–796.

Hertog, M. G. L., Hollman, P. C. H., & Venema, D. P. (1992). Optimization of a quantitative HPLC determination of potentially anticarcinogenic flavonoids in vegetables and fruits. *Journal of Agricultural and Food Chemistry, 40*, 1591–1598.

Hyun-Hee, L., Seok-In, H., Young-Sook, H., & Dongman, K. (2003). Effect of hot water treatment on biochemical changes in minimally processed onion. *Food Science and Biotechnology, 12*, 445–450.

Kaack, K., Christensen, L. P., Hansen, S. L., & Grevsen, K. (2004). Non-structural carbohydrates in processed soft fried onion (*Allium cepa* L.). *European Food Research and Technology, 218*, 372–379.

Kim, J. C. (2006). Firmness of thermal processed onion as affected by blanching. *Journal of Food Processing and Preservation, 30*, 659–669.

Krebbers, B., Matser, A. M., Hoogerwerf, S. W., Moezelaar, R., Tomassen, M. M. M., & van den Berg, R. W. (2003). Combined high-pressure and thermal treatments for processing of tomato puree: evaluation of microbial inactivation and quality parameters. *Innovative Food Science and Emerging Technologies, 4*, 377–385.

Kubec, R., Hrbacova, M., Musah, R. A., & Velisek, J. (2004). *Allium* discoloration: precursors involved in onion pinking and garlic greening. *Journal of Agricultural and Food Chemistry, 52*, 5089–5094.

Lee, S. U., Lee, J. H., Choi, S. H., Lee, J. S., Ohnisi-Kameyama, M., Kozukue, N., Levin, C. E., & Friedman, M. (2008). Flavonoid content in fresh, home-processed, and light-exposed onions and in dehydrated commercial onion products. *Journal of Agricultural and Food Chemistry, 56*, 8541–8548.

Makris, D. P., & Rossiter, J. T. (2001). Domestic processing of onion bulbs (*Allium cepa*) and asparagus spears (*Asparagus officinalis*): effect on flavonol content and antioxidant status. *Journal of Agricultural and Food Chemistry, 49*, 3216–3222.

MAPA (Anuario de Estadística Agroalimentaria) (2006). Available from <http://www.mapa.es/estadistica/Anu_06/metodologia/Anuario_2006.pdf>; Accessed April 2008.

McInerney, J. K., Seccafien, C. A., Stewart, C. M., & Bird, A. R. (2007). Effects of high pressure processing on antioxidant activity, and total carotenoid content and availability, in vegetables. *Innovative Food Science and Emerging Technologies, 8*, 543–548.

Mogren, L. M., Olssen, M. E., & Gertsson, U. E. (2007). Effects of cultivar, lifting time and nitrogen fertiliser level on quercetin content in onion (*Allium cepa* L.) at lifting. *Journal of the Science of Food and Agriculture, 87*, 470–476.

Moskaug, J.Ø., Carlsen, H., Myhrstad, M., & Blomhoff, R. (2004). Molecular imaging of the biological effects of quercetin and quercetin-rich foods. *Mechanisms of Ageing and Development, 125*, 315–324.

Murota, K., Mitsukuni, Y., Ichikawa, M., Tsushida, T., Miyamoto, S., & Terao, J. (2004). Quercetin-4′-glucoside is more potent than quercetin-3-glucoside in protection of rat intestinal mucosa homogenates against iron ion-induced lipid peroxidation. *Journal of Agricultural and Food Chemistry, 52*, 1907–1912.

Nemeth, K., & Piskula, M. K. (2007). Food content, processing, absorption and metabolism of onion flavonoids. *Critical Reviews in Food Science and Nutrition, 47*, 397–409.

Plaza, L., Sánchez-Moreno, C., De Ancos, B., & Cano, M. P. (2006a). Carotenoid content and antioxidant capacity of Mediterranean vegetable soup (*gazpacho*) treated by high-pressure/temperature during refrigerated storage. *European Food Research and Technology, 223*, 210–215.

Plaza, L., Sánchez-Moreno, C., Elez-Martínez, P., De Ancos, B., Martín-Belloso, O., & Cano, M. P. (2006b). Effect of refrigerated storage on vitamin C and antioxidant activity of orange juice processed by high-pressure or pulsed electric fields with regard to low pasteurization. *European Food Research and Technology, 223*, 487–493.

Prakash, D., Singh, B. N., & Upadhyay, G. (2007). Antioxidant and free radical scavenging activities of phenols from onion (*Allium cepa*). *Food Chemistry, 102*, 1389–1393.

Price, K. R., Bacon, J. R., & Rhodes, M. J. C. (1997). Effect of storage and domestic processing on the content and composition of flavonol glucosides in onion (*Allium cepa*). *Journal of Agricultural and Food Chemistry, 45*, 938–942.

Rastogi, N. K., Raghavarao, K., Balasubramaniam, V. M., Niranjan, K., & Knorr, D. (2007). Opportunities and challenges in high pressure processing of foods. *Critical Reviews in Food Science and Nutrition, 47*, 69–112.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine, 20*, 933–956.

Rohn, S., Buchner, N., Driemel, G., Rauser, M., & Kroh, L. W. (2007). Thermal degradation of onion quercetin glucosides under roasting conditions. *Journal of Agricultural and Food Chemistry, 55*, 1568–1573.

Roldán, E., Sánchez-Moreno, C., De Ancos, B., & Cano, M. P. (2008). Characterisation of onion (*Allium cepa* L.) by-products as food ingredients with antioxidant and antibrowning properties. *Food Chemistry, 108*, 907–916.

Sánchez-Moreno, C., Larrauri, J. A., & Saura-Calixto, F. (1998). A procedure to measure the antiradical efficiency of polyphenols. *Journal of the Science of Food and Agriculture, 76*, 270–276.

Sánchez-Moreno, C., Plaza, L., De Ancos, B., & Cano, M. P. (2003). Effect of high-pressure processing on health-promoting attributes of freshly squeezed orange juice (*Citrus sinensis* L.) during chilled storage. *European Food Research and Technology, 216*, 18–22.

Sánchez-Moreno, C., Plaza, L., De Ancos, B., & Cano, M. P. (2004). Effect of combined treatments of high-pressure and natural additives on carotenoid extractability and antioxidant activity of tomato puree (*Lycopersicum esculentum* Mill.). *European Food Research and Technology, 219*, 151–160.

Sánchez-Moreno, C., Plaza, L., De Ancos, B., & Cano, M. P. (2006). Impact of high-pressure and traditional thermal processing of tomato purée on carotenoids, vitamin C and antioxidant activity. *Journal of the Science of Food and Agriculture, 86*, 171–179.

Sánchez-Moreno, C., Plaza, L., Elez-Martínez, P., De Ancos, B., Martín-Belloso, O., & Cano, M. P. (2005). Impact of high pressure and pulsed electric fields on bioactive compounds and antioxidant activity of orange juice in comparison with traditional thermal processing. *Journal of Agricultural and Food Chemistry, 53*, 4403–4409.

Slimestad, R., Fossen, T., & Vågen, I. M. (2007). Onions: a source of unique dietary flavonoids. *Journal of Agricultural and Food Chemistry, 55*, 10067–10080.

Toivonen, P. M. A., & Brummell, D. A. (2008). Biochemical bases of appearance and texture changes in fresh-cut fruit and vegetables. *Postharvest Biology and Technology, 48*, 1–14.

Torres, J. A., & Velazquez, G. (2005). Commercial opportunities and research challenges in the high pressure processing of foods. *Journal of Food Engineering, 67*, 95–112.

Vinson, J. A., Hao, Y., Su, X. H., & Zubik, L. (1998). Phenol antioxidant quantity and quality in foods: vegetables. *Journal of Agricultural and Food Chemistry, 46*, 3630–3634.

Wenzel, U., Herzog, A., Kuntz, S., & Daniel, N. (2004). Protein expression profiling identifies molecular targets of quercetin as a major dietary flavonoid in human colon cancer cells. *Proteomics, 4*, 2160–2174.

Wolbang, C. M., Fitos, J. L., & Treeby, M. T. (2008). The effect of high pressure processing on nutritional value and quality attributes of *Cucumis melo* L. *Innovative Food Science and Emerging Technologies, 9*, 196–200.

Chapter 6

Effects of an onion by-product on bioactivity and safety markers in healthy rats

Eduvigis Roldán-Marín, Britta N. Krath, Morten Poulsen, Mona-Lise Binderup, Tom H. Nielsen, Max Hansen, Thaer Barri, Søren Langkilde, M. Pilar Cano, Concepción Sánchez-Moreno, Lars O. Dragsted

British Journal of Nutrition **(2009) In press**
(doi:10.1017/S0007114509990870)

British Journal of Nutrition (2009), page 1 of 9
doi:10.1017/S0007114509990870

Effects of an onion by-product on bioactivity and safety markers in healthy rats

Eduvigis Roldán-Marín[1,3], Britta N. Krath[1,2], Morten Poulsen[1], Mona-Lise Binderup[1], Tom H. Nielsen[4], Max Hansen[1], Thaer Barri[2], Søren Langkilde[1], M. Pilar Cano[3], Concepción Sánchez-Moreno[3] and Lars O. Dragsted[2]*

[1]*Department of Toxicology and Risk Assessment, National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, 2860 Søborg, Denmark*
[2]*Department of Human Nutrition, University of Copenhagen, 30 Rolighedsvej, 1958 Frederiksberg, Denmark*
[3]*Department of Plant Foods Science and Technology, Instituto del Frío, Consejo Superior de Investigaciones Científicas (CSIC), José Antonio Novais 10, Ciudad Universitaria, E-28040 Madrid, Spain*
[4]*Department of Plant Biology and Biotechnology, University of Copenhagen, 40 Thorvaldsensvej, 1871 Frederiksberg, Denmark*

(Received 10 November 2008 – Revised 18 May 2009 – Accepted 26 May 2009)

Onions are excellent sources of bioactive compounds including fructo-oligosaccharides (FOS) and polyphenols. An onion by-product was characterised in order to be developed as a potentially bioactive food ingredient. Our main aim was to investigate whether the potential health and safety effects of this onion by-product were shared by either of two derived fractions, an extract containing the onion FOS and polyphenols and a residue fraction containing mainly cell wall materials. We report here on the effects of feeding these products on markers of potential toxicity, protective enzymes and gut environment in healthy rats. Rats were fed during 4 weeks with a diet containing the products or a control feed balanced in carbohydrate. The onion by-product and the extract caused anaemia as expected in rodents for *Allium* products. No other toxicity was observed, including genotoxicity. Glutathione reductase (GR) and glutathione peroxidase (GPx1) activities in erythrocytes increased when rats were fed with the onion extract. Hepatic gene expression of *Gr*, *Gpx1*, catalase, 5-aminolevulinate synthase and NAD(P)H:quinone oxidoreductase was not altered in any group of the onion fed rats. By contrast, γ-glutamate cysteine ligase catalytic subunit gene expression was upregulated but only in rats given the onion residue. The onion by-products as well as the soluble and insoluble fractions had prebiotic effects as evidenced by decreased pH, increased butyrate production and altered gut microbiota enzyme activities. In conclusion, the onion by-products have no *in vivo* genotoxicity, may support *in vivo* antioxidative defence and alter the functionality of the rat gut microbiota.

Onion by-products: Fructo-oligosaccharides: Hb: DNA damage: Antioxidant enzymes: Gene expression: Gut health

Fruits and vegetables are excellent sources of fibres, vitamins and minerals, but they also contain components like polyphenols, terpenes and alkaloids that may provide substantial health benefits beyond basic nutrition[1].

The *Allium* genus includes approximately 500 species. Commonly used *Allium* vegetables include onion, garlic, leeks, chives and scallions, which are used all over the world[2]. It has been shown that *Allium* species may help to prevent tumour promotion, CVD and ageing; all processes that are associated with free radicals[3]. Onions are regularly consumed forming part of the basic diet of many subjects. They have been recognised as an important source of valuable phytonutrients, such as flavonoids, fructo-oligosaccharides (FOS), thiosulphinates and other sulphur compounds[4]. These compounds have been implicated in providing health-promoting as well as adverse attributes to onions. Other interesting biological onion properties reported are potential antioxidant, anticarcinogenic, antimutagenic, anti-asthmatic, immunomodulatory, antimicrobial and prebiotic characteristics[5].

The constant increase in onion consumption and production in many countries has triggered a worldwide disposal of onion wastes in large amounts. Onion wastes could be processed and stabilised in order to obtain useful onion by-products for the food industry. Several onion by-products were characterised in a previous study as potential functional food ingredients with antioxidant and antibrowning properties[6]. An onion powder could be easily added into many foods to improve these technological properties, while also adding prebiotic or other health-related effects[7].

Onion flavonoids, particularly the flavonol, quercetin and its glycosides, have been the target of a wide range of

Abbreviations: BGL, β-glucosidase; FOS, fructo-oligosaccharides; *Gclc*, γ-glutamate cysteine ligase catalytic subunit; GPx1, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GUS, β-glucuronidase; OSC, organosulphur compounds.

* **Corresponding author:** Lars O. Dragsted, fax +45 35 33 24 69, email ldra@life.ku.dk

British Journal of Nutrition

in vitro and *in vivo* investigations, including actions on redox homeostasis[8–12]. Organosulphur compounds (OSC) are also becoming target of many investigations due to their potential chemopreventive and antioxidant effects, but their toxic properties in animals and birds are also a cause of concern and limit the usefulness of onion wastes as animal feeds[13–15].

Few, if any, *in vivo* studies have been done focusing on the fibre fractions in onions such as FOS. Onions have characteristic high contents of certain dietary fibres, particularly fructans and FOS[16–19], but their chain length distribution differs from most other sources such as chicory. Inulin-type fructans have a potential role in colorectal cancer prevention, associated with their 'bifidogenic' prebiotic effect in animal models[20]. The use of FOS as food ingredients has triggered much research on their possible health effects. Functionally, they are used as non-digestible dietary fibre and technologically they are used for their texturing properties in many foodstuffs[21]. Recently, other interesting uses of FOS have been described, including their use as sweeteners for diabetics[22].

To the present knowledge, no studies have investigated the potential antioxidant and prebiotic *in vivo* effect of onion or onion by-products as FOS sources. Effects of onion components on gut health parameters including changes in pH, transit time, bacterial activities and SCFA production need to be investigated. Previous studies with onions have provided evidence for some adverse effects on Hb biosynthesis and anaemia due to the formation of Heinz bodies in birds and some animal species, including rodents. The effects seem to be caused by some of the sulphur compounds, but details of the mechanism are lacking. As a consequence, haeme and redox homeostasis might be affected, and we therefore determined hepatic gene expression of the rate-limiting gene in haeme biosynthesis along with expression of several antioxidant enzymes.

We present here a rat model study aimed to evaluate the potential effect of an onion by-product and two derived onion fractions, a soluble onion extract rich in FOS and polyphenols and an onion residue fraction, on selected effects related to onion-containing compounds, including anaemia, antioxidant defence, phase 2 enzyme induction, gut health and related gene expression.

Materials and methods

Chemicals

All chemical reagents used are analytical grade from Fluka (Steinheim, Germany), Merck (Darmstadt, Germany) and Sigma-Aldrich (Steinheim, Germany). Ethanol (96 %) was purchased from De Danske Spritfabrikker (Aalborg, Denmark). Water is MilliQ (Millipore, Bedford, MA, USA) with $>18\,M\Omega$ resistivity.

Dietary substances

Onion powder was freeze-dried from an onion paste[6] at Instituto del Frío-CSIC in Madrid. The onion paste was a 'Recas' cultivar onion by-product that was pasteurised before shipment. It was kept at 4°C until the preparation of animal diets. In addition, two onion fractions were produced from the onion powder; an onion extract rich in FOS (water/ethanol soluble) and an onion residue (dry residue). They were produced at the National Food Institute, Technical University of Denmark as described later.

Fructan and fructo-oligosaccharides extraction

Fructans and FOS extraction from the onion powder was carried out following the modified Shiomi method with minor modifications described by Jaime *et al.*[23]. The total amount of onion powder used was 1·5 kg. The yield of this powder was 60 % onion extract rich in FOS and percentage of onion residue. The procedure used was the following: portions of 200 g freeze-dried, finely milled material was homogenised in 1 litre of 70 % ethanol and immediately heated to the boiling point for 10 min. Subsequently, the mixture obtained was centrifuged at 2930 ***g*** for 15 min at room temperature. The supernatant was decanted and the pellet was extracted one more time with 40 ml of 70 % boiling ethanol and centrifuged again after cooling. Supernatants were pooled and vacuum evaporated at 30–33°C to dryness obtaining an ethanolic extract. Pellets were combined as a residue fraction and lyophilised. The residue fraction contained 30 % by weight of the starting material.

Analysis for sugars, starch, fructo-oligosaccharides and quercetin in the onion fractions

Samples were extracted at 80°C with 80 % (v/v) ethanol, 20 % (v/v) ethanol and finally water. The combined extracts were lyophilised and redissolved in water. Soluble sugars were determined by standard methods[24–26]. Starch was degraded to glucose units as previously described[27] and fructans were determined after fructanase treatment according to the protocol of the manufacturer (Megazyme Intl., Bray, Ireland). All assays were performed in microplates using a Spectra-Max 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA). High-performance anion exchange chromatographic analysis of fructo-oligosaccharide size distribution was performed as described for glucans[28]. For analysis of quercetin, each fraction was extracted twice with 96 % ethanol, 70 % ethanol, methanol and water, successively. The extracts were combined and concentrated by evaporation, added with genistein as an internal standard and analysed on a 2·1 mm × 10 cm C_{18} BEH column (1·7 μm particle size), using a UPLC-TQD system (Waters, Milford, MA, USA) operated in the multiple reaction mode. A gradient from 10 to 100 % acetonitrile–methanol (1:1) with 0·1 % formic acid with 0·7 ml/min flow rate was used, and quantification multiple reaction mode transitions for quercetin and genistein were $301 > 151$ and $269 > 133$, respectively. Analysis of each fraction was performed with and without preceding hydrolysis of glucosides in 1·2 M HCl at 90°C for 2 h.

Animals

Thirty-two male Fisher 344 rats were obtained from Charles River (Sulzfeld, Germany). The animals were housed 2 × 2 in Macrolon cages with stainless steel wire lids. During the study the animals were maintained on a 12 h light and 12 h dark cycle at an average temperature and relative humidity of 22 ± 1°C and 55 ± 5 %, respectively, and air was changed

8–10 times/h. Diets and tap water were provided *ad libitum*. Animals were divided into four groups of eight rats with equal mean body weights. After 12 d of adaptation to the control diet, the rats were fed either (1) control diet; (2) control diet added 10 % of onion by-product powder; (3) control diet added 7 % of onion extract or (4) control diet added 3 % onion residue for a period of 4 weeks until euthanisation (Table 1). Every diet was based on a purified rodent diet produced at the National Food Institute, Technical University of Denmark, according to Table 1. Animal experiments were carried out under the supervision of the Danish National Agency for the Protection of Experimental Animals. All animal study procedures have been approved by the Institutional Committee for Animal Experimentation, and the Institute has been approved for this type of experiment with rodents by the Danish Ministry of Justice.

Transit time measurement

One week before sacrifice, the rats were dosed with 5 % of carmine solution. Each animal was dosed with 0·7 ml/100 g body weight. At dosing, the time, animal number and animal weight were recorded. One piece of faeces was collected from each cage once before dosing. After dosing, faeces were examined every hour for the appearance of red colour. At the time red faeces were observed, time was recorded and faeces collected in a tube. Afterwards, 30 mg of the red faeces were suspended in 3 ml 0·1 M NaOH, centrifuged at 2000 ***g*** for 30 min, and absorbance was read at 450 and 550 nm.

Sacrifice and sampling

After 4 weeks on the experimental diets, the animals were fasted overnight. The next day, after recording the body weight, the rats were anaesthetised in CO_2/O_2 and sacrificed by decapitation. Immediately after the decapitation, samples of blood were collected as detailed later. The liver was removed, weighed and grinded in liquid N_2 to a fine powder. Three portions of 30 mg each were stored separately for later analysis of antioxidant enzyme activities, gene expression and comet assay. The caecum was washed in ice-cold saline and weighed. The pH in the caecum content near the colon outlet was determined using a microelectrode (Knick, Portamess 751 calimatic pH meter, Berlin, Germany) equipped with a Hamilton biotrode (Reno, NV, USA). Caecum was opened and approximately 0·5 g of the content from the same area was sampled and stored at −80°C until analysis for β-glucosidase (BGL) and β-glucuronidase (GUS) activity. Of the remaining content, one part (approximately 0·1 g) was suspended in nine parts of alkaline buffer (0·1 M Tris, pH 9·6, and 5 mg/ml malonic acid), centrifuged (14 000 ***g***, 10 min, 4°C) and filtrated using a sterile 0·2 μm filter. Samples were kept at −80°C until analysis for SCFA. The caecum was rinsed in 0·9 % NaCl and weighed again empty. After the measurement of pH, samples of caecal contents were taken and treated as described later. The caecum was weighed again empty. One millilitre of blood was collected into a PAXgene blood RNA tube for purification of RNA from the leucocytes (BD Denmark A/S, Brøndby, Denmark). The rest of the blood was collected in vacutainer tubes with heparin as anticoagulant. After 10 min of incubation on ice, samples were centrifuged at 1500 ***g*** for 10 min at 4°C. Plasma was removed for later analysis. The leucocytes were carefully aspirated into 10 % dimethyl sulphoxide in Histopaque-1077 (1:2) for comet analysis. The erythrocyte fraction was haemolysed by adding an equal volume of ice-cold water. All collected fractions were immediately frozen at −80°C.

Hb and antioxidant enzymes

On the day of analysis, the 50 % haemolysates were thawed slowly on ice and diluted 2·5 × in water and sonicated 10 s on ice. For the analysis of Hb, glutathione reductase (GR; *EC* 1.8.1.7) and catalase (*EC* 1.11.1.6), the samples were further diluted to 40 × in 100 mM KH_2PO_4 buffer (pH 7·4) containing 1 mM dithiothreitol and 1 mM EDTA. Glutathione peroxidase (GPx1; *EC* 1.11.1.9) was analysed in 400 × diluted samples. The 30 mg liver powder was homogenised in 1 ml PBS pH 7·4 for 20 s, and centrifuged at 10 000 ***g*** for 20 min at 4°C. The supernatant was used for measuring GR and GPx1 activities, together with total protein in 200 × diluted samples.

Table 1. Composition of animal diets

Ingredient (g/kg feed)	Control (g)	Onion by-product (g)	Onion extract (g)	Onion residue (g)
Onion by-product		100		
Onion extract			70	
Onion residue				30
Protein (casein)	200	200	200	200
Sucrose	100	100	100	100
Maize starch	456	384	386	454
Soya oil with AEDK vitamins	50	50	50	50
Soya oil	20	20	20	20
Maize oil	80	80	80	80
Mineral mixture*	32	32	32	32
B-Vitamin mixture†	12	12	12	12
Cellulose powder	50	22	50	22

* Containing in mg/kg diet: 3000 Ca; 1900 P; 3600 K; 300 S; 2500 Na; 1500 Cl; 600 Mg; 34 Fe; 30 Zn; 10 Mn; 0·20 iodine; 0·15 Mo; 0·15 Se; 2·5 Si; 1·0 Cr; 1·0 F; 0·5 Ni; 0·5 B; 0·1 Li; 0·1 V; 0·07 Co.

† Composition in mg/kg: 5000 (IU) vitamin A; 1000 (IU) vitamin D_3; 50 (IU) vitamin E; 5 thiamine; 6 riboflavin; 8 pyridoxol; 2 folic acid; 0·3 D-biotin; 0·03 vitamin B_{12}; 20 pantothenate; 2600 cholinhydrogentartrat; 400 inositol; 40 nicotinic acid; 1 phylloquinine; 40 *p*-aminobenzoic acid; 1000 methionine; 2000 L-cystine.

The enzyme activities of GR, GPx1 and catalase, including Hb and total protein, were determined spectrophotometrically on an Automated Roche/Hitachi 912 Analyzer (Roche Diagnostic A/S, Hvidovre, Denmark) at 37°C. The activity of GR was measured by following the consumption of NADPH at 340 nm by the method of Goldberg & Spooner[29]. The GPx1 activity was determined by the coupled enzyme method described by Paglia & Valentine[30]. The peroxidative activity of catalase was measured by the reaction of formaldehyde (HCHO) as described earlier elsewhere[31].

RNA isolation and quantitative real-time PCR

Total RNA was isolated from 30 mg liver powder using Qiagen RNeasy Mini kit according to the protocol described by the manufacturer (Qiagen, Hilden, Germany). Reverse transcriptase reactions were performed using Random Hexamer and SuperScript II Reverse Transcriptase kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Relative mRNA expression was quantified by Real-time PCR on an ABI 7900HT FAST System using the comparative ΔCt method according to ABI manual (TaqMan Gene Expression Master Mix Protocol, Applied Biosystems, Foster City, CA, USA). PCR amplification for each gene target was performed in triplicate with cDNA samples equivalent to 3 ng RNA. The eukaryotic 18S rRNA was used as an internal normalisation standard and data were expressed as fold difference in gene expression relative to a calibrator. Control group samples were pooled and used as a calibrant. TaqMan Gene Expression Assays used were the following: eukaryotic 18S rRNA endogenous control (catalogue number 4352930E); rat *Alas1* (catalogue number Rn00675323_g1); rat *Cat* (catalogue number Rn00680386_m1); rat *Gclc* (catalogue number Rn00689048_m1); rat *Gpx1* (catalogue number Rn00577994_g1); rat *Gr* (catalogue number Rn01482160_m1); rat *Nqo1* (catalogue number Rn00566528_m1).

Comet assay

The single-cell gel electrophoresis (Comet assay) was performed according to the recommendations of Tice *et al.*[32], with some minor modifications. Observations were made at magnification of 400 × with a fluorescent microscope (Leica microsystems A/S, Herlev, Denmark) coupled via a CCD camera to an imagine analysis system (Kinetic Imaging 5.0, Bromborough, UK). The data were based on 100 randomly selected cells per sample, fifty cells per each of the two replicate slides. Positive and negative controls were included for each assay. The Caco-2 colon cancer cell line was used as negative control, and for positive control Caco-2 cells were exposed to 4 % of ethyl methanesulphonate in water. DNA damage was measured with the parameters of tail length, olive tail moment, tail extent moment and percentage tail DNA.

β-Glucosidase and β-glucuronidase enzymes

Samples of caecal content (0·2 g) were homogenised in 1 ml PBS, 0·1 % NaN_3 pH 7·4, centrifuged at 10 000 ***g*** for 20 min at 4°C. The supernatant was used to determine the activity of bacterial BGL and GUS on an Automated Roche/Hitachi 912 Analyzer (Roche Diagnostic GmbH, Mannheim, Germany) at 37°C.

BGL (*EC* 3.2.1.21) was assayed by determining the rate of hydrolysis of the substrate *p*-nitrophenyl-β-D-glucopyranoside. The amount of *p*-nitrophenol released was measured at 415 nm. *p*-Nitrophenol was used as standard. GUS (*EC* 3.2.1.31) was measured by determining the rate of release of phenolphthalein from phenolphthalein-β-D-glucuronide at 540 nm with phenolphthalein as standard. The specific activity is defined as U/g caecal content.

SCFA in caecal content

Propionic and butyric acids in caecal contents were analysed using capillary electrophoresis with indirect UV detection as described previously[33].

Statistical analysis

The data were analysed for normal distribution using the Shapiro–Wilks' *W* test and for homogeneity of variance using Levene's test ($P > 0{\cdot}05$). Some data had to be log-transformed in order to meet these criteria. The normally distributed and variance homogenous data were analysed by ANOVA. If significant differences were found between groups, further comparisons were done using least-square means. Other data were analysed using the non-parametric Wilcoxon rank-sums test. We used the Statistical Analysis Systems statistical package v. 9.1 (SAS Institute, Cary, NC, USA) and consider a *P*-value below 0·05 significant.

Results

Onion powder and extracts

The contents of sugars, fructans and quercetin were determined in the onion powder and its fractions in order to determine the exposures in the different rat groups. Table 2 shows the measured contents in each of the fractions. Recovery of sugars after extraction was 96·3 % and recovery of quercetin was 105·6 %, whereas the apparent recovery of fructans was 156 %, indicating that extraction of fructans from the solid onion by-product was more efficient in the preparatory procedure than in the subsequent procedure used for quantitative analysis. A semiquantitative size distribution analysis of the fructans in the extract indicated that more than 90 % had ten fructose residues or less and more than 60 % had five residues or less. Very small amounts of longer chain fructans were present, very unlike the pattern seen for a reference chicory extract (data not shown). The quercetin was analysed before and after hydrolysis of glycosides, and 25–30 % of the quercetin was present as the aglycone in each fraction (data not shown). Starch was not found in any of the samples (data not shown). The results of these analyses show that as a percentage of the total recovered materials, an amount corresponding to 85 % of the sugars, 88 % of the fructans and 91 % of the flavonoid in the feed containing onion by-product was found in the feed with extract and the remaining 9–15 % in the feed with residue.

Table 2. Contents (mg/g) of sugars, fructans, quercetin and total DM in the onion by-product and its fractions (Mean values and standard deviations)

	Glucose		Fructose		Sucrose		Fructans		Quercetin		Other DM	
Fraction	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Onion by-product	205·6	4·9	189·4	9·2	96·9	4·4	42·5	4·8	3·37	0·52	264	8
Onion extract	215·8	4·8	199·3	5·6	85·7	2·1	71·7	8·8	3·97	0·01	315*	3
Onion residue	102·8	8·6	95·5	8·6	53·2	3·9	30·8	2·9	1·22	0·33	643	2

*Estimated on the basis of measured water content in this fraction.

Hb and antioxidant enzymes

The concentration of Hb expressed as g/l of erythrocytes is shown in Fig. 1. There was a significant decrease ($P<0·05$) in the Hb concentration in the rats fed with the three onion products compared with the rats in the control group. Antioxidant enzyme activities were measured in erythrocytes and in liver. There was a significant increase ($P<0·05$) in GR and GPx1 activities in erythrocytes of rats fed with the onion extract rich in FOS. In contrast, hepatic GPx1 activity was significantly decreased ($P<0·01$) in the onion extract fed rats, but not in the other onion groups compared with the control group. Hepatic GR was not affected by any of the three onion products (Table 3).

Gene expression of phase 2 enzymes and haeme synthesis

The relative expression quantification method was used for gene expression quantification. The eukaryotic 18S rRNA was used as endogenous reference and data were expressed as fold difference in gene expression relative to a calibrant. Control group samples were pooled and used as calibrant. Rat β-actin was also used as endogenous reference, but gave the same results as obtained with eukaryotic 18S rRNA (not shown).

Hepatic gene expression revealed that *Gr*, *Gpx1* and *Cat* were not affected in onion fed rats. The gene expression of the phase 2 enzyme *Gclc* involved in glutathione (GSH) synthesis was significantly upregulated by a factor of more than 2, but only in the rats given the onion residue. The expression of *Nqo1*, which is also a phase 2 enzyme, was numerically increased to the same extent in this fraction, but the increase was not significant ($P=0·14$). We also explored whether hepatic haeme biosynthesis was affected by measuring the gene expression of *Alas1*, the rate-limiting step in porphyrin biosynthesis. Feeding onion by-product powder or any of the two onion fractions had no significant effect on expression of this gene (Table 4).

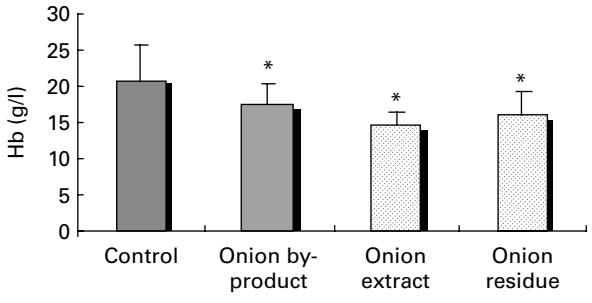


Fig. 1. Hb concentration of rats fed with an onion by-product and two derived onion fractions. Values are means of eight measurements performed in each rat group, with standard deviation depicted by vertical bars. * Significant difference between the onion groups and the control group at $P<0·05$.

DNA damage

Liver and leucocytes samples were used to measure DNA damage by the comet assay. At least 100 scores per sample were analysed, two samples from each animal were used. Internal positive and negative controls were included in each assay performed. Their values were within the laboratory historical control range. There were no significant differences ($P>0·05$) in any of the comet parameters analysed between the control and the three onion groups in liver and leucocyte samples (data not shown).

Caecum weight, pH and transit time

Data on animal caecal weights, caecal pH and transit time values are reported in Table 5. The caecal weight of rats fed with onion by-product was not significantly higher ($P=0·12$) compared to the control group and the two onion fractions groups. Caecal pH was significantly decreased by the onion by-product and both fractions ($P<0·01$). No significant difference ($P>0·05$) was found when gastrointestinal transit time was measured in the three onion groups compared to the control group.

Bacterial activities

BGL and GUS activities were measured in the caecal contents (Fig. 2). Both activities were significantly increased ($P<0·05$) in the three onion groups compared with the control group.

SCFA

There was a significant increase ($P<0·05$) in the formation of caecal propionate and butyrate in all onion fed rats (Fig. 3). The effect was significantly stronger in the onion residue fed group compared with the onion by-product and the onion extract fed groups.

Discussion

In the present study, we have investigated the biological responses of healthy rats fed with an onion by-product and two derived fractions. The onion powder was obtained from

NS British Journal of Nutrition

Table 3. Effect of an onion by-product and two derived onion fractions on rat erythrocytes and hepatic antioxidant enzyme activities

(Mean values and standard deviations, *n* 8)

	Erythrocytes						Liver			
	GR (U/g Hb)		GPx1 (U/g Hb)		CAT (U/g Hb)		GR (U/mg protein)		GPx1 (U/mg protein)	
Group	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	1·9	0·5	1521	182	130	12	70	17	2013	408
Onion by-product	1·7	0·2	1507	192	124	32	78	24	1770	498
Onion extract	2·3*	0·3	1810*	127	153	25	69	9	1470**	225
Onion residue	1·9	0·2	1677	252	147	25	70	23	1832	522

GR, glutathione reductase; GPx1, glutathione peroxidase; CAT, catalase.
Mean values were significantly different from those of the control group: *$P<0·05$, **$P<0·01$.

a pasteurised 'Recas' paste, which was chosen among a battery of stabilised onion by-products (juices, bagasses and pastes) from different cultivars. From a technological and nutritional point of view, stabilised onion by-products from the 'Recas' cultivar showed good characteristics to be developed as antioxidant food ingredients. Pasteurisation applied as stabilisation treatment and paste as a form of onion by-product kept the bioactive and technological characteristics of fresh onion. The by-product chosen in the previous study showed several advantages: a remarkable antioxidant activity; a high content of polyphenols; an excellent anti-browning effect[6].

We extracted the soluble fibre from this onion by-product powder in order to elucidate whether fructans and FOS are partly responsible for the potential health-promoting effects of onion. Our analyses show that a very large part of the water or ethanol soluble compounds, including sugars, FOS and flavonoids were successfully extracted into this fraction. For FOS, we succeeded mainly in extracting shorter chain length fructans including kestose. It is possible that some longer chain FOS remained in the residue fraction obtained after extraction, but onion is known to be mainly composed of short chain FOS, so we find this unlikely. The residue fraction seems therefore to be mostly composed of insoluble cell wall material. Quercetin was used as a marker for ethanol soluble onion compounds, and also these compounds were only left in small amounts in the residue. We have no direct data on the concentration of OSC in the fractions; but in a different study, we have analysed the urine from these rats by NMR and observed that organosulphur metabolites were distributed between the extract and the residue groups according to the fractions of onion materials in their diets (7:3, data to be published elsewhere), indicating that the doses to the rats in the three groups would be approximately 3:2:1 for onion by-product, extract and residue, respectively.

A significant decrease in the Hb concentration of the rats fed with onion compared with rats in the control group was a main finding (Fig. 1). In agreement with the present results, several studies using different animals as models concluded that onion supplementation resulted in dose-dependent reductions in erythrocyte counts and Hb levels involving oxidative damage to erythrocytes and consequent haemolytic anaemia and Heinz body formation[34,35]. As far as we are aware, a similar response has not been described in human subjects, indicating that this toxic effect is not present or at least much weaker in human subjects. Onion OSC have been proposed to be responsible of this toxic effect in rats due to their ability to generate reactive oxygen species in the presence of GSH. Particularly, the relatively lipophilic dipropyl tri- and tetrasulphides and dipropenyl disulphide may be largely responsible for the onion toxicity observed in some animals[36,37]. This would be in accordance with our observation that the residue was also toxic, so hot ethanol was not sufficient for full extraction of OSC.

With respect to onion antioxidant properties, several rat studies have related these properties with the onion antihyperglycaemic or antihypertensive effects[35,38]. Some recent studies have described an enhancement of the total antioxidant capacity of plasma in rats fed with onions[39,40]. We found that antioxidant enzyme activities in erythrocytes showed

Table 4. Effect of an onion by-product and two derived fractions on rat hepatic gene expression of antioxidant enzymes†

(Mean values and standard deviations, *n* 5)

	Gr		*Gpx1*		*Cat*		*Gclc*		*Nqo1*		*Alas1*	
Group	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	0·73	0·27	0·80	0·30	0·95	0·36	0·76	0·42	0·79	0·25	0·82	0·17
Onion by-product	0·72	0·38	0·73	0·29	0·88	0·57	0·90	0·44	0·97	0·24	0·67	0·53
Onion extract	0·83	0·40	1·13	0·47	0·87	0·40	1·02	0·38	0·91	0·32	0·52	0·22
Onion residue	1·06	1·16	1·27	0·82	0·76	0·54	2·12*	1·59	2·11	2·32	0·70	0·45

Gr, glutathione reductase; *Gpx1*, glutathione peroxidase; *Cat* catalase. Phase 2 enzymes: *Gclc*, γ-glutamate cysteine ligase catalytic subunit; *Nqo1*, NAD(P)H: quinone oxidoreductase; *Alas1*, 5-aminolevulinate synthase.
Mean value was significantly different from those of the control group: *$P<0·05$.
† Gene expression of target genes is related to the endogenous reference 18S rRNA and to a calibrant (relative expression quantification).

British Journal of Nutrition

Table 5. Caecal weight, caecal pH and transit time in rats fed with an onion by-product and two derived onion fractions

(Mean values and standard deviations, *n* 8)

	Caecal weight (g)		Caecal pH		Transit time (min)	
Group	Mean	SD	Mean	SD	Mean	SD
Control	2·2	0·5	7·0	0·1	609	56
Onion by-product	3·0	1·1	6·7*	0·1	606	95
Onion extract	2·2	0·4	6·7*	0·1	577	102
Onion residue	2·1	0·9	6·6*	0·1	636	87

Mean value was significantly different from those of the control group: *$P<0·01$.

a significant increase in GR and GPx1 activities when rats were fed with the onion extract rich in FOS (Table 3). This effect might be a result of erythrocyte GSH depletion leading to an increased demand for GSH-dependent enzymes and especially for reduced GSH. In agreement with this assumption, the hepatic gene expression of *Gclc* was higher after feeding with onion fractions (Table 4), but only significantly so in the residue fraction. GPx1 activity in liver tissue of rats fed with the onion extract was significantly decreased compared with the activities found in the rest of the groups (Table 3). We speculate that the antioxidant flavonols, quercetin and its glycosides may have acted as inhibitors.

Onions have also been proposed to be chemoprotective due to the ability of their OSC to increase activities of phase 2 detoxification enzymes and proteins, including GSH *S*-transferase, epoxide hydrolase, NAD(P)H:quinone oxidoreductase1 and UDP-glucuronosyltransferase[41,42]. Moreover, the combination of OSC and the glycosides of quercetin found in onions have been reported to exert chemoprotective action by enhancing the phase 2 enzymes and inhibiting phase 1 enzyme activities such as cytochromes P450. It has been shown by others that consumption of onion decreased the activity of CYP2E1[43].

Hepatic gene expression evaluated in the present study revealed an upregulation of the phase 2 enzyme *Gclc* in rats fed with the onion residue fraction, but not in rats fed with the onion by-product or the onion extract fraction giving higher doses of OSC. By contrast, none of the other antioxidant and phase 2 genes evaluated in the present study was significantly up- or downregulated by onion supplementation, although the expression of *Nqo1* apparently increased

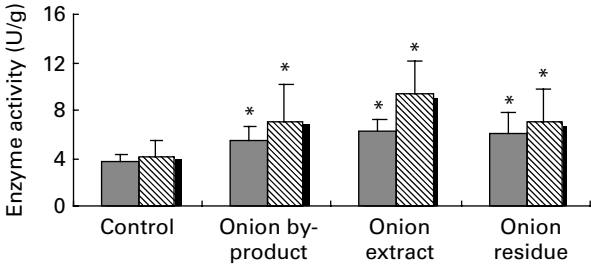


Fig. 2. Effect of an onion by-product and two derived onion fractions on bacterial β-glucosidase (▩, BGL) and β-glucuronidase (▧, GUS) activities in caecal content. Values are means of eight measurements performed in each rat group, with standard deviation depicted by vertical bars. * Significant difference between the onion groups and the control group at $P<0·05$.

Fig. 3. Effect of an onion by-product and two derived onion fractions on SCFA content: propionic (▩) and butyric (▨) acids in caecum. Values are means of eight measurements performed in each rat group, with standard deviation depicted by vertical bars. * Significant difference between the onion groups and the control group at $P<0·05$.

with a pattern similar to that of *Gclc* (Table 4). If the OSC compounds are responsible for *Gclc* induction, our data indicate that the more lipophilic OSC may be the more efficient enzyme inducers since only the residue fraction was significantly active. Expression of hepatic *Alas1* did not follow these same patters and was unaffected by feeding rats with onion products, indicating that toxicity is not due to global downregulation of haeme biosynthesis. On the other hand, upregulation to compensate for haeme loss does not take place in the liver, but may happen in the red bone marrow. Additional studies with further fractionation will have to be performed in order to study which onion compounds with or without thiol groups in their composition play a role in onion toxicity or in modulating phase 2 enzyme activities.

Since GSH depletion might cause increased formation of reactive chemical species, including oxygen radicals, the potential genotoxicity of onion was also evaluated by performing the comet assay. Our results show that the onion products did not decrease the background level of DNA damage (data not shown). Therefore, it could be inferred that the three onion products were not genotoxic. Recent studies have indicated that OSC protect human-derived cells against oxidative DNA damage[44,45]. With the present study, it cannot be verified that these onion products possess an antigenotoxic effect.

We observed a significant lowering effect by feeding the onion by-product or either of the two onion fractions on rat caecal pH without an effect on transit time (Table 5). A marked effect was also shown on bacterial BGL and GUS activities (Fig. 2) and on gut fermentation to SCFA (Fig. 3).

BGL and GUS enzymes are the principal glycosidases produced by the intestinal microbiota, which hydrolyse glycosidic bonds in the gut originating either from the diet or from compounds excreted with the bile. As a result, there is a release of aglycones or metabolites some of which are potentially toxic or carcinogenic. The health significance of increases in BGL or GUS is uncertain. Interestingly, slightly higher BGL and GUS activities were found when rats were fed with the onion extract fraction compared with the other rat groups fed with onion by-product or the residue. A significant increase in caecal lactobacilli and bifidobacteria could partly explain the increase in the BGL activity due to the fact that these two colonic bacterial genera possess higher levels of BGL activities. However, the *Bifidobacterium* genus expresses a low GUS activity, and consequently other bacterial genera might be involved in the observed rise in GUS activity[46]. Moreover, in accordance with Lara-Villoslada *et al.*[47], short-chain FOS with a high content of kestose promoted

a more favourable intestinal microbiota increasing caecal lactobacilli and bifidobacteria counts as well as SCFA production in healthy rats. Thus, we expect similar results in the present study since we confirmed the findings of others of a high presence of this trisaccharide in the onion extract[23].

SCFA are important products formed by fermentation of inulin-type fructans with rat or human gut microbiota. We observed increased levels of propionate and butyrate with all three products, including the group of rats fed with the low FOS onion residue (Fig. 3), indicating that the insoluble fibres contribute significantly to the fermentation. Butyrate has been shown to increase apoptosis in colon cell lines and to protect from genotoxic carcinogens by enhancing expression of genes involved in detoxification[48]. Our results, therefore, show that onion by-products possess additional fermentable fibres compared to FOS-rich additives. Decreased pH is often seen as a consequence of caecal fermentation, and we have previously found that decreased pH was the best predictor of subsequent lower risk of colon cancer in rats fed sugars, oligofructose and inulin[49]. Overall, these effects in the caecum would clearly indicate that increased fermentation of onion fibres is taking place, and that this has altered the functionality and possibly also the composition of the gut microbiota leading to a healthier phenotype.

Conclusion

The present study represents a first step assuring the safety of onion by-products as a food ingredient. Feeding rats with an onion by-product and two derived onion fractions did not involve any genotoxic risk despite our reproduction of the well-known effect of onion on anaemia in rodents. The soluble fraction of the onion by-product seems to affect *in vivo* antioxidant properties, whereas the residue fraction caused phase 2 induction. Moreover, the onion by-product and the two derived onion fractions exert prebiotic properties as evidenced by decreased pH, increased butyrate production and altered gut microbiota enzyme activities. Additional model studies would have to be done with additional well-characterised subfractions in order to further explore these effects and to relate them with specific onion components. Human studies would be needed to ascertain that the effects are related to improved health.

Acknowledgements

The present study was supported by funding from the Spanish Ministry of Science and Innovation (AGL2003-09 138-C04-01; 200670I08; Consolider-Ingenio Programme 2010, FUN-C-FOOD, CSD2007-00 063; and AGL2008-04 798-C02-01/ALI) and from the Danish Ministry of Food, Agriculture and Fisheries (NuBI, 3304-FVFP-060696-01). E. R.-M. wishes to thank the Spanish Ministry of Science and Innovation for a Predoctoral Fellowship. We thank Vibeke Kegel and Margit Wagtberg Hansen for their excellent technical assistance. The cooperation of Anis Arnous with the onion fractions' freeze-drying process is also gratefully appreciated. The author's responsibilities were as follows: E. R.-M., C. S.-M. and M. P. C. provided the onion by-product and produced the onion powder. C. S.-M. proposed the evaluation of onion by-product bioactivity, suggesting the collaboration between the groups. T. H. N. and T. B. analysed the products for contents of sugars, FOS and quercetin. L. O. D. planned the rat study. M. P. was responsible for the animal study protocol and diets. E. R.-M. was responsible for the onion by-product extraction into two fractions, comet assay and antioxidant enzymes' analyses. B. N. K. was responsible for gene expression, bacterial enzymes and antioxidant enzymes' analyses. M. L. B. supervised the comet assay performance and results' data. M. H. was responsible for the SCFA and rat caecal pH; S. L. for the transit time measurement. L. O. D. provided statistical support. E. R.-M. wrote the first draft of the manuscript. L. O. D. and C. S.-M. helped E. R.-M. with the first manuscript draft. M. P. C., C. S. -M., and L. O. D. supervised E. R. -M. manuscript drafts. All authors approved the final manuscript. All authors declared that they had no conflict of interest.

References

1. Aggarwal BB & Shishodia S (2006) Molecular targets of dietary agents for prevention and therapy of cancer. *Biochem Pharmacol* **71**, 1397–1421.
2. Sengupta A, Ghosh S & Bhattacharjee S (2004) *Allium* vegetables in cancer prevention: an overview. *Asian Pac J Cancer Prev* **5**, 237–245.
3. Stajner D, Miliç N, Canadanoviç-Brunet J, *et al.* (2006) Exploring *Allium* species as a source of potential medicinal agents. *Phytother Res* **20**, 581–584.
4. Slimestad R, Fossen T & Vågen IM (2007) Onions: a source of unique dietary flavonoids. *J Agric Food Chem* **55**, 10067–10080.
5. Griffiths G, Trueman L, Crowther T, *et al.* (2002) Onions – a global benefit to health. *Phytother Res* **16**, 603–615.
6. Roldán E, Sánchez-Moreno C, de Ancos B, *et al.* (2008) Characterisation of onion (*Allium cepa* L.) by-products as food ingredients with antioxidant and antibrowning properties. *Food Chem* **108**, 907–916.
7. Corzo-Martínez M, Corzo N & Villamiel M (2007) Biological properties of onions and garlic. *Trends Food Sci Technol* **18**, 609–625.
8. Price KR & Rhodes MJC (1997) Analysis of the major flavonol glycosides present in four varieties of onion (*Allium cepa*) and changes in composition resulting from autolysis. *J Sci Food Agric* **74**, 331–339.
9. Boyle SP, Dobson VL, Duthie SJ, *et al.* (2000) Absorption and DNA protective effects of flavonoid glycosides from an onion meal. *Eur J Nutr* **39**, 213–223.
10. Femia AP, Caderni G, Ianni M, *et al.* (2003) Effect of diets fortified with tomatoes or onions with variable quercetin-glycoside content on azoxymethane-induced aberrant crypt foci in the colon of rats. *Eur J Nutr* **42**, 346–352.
11. Taché S, Ladam A & Corpet DE (2007) Chemoprevention of aberrant crypt foci in the colon of rats by dietary onion. *Eur J Cancer* **43**, 454–458.
12. Nemeth K & Piskula MK (2007) Food content, processing, absorption and metabolism of onion flavonoids. *Crit Rev Food Sci Nutr* **47**, 397–409.
13. Bianchini F & Vainio H (2001) Allium vegetables and organosulfur compounds: do they help prevent cancer? *Environ Health Perspect* **109**, 893–902.
14. Lanzotti V (2006) The analysis of onion and garlic. *J Chromatogr A* **1112**, 3–22.

British Journal of Nutrition

15. Stan SD, Kar S, Stoner GD, *et al.* (2008) Bioactive food components and cancer risk reduction. *J Cell Biochem* **104**, 339–356.
16. Jaime L, Martín-Cabrejas MA, Mollá E, *et al.* (2001) Effect of storage on fructan and fructooligosaccharide of onion (*Allium cepa* L.). *J Agric Food Chem* **49**, 982–988.
17. Jaime L, Mollá E, Fernández A, *et al.* (2002) Structural carbohydrate differences and potential source of dietary fiber of onion (*Allium cepa* L.) tissues. *J Agric Food Chem* **50**, 122–128.
18. Kaack K, Christensen LP, Hansen SL, *et al.* (2004) Non-structural carbohydrates in processed soft fried onion (*Allium cepa* L.). *Eur Food Res Technol* **218**, 372–379.
19. Shiomi N, Benkeblia N & Onodera S (2005) The metabolism of the fructo-oligosaccharides in onion bulbs: a comprehensive review. *J Appl Glycosci* **52**, 121–1277.
20. Pool-Zobel BL (2005) Inulin-type fructans and reduction in colon cancer risk: review of experimental and human data. *Br J Nutr* **93**, S73–S90.
21. Benkeblia N & Shiomi N (2006) Hydrolysis kinetic parameters of DP 6, 7, 8, and 9-12 fructooligosaccharides (FOS) of onion bulb tissues. Effect of temperature and storage time. *J Agric Food Chem* **54**, 2587–2592.
22. Mabel MJ, Sangeetha PT, Platel K, *et al.* (2008) Physicochemical characterization of fructooligosaccharides and evaluation of their suitability as a potential sweetener for diabetics. *Carbohydr Res* **343**, 56–66.
23. Jaime L, Martínez F, Martín-Cabrejas MA, *et al.* (2001) Study of total fructan and fructooligosaccharide content in different onion tissues. *J Sci Food Agric* **81**, 177–182.
24. Beutler HO (1984) Monosaccharides and derivatives. D-Fructose. In *Methods of Enzymatic Analysis*, vol. 6, pp. 321–327 [HU Bergmeyer, editor]. Weinheim: Verlag Chemie.
25. Kunst A, Draeger B & Ziegenhorn J (1984) Monosaccharides and derivatives. D-Glucose. In *Methods of Enzymatic Analysis*, vol. 6, pp. 163–172 [HU Bergmeyer, editor]. Weinheim: Verlag Chemie.
26. Outlaw WH & Tarczynski MC (1984) Poly-, oligo- and disaccarides. Sucrose. In *Methods of Enzymatic Analysis*, vol. 6, pp. 96–103 [HU Bergmeyer, editor]. Weinheim: Verlag Chemie.
27. Nielsen TH, Skjærbæk HC & Karlsen P (1991) Carbohydrate metabolism during fruit development in sweet pepper (Capsicum annuum L.) plants. *Physiol Plantarum* **82**, 311–319.
28. Blennow A, Bay-Smidt AM, Olsen CE, *et al.* (1998) The degree of starch phosphorylation is related to the chain length distribution of the neutral and the phosphorylated chains of amylopectin. *Carbohydr Res* **307**, 45–54.
29. Goldberg DM & Spooner RJ (1983) Assay of glutathione reductase. In *Methods in Enzymology*, vol. 3, pp. 258–265 [HU Bergmayer, editor]. New York: Academic Press.
30. Paglia DE & Valentine WN (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* **70**, 158–169.
31. Johansson LH & Borg HLA (1988) A spectrophotometric method for determination of catalase activity in small tissue samples. *Anal Biochem* **174**, 331–336.
32. Tice RR, Agurell E, Anderson D, *et al.* (2000) Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environ Mol Mutagen* **35**, 206–221.
33. Hansen M, Baunsgaard D, Autrup H, *et al.* (2008) Sucrose, glucose and fructose have similar genotoxicity in the rat colon and affect the metabolome. *Food Chem Toxicol* **46**, 752–760.
34. Ostrowska E, Gabler NK, Sterling SJ, *et al.* (2004) Consumption of brown onions (*Allium cepa* var. cavalier and var. density) moderately modulates blood lipids, haematological and haemostatic variables in healthy pigs. *Br J Nutr* **91**, 211–218.
35. Yamamoto Y, Aoyama S, Hamaguchi N, *et al.* (2005) Antioxidative and antihypertensive effects of Welsh onion on rats fed with a high-fat high-sucrose diet. *Biosci Biotechnol Biochem* **69**, 1311–1317.
36. Yamato O, Hayashi M, Kasai E, *et al.* (1999) Reduced glutathione accelerates the oxidative damage produced by sodium n-propylthiosulfate, one of the causative agents of onion-induced hemolytic anemia in dogs. *Biochim Biophys Acta* **1427**, 175–182.
37. Munday R, Munday JS & Munday CM (2003) Comparative effects of mono-, di-, tri-, and tetrasulfides derived from plants of the Allium family: redox cycling in vitro and hemolytic activity and phase II enzyme induction in vivo. *Free Radic Biol Med* **34**, 1200–1211.
38. El-Demerdash FM, Yousef MI & El-Naga NIA (2005) Biochemical study on the hypoglycemic effects of onion and garlic in alloxan-induced diabetic rats. *Food Chem Toxicol* **43**, 57–63.
39. Son Y, Jung W-K, Jeon Y-J, *et al.* (2008) Protective effects of fermented onion juice containing higher amount of querectin aglycone against oxidative stress by 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) treatment in Sprague–Dawley rats. *Eur Food Res Technol* **226**, 473–482.
40. Park J, Kim J & Kim MK (2007) Onion flesh and onion peel enhance antioxidant status in aged rats. *J Nutr Sci Vitaminol* **53**, 21–29.
41. Munday R, Munday CM, Helmut Sies, *et al.* (2004) Induction of phase II enzymes by aliphatic sulfides derived from garlic and onions: an overview. In *Methods in Enzymology*, pp. 449–456. New York: Academic Press.
42. Guyonnet D, Belloir C, Suschetet M, *et al.* (2001) Antimutagenic activity of organosulfur compounds from Allium is associated with phase II enzyme induction. *Mutat Res Genet Toxicol Environ Mutagen* **495**, 135–145.
43. Teyssier C, Amiot MJ, Mondy N, *et al.* (2001) Effect of onion consumption by rats on hepatic drug-metabolizing enzymes. *Food Chem Toxicol* **39**, 981–987.
44. Arranz N, Haza AI, García A, *et al.* (2007) Protective effects of organosulfur compounds towards N-nitrosamine-induced DNA damage in the single-cell gel electrophoresis (SCGE)/HepG2 assay. *Food Chem Toxicol* **45**, 1662–1669.
45. Belloir C, Singh V, Daurat C, *et al.* (2006) Protective effects of garlic sulfur compounds against DNA damage induced by direct- and indirect-acting genotoxic agents in HepG2 cells. *Food Chem Toxicol* **44**, 827–834.
46. De Preter V, Raemen H, Cloetens L, *et al.* (2008) Effect of dietary intervention with different pre- and probiotics on intestinal bacterial enzyme activities. *Eur J Clin Nutr* **62**, 225–231.
47. Lara-Villoslada F, de Haro O, Camuesco D, *et al.* (2006) Short-chain fructooligosaccharides, in spite of being fermented in the upper part of the large intestine, have anti-inflammatory activity in the TNBS model of colitis. *Eur J Nutr* **45**, 418–425.
48. Pool-Zobel BL & Sauer J (2007) Overview of experimental data on reduction of colorectal cancer risk by inulin-type fructans. *J Nutr* **137**, S2580–S2584.
49. Jacobsen H, Poulsen M, Dragsted LO, *et al.* (2006) Carbohydrate digestibility predicts colon carcinogenesis in azoxymethane treated rats. *Nutr Cancer* **55**, 163–170.

NS British Journal of Nutrition

Chapter 7

An exploratory NMR nutri-metabonomic investigation reveals dimethyl sulfone as a dietary biomarker for onion intake

Hanne Winning, Eduvigis Roldán-Marín, Lars O. Dragsted, Nanna Viereck, Morten Poulsen, Concepción Sánchez-Moreno, M. Pilar Cano, Søren B. Engelsen

Analyst **(2009) 134, 2344-2351**

An exploratory NMR nutri-metabonomic investigation reveals dimethyl sulfone as a dietary biomarker for onion intake

Hanne Winning,*[a] Eduvigis Roldán-Marín,[bc] Lars O. Dragsted,[b] Nanna Viereck,[a] Morten Poulsen,[d] Concepción Sánchez-Moreno,[c] M. Pilar Cano[c] and Søren B. Engelsen[a]

Received 3rd September 2009, Accepted 14th September 2009
First published as an Advance Article on the web 24th September 2009
DOI: 10.1039/b918259d

The metabolome following intake of onion by-products is evaluated. Thirty-two rats were fed a diet containing an onion by-product or one of the two derived onion by-product fractions: an ethanol extract and the residue. A 24 hour urine sample was analyzed using ^{1}H NMR spectroscopy in order to investigate the effects of onion intake on the rat metabolism. Application of interval extended canonical variates analysis (ECVA) proved to be able to distinguish between the metabolomic profiles from rats consuming normal feed and rats fed with an onion diet. Two dietary biomarkers for onion intake were identified as dimethyl sulfone and 3-hydroxyphenylacetic acid. The same two dietary biomarkers were subsequently revealed by interval partial least squares regression (PLS) to be perfect quantitative markers for onion intake. The best PLS calibration model yielded a root mean square error of cross-validation (RMSECV) of 0.97% (w/w) with only 1 latent variable and a squared correlation coefficient of 0.94. This indicates that urine from rats on the by-product diet, the extract diet, and the residue diet all contain the same dietary biomarkers and it is concluded that dimethyl sulfone and 3-hydroxyphenylacetic acid are dietary biomarkers for onion intake. Being able to detect specific dietary biomarkers is highly beneficial in the control of nutritionally enhanced functional foods.

Introduction

Onions (*Allium cepa*) constitute a part of the daily diet for most of the world's population. Nutritionally, onion properties have been widely reported, indicating beneficial health effects. Most of the beneficial health effects have been related to the onion anti-oxidantic, anticarcinogenic, antimutagenic, antiasthmatic, immunomodulatory, antimicrobial, prebiotic and cardiovascular protective properties.[1–3] The main bioactive compounds in onion related to the onion beneficial health effects include flavonols, particularly quercetin and quercetin glucosides,[4] soluble fibers, fructooligosaccharides and organosulfur compounds.[5–9] Organosulfur compounds have become subject of many investigations due to their potential chemopreventive and antioxidant effects.[9–11] For example, the *S*-methyl sulfoxide isolated from *Allium cepa* has been shown to have a lipid-lowering effect in cholesterol-fed rats.[12] The metabolism of onion is not yet fully understood, but cycloalliin, an organosulfur compound found in garlic and onion, initiates several biological activities and its metabolite, (3*R*,5*S*)-5-methyl-1,4-thiazane-3-carboxylic acid, has been found in urine after intravenous or oral administration to rats.[13] Boyle and co-workers found a significant decrease in the level of human urinary 8-hydroxy-2′-deoxyguanosine after ingestion of an onion meal.[6]

Worldwide, large amounts of onion disposal are produced from the production of onions. Because onion is toxic for many animals, this waste product cannot be utilized in the general feeding industry. Onion waste can be stabilized as a useful onion by-product which can act as an antioxidant or antibrowning agent. With respect to the health beneficial effect of onion, developing dietary supplements or nutritionally enhanced functional foods including onion could be highly beneficial. When developing nutritionally enhanced functional foods, it requires understanding of the mechanisms of prevention and of protection in order to utilize and document the potential nutritional effects of an onion supplement. The identification of biologically active molecules as potential dietary biomarkers leads to a greater understanding of biochemical pathways and potentially allows objective quantification of onion intake in mammalian metabolomic studies[14] and eventually in human metabolomic studies.

In contrast to **metabolomics**, which focuses on high-throughput characterization of low molecular weight metabolites in order to obtain a complete molecular profile of the measured biological sample, **metabonomics** focuses at measuring how the molecular profile (metabolome) responds to external factors. High-resolution nuclear magnetic resonance (NMR) (most often proton, ^{1}H, NMR) has emerged as a powerful non-invasive technique for metabonomic studies due to its ability to simultaneously detect a large number of compounds in a rapid high-throughput manner that requires little sample manipulation.[15] ^{1}H NMR spectroscopy is widely used to study the metabolic

[a]*University of Copenhagen, Faculty of Life Sciences, Dept. of Food Science, Quality & Technology, Rolighedsvej 30, 1958 Frederiksberg C, Denmark*
[b]*University of Copenhagen, Faculty of Life Sciences, Dept. of Human Nutrition, Centre for Advanced Food Studies, Rolighedsvej 30, 1958 Frederiksberg C, Denmark*
[c]*Department of Plant Foods Science and Technology, Instituto del Frío, Consejo Superior de Investigaciones Científicas (CSIC), Ciudad Universitaria, José Antonio Novais 10, E-28040 Madrid, Spain*
[d]*National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, 2860 Søborg, Denmark*

variation in biofluids and its capabilities for metabonomics is well established.[16] It has become more and more common to combine investigations of complex NMR spectra with advanced multivariate data analysis such as chemometrics in order to extract systematic latent information from the complex biological NMR spectra. Such analysis requires a minimum of assumptions and the relationships may be visualized intuitively. Already in the early nineties, principal component analysis (PCA)[17] was introduced to classify ^{1}H NMR spectra of urine by the Nicholson group at Imperial College (UK).[18] This group also introduced the definition of metabonomics as: 'understanding the metabolic responses of living systems to pathophysiological stimuli *via* multivariate statistical analysis of biological ^{1}H NMR spectroscopic data'.[19] Subsequently, the term metabonomics has been broadened to 'metabolic processes studied by ^{1}H NMR spectroscopy of biofluids' and thus also includes nutrition studies.[20]

Urine is often used as a biological fluid for metabonomic investigations due to the easy and non-invasive possibilities of collecting repeated samples, the variable metabolite composition, and the often higher metabolite concentrations achieved relative to blood plasma.[21] The fact that the urine profiles are generated and analyzed without *a priori* assumptions about the metabolic and physiological processes involved allows several hypotheses to be tested simultaneously, as well as new hypotheses to be generated from unexpected associations. In nutritional metabonomics, one often has to deal with large variability in the samples compared to the changes of interest induced by the nutritional intervention. This often large variation is caused by biological variation, experimental inhomogeneity or inadequate sample procedure (sample preparation, time of sampling and storage). Therefore, the inter- and intra-individual metabolite variance within a normal population has to be evaluated qualitatively and quantitatively before conclusions can be made. In rodents, it has been determined that species, strain, genetics, sex, age, hormone concentrations, diurnal cycles, diet, temperature, stress and gut microflora all contribute to the metabolic composition of the urine of the animals.[22] However, it is known that human volunteers in dietary metabonomic studies frequently do not report all their medication or food supplements[23] and it is therefore highly desirable to gain objective knowledge about the true diet of a test person. Investigations using animals as a model-system make it possible to investigate biomarkers under controlled conditions.

A rodent study was recently conducted to evaluate possible health effects after feeding with an onion by-product and two derived onion fractions.[24] In this study, the effect of onion intake on antioxidant enzymes, DNA damage, and gut environment in healthy rats was investigated and it was found that the onion by-product and the onion sub-fractions have no genotoxicity, may support antioxidative defense and alter the functionality of the rat gut microbiota. The purpose of the work presented here is to investigate the effect of onion intake on urine composition of the same rats with explorative metabonomic analysis using ^{1}H NMR spectroscopy and chemometrics. Onion contains both soluble and insoluble compounds. Therefore, the onion product was fractionated into two fractions: an ethanol extract, rich in fructooligosaccharides; and the residue, the insoluble matrix. In this way, potential onion dietary biomarkers in the onion by-product investigated can be either located in the extract or left in the residue.

Experimental

Onion and rat study

The onion product used to feed the studied animals is a freeze-dried onion by-product produced from a pasteurized onion paste (*Allium cepa* L. *cepa*, 'Recas') produced at Instituto del Frío (CSIC, Madrid, Spain). The onion by-product was fractionated into an ethanol/water soluble extract which is 70% (w/w) of the by-product and the rest, the dry residue, which is 30% (w/w) of the by-product. The extraction and the rat study were carried out at the National Food Institute, Technical University of Denmark (Søborg, Denmark). The onion extraction procedure and the animal study are detailed as described elsewhere.[24] Briefly, 32 male rats of the inbred strain Fisher 344 were divided into four groups of eight rats and fed four weeks either a control feed, a control feed supplemented with 10% of onion by-product, a control feed supplemented with 7% of onion extract or a control feed supplemented with 3% onion residue (Table 1). The 10% dose was sufficient to elicit physiological effects but not high enough to cause any adverse effects in the rats. The amounts of onion fractions to be added to the feed were chosen taking into account the content of the dietary fiber fructans in each fraction obtained in the extraction of the onion by-product.[24] Consequently, the two onion fractions are supplemented to the feed in concentrations which match the concentration of the by-product. Due to the experimental design, the extract and the residue added should be similar to the by-product, provided that the extraction is complete. The animals were housed 2 × 2 in Macrolon cages, in the same room under the same experimental conditions. The control group was fed with an isocaloric diet, substituting onion sugars with sucrose and onion fiber with starch.

Table 1 Composition of rat feed. For detailed composition of diet see Roldán-Marín *et al.*[24]

	Composition/g per kg feed			
	Control group	Onion by-product group	Onion extract group	Onion residual group
Onion by-product	0	100	0	0
Onion extract	0	0	70	0
Onion residue	0	0	0	30
Control feed	1000	900	930	970
Total feed	1000	1000	1000	1000

Urine samples

Urine samples were collected for a period of 24 h. Two milliliters of 1 mM NaN_3 were added to the urine sample test tubes before the urine was collected and the tubes were kept at 0 °C. The urine volume was recorded and samples were frozen in portions at −80 °C for further analysis. Prior the ^{1}H NMR analysis, the thawed urine samples were prepared by centrifugation at 1200 *g* for 10 min, 340 μl of the supernatant were transferred to NMR tubes followed by addition of 170 μl of 100 mM phosphate buffer solution (H_2O, D_2O, TSP-d4 (per-deuterated 3-trimethylsilyl propionate sodium salt), NaN_3, pH 7.4) to reduce the pH range of the samples. TSP-d4 was added to act as an internal chemical shift reference (δ^1H 0.0), D_2O was added to provide a lock signal for the NMR spectrometer and NaN_3 was added as a preservative. The urine samples were prepared to run in a random order.

^{1}H NMR measurements

^{1}H NMR spectra were recorded for the 32 urine samples. The spectra were acquired on a Bruker Avance Ultra Shield 400 spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) operating at 400.13 MHz using a broad band inverse probe head. Data were accumulated at 300 K employing a pulse sequence using pre-saturation of the water resonance during the recycle period followed by a composite 90° pulse[25] with an acquisition time of 2.04 s, a recycle delay of 5 s, 128 scans and a sweep width of 8012.82 Hz, resulting in 32 000 complex data points. All samples were automatically tuned, matched and shimmed. Prior to Fourier transformation, each FID was apodized by Lorentzian line broadening of 1.0 Hz and zerofilled once and the corresponding spectra were manually phased and automatically baseline corrected. Receiver gain was automatically set. Prior to the chemometric analysis the raw proton NMR spectra data matrix to be investigated had the dimensions (32 × 65 536) but was reduced to 32 202 data points (10–0.2 ppm) excluding spectral areas with no signals. All spectra were aligned (rigid movement) in proportion to the TSP signal at 0.0 ppm. Furthermore, due to insufficient (unequal) depression of the water signal, the area from 5.00 to 4.50 ppm was removed. It also proved necessary to normalize spectra in proportion to the total sum of the spectra in order to remove the large concentration differences of the urine samples. Normalization of urinary metabolic data is best considered as a data transformation which minimizes inter-sample variation due to differences in gross urinary concentration between samples caused by volume and dry matter differences. Furthermore, two 2D NMR experiments – total correlation spectroscopy (TOCSY) and heteronuclear single quantum coherence (HSQC) spectra – were acquired on urine from a rat fed the onion by-product supplement. These experiments were used for assignment of selected signals. The 2D NMR spectra were recorded using the Bruker pulse sequences mlewphpr and hsqcgpph (mixing time of 60 ms).[26,27] The 2D NMR spectra were referenced to TSP-d4 at 0.0 ppm before data analysis. Besides the spectra of the rat urine, ^{1}H NMR spectra of the three different onion fractions were obtained. One milligram of each of the onion fractions was suspended in 1 ml D_2O solution with added 10% TSP-d4. Acquisition parameters were similar to the one used for the urine NMR spectra. The ^{1}H NMR spectrum of dimethyl sulfone was measured with the pure chemical compound dissolved in the 100 mM phosphate buffer solution (pH 7.4) and with the same acquisition parameters as the urine ^{1}H NMR spectra.

Chemometric analysis and software

Multivariate data analysis in the form of PCA and partial least squares regression (PLS)[28] was applied to obtain optimal quantitative and qualitative information from the measured spectra. PCA is the primary tool for investigation of large bilinear data structures for the study of trends, groupings and outliers. By means of PCA it is possible to find the main variation in a multidimensional data set by creating new linear combinations, principal components (PCs), from the underlying latent structures in the raw data. PLS is a multivariate calibration method by which two sets of data, X and y, are related by means of regression. The purpose of PLS is to establish a linear model of latent variables (LVs), which enables the prediction of a reference value y (slow measurement) from the measured spectrum X (fast measurement). Furthermore, extended canonical variates analysis (ECVA)[29] models were applied for classification of feed groups. Canonical variates analysis (CVA)[30,31] is a method for estimation of directions in space that maximizes the differences between groups of samples. However, CVA cannot deal with highly collinear data such as spectroscopic data, where the number of variables is much larger than the number of samples. The ECVA method solves this problem by the use of PLS in the inner part of CVA and thereby allowing for the analysis of highly collinear data.[29] In order to improve the calibration models and to investigate the influential areas of the spectra, interval PLS (iPLS) and interval ECVA (iECVA) were employed.[32] iPLS is an extension of PLS which develops local PLS models on a number of sub-intervals of the full-spectrum region. The main advantage of iPLS is that it provides an overall picture of the relevant information in different spectral sub-divisions, thereby facilitating interpretations and removing interferences from other regions. iECVA works similarly to the iPLS model.

Scaling or other pre-transformations of NMR data are often necessary before the data analysis in order to assure that all signals are influencing the model. In this study, pareto-scaling was used as scaling method applied to the NMR data before the further data analysis. Pareto-scaling reduces the relative importance of large values, but keeps the data structure partially intact. Each variable is divided by the square root of the standard deviation of the column values.[33] Due to the low number of samples, all of the calibration models were validated using cross-validation (CV) with five segments, leaving out one segment at a time from which the root mean square error of cross-validation (RMSECV) was calculated as a measure of the prediction error. This validation method without using an independent test set is known to be slightly optimistic.[34] However, great consistency with permutation tests was obtained for all models, and besides, it was not the scope of the current study to optimize PLS correlations.

The spectra were analyzed using the chemometric software LatentiX 2.0 (www.latentix.com, Latent5, Copenhagen, Denmark), PLS Toolbox 4.11 (Eigenvector Research, Manson, Washington, USA), and MATLAB 7.6 2008a (The MathWorks,

Inc., Natrick, Massachusetts, USA). Regression (iPLS) and the iECVA model were performed in MATLAB using iToolbox and the ECVA Toolbox version 2.02, respectively (all available at www.models.life.ku.dk).

Results and discussion

The ^{1}H NMR spectra of the three onion products fed to the rats are shown in Fig. 1. There are both similarities and differences in the ^{1}H NMR spectra of the three onion products, illustrating both the complexity and the difference between the three onion fractions before they are metabolized by the rats. The spectra reveal diets high in fructans (3.5–6 ppm) with significant amount of aromatic (6–9 ppm) compounds. The ^{1}H NMR spectrum of the extract differs from the spectrum of the residue by more intense signals in the aromatic region. On the other hand, the residue spectrum has slightly more intense signals in the high-field region of the spectrum compared to the ^{1}H NMR spectra of the two other fractions. The spectrum of the (ethanol) extract differs from the spectra of the by-product and the residue by a triplet at δ 1.18 ppm which is assigned to the CH_3 signal from residual ethanol. From the spectra, it is difficult to assure that the by-product spectrum equals the residue plus the extract spectrum.

Fig. 2 shows the average of ^{1}H NMR rat urine spectra of each of the feed groups. The spectra appear very similar despite the different feeding schemes. The ^{1}H NMR spectra of urine typically contain thousands of sharp lines from predominantly low molecular weight metabolites except for one broad band at δ 5.8 ppm from urea.[35–44] The spectra display a wide range of metabolites such as aromatics, aliphatic compounds, sugars, amino acids and other metabolites. However, from this global investigation of the raw data, no obvious difference in the urine profile of the three onion diets can be detected.

In order to investigate possible metabolic differences between the different feeding schemes, a PCA model was established on the full ^{1}H NMR spectra of the 32 urine samples. However, the PCA model was not able to distinguish between the four different feeding groups or to group the samples in an onion and a control group (Fig. 3). No separate clustering was observed for any of the four classes, indicating that the variance between and within classes is similar at least for the two most important principal components.

In order to scrutinize the spectra for signals able to distinguish between the control feed and the different onion-fed groups (by-product, extract and residue), iECVA was carried out on the urine spectra (Fig. 4) using 20 equally sized sub-intervals. Indeed, two interesting intervals were found by iECVA which were able to improve the misclassifications rate significantly. The best interval, 6.50–6.95 ppm, was able to reduce the number of misclassifications from 11 to 2 (Fig. 4). The interval includes signals from some of the aromatic compounds in the urine. The second best interval from 2.98–3.42 ppm was able to decrease the number of misclassifications to 3. The signals in two intervals selected by iECVA are shown in Fig. 5, colored according to the feed group.

The signal at δ 3.15 ppm in one of the selected regions in Fig. 5 shows that the urine spectra with the highest signal intensity at 3.15 ppm is the urine from rats fed with a diet which contained a 10% supplement of onion by-product. In contrast, the urine from rats fed the control diet (without onion) shows no signal in this area. This indicates that the signal also contains quantitative information on onion *dose*. The signal with a chemical shift of δ 6.8 ppm shows (to a lesser extent) the same pattern. This signal matches the spectral profile of 3-hydroxyphenylacetic acid, when matched in an NMR spectral base (BBIOREFCODE) containing 535 compounds found in urine. The correlation between the signal at δ 3.15 ppm and one of the signals from the aromatic protons in 3-hydroxyphenylacetic acid at 6.8 ppm is 0.94, which indicates a connection between the signals. They could be from protons within the same metabolite; however, the signal from the CH_2 group in 3-hydroxyphenylacetic acid is expected to be located at approximately 3.75 ppm. Instead, this could indicate that the 3-hydroxyphenylacetic acid and the compound which has signal at δ 3.15 ppm are both involved in the metabolism of onion. The correlation coefficient between the signal at 6.80 ppm

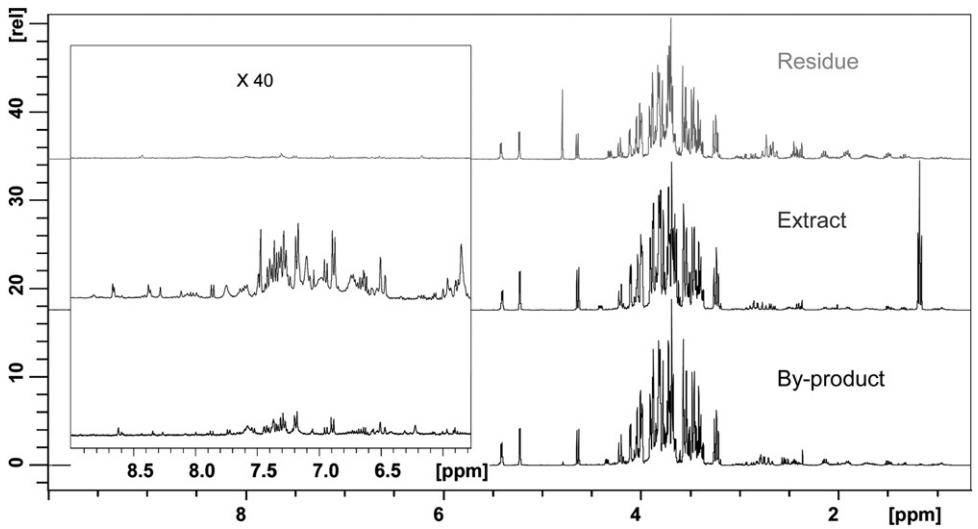


Fig. 1 ^{1}H NMR spectra of onion by-product, onion extract and onion residue dissolved in D_2O included 1 mg/ml TSP-d4.

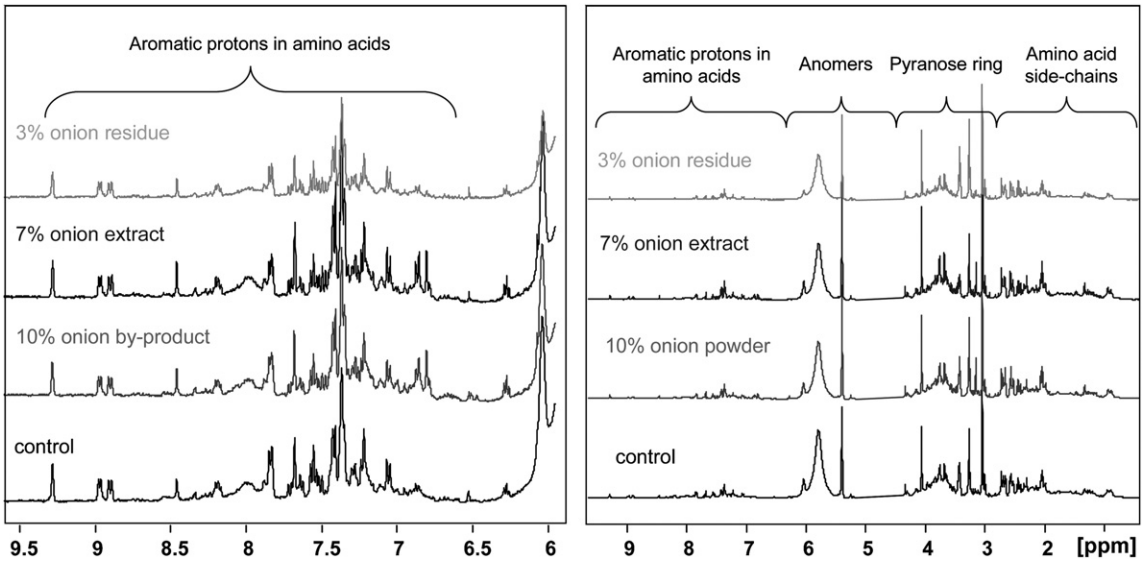


Fig. 2 ^{1}H NMR spectra of averaged rat urine from each of the four dietary groups. The water signal is removed and the aromatic region magnified by a factor of 100.

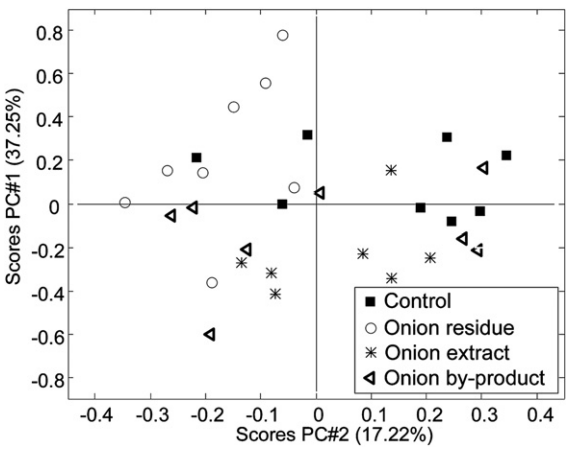


Fig. 3 PCA model (PC1 *versus* PC2) of pareto-scaled ^{1}H NMR spectra of 32 urine samples of rats from the four different feeding schemes.

and the doublet at 6.86 ppm is slightly lower: 0.89, which might indicate that other compounds also have a signal at this chemical shift.

In order to investigate quantitative information regarding onion intake (*onion dose*) the ^{1}H NMR spectra were analyzed using PLS relating to the ^{1}H NMR spectra and the onion dose (0, 3, 7 and 10%). Variable selection using iPLS was applied to find which regions of the ^{1}H NMR spectra of urine that include quantitative information about the onion dietary biomarkers. The prediction error of the full-spectrum model was 1.56% (w/w), as illustrated by the dashed line in Fig. 6A. Two intervals were found which were able to improve the prediction error significantly: 6.50–6.95 ppm and 2.98–3.42 ppm (marked in Fig. 6A). The optimal interval is 2.98–3.42 ppm which results in a prediction error of 1.12% using only 3 LVs. Adding the interval around 6.8 ppm results in a further reduction of the error to 0.97% (w/w) using only one LV. The actual *versus* predicted plot in Fig. 6B shows a simple one-component PLS model obtained on the two selected intervals, revealing that the NMR urine spectra contain univocal quantitative information about onion dose.

The two optimal spectral regions found by iPLS are exactly the same intervals as found by the iECVA. This indicates that it is the same quantitative information which is extracted by the iECVA and iPLS. Unfortunately, the experimental design used has dose and fraction confounded which makes it impossible to decide which effect is modeled, even if the ^{1}H NMR spectra should hold information about both features. In theory, it should be possible to mathematically remove the information about dose and retain the information about fraction. This can be done by orthogonalization where the vector describing the dose response is withdrawn from the data.[45] Indeed, it was tested in this study, but the orthogonalization approach led to a rather overfitted classification (results not shown).

In the interval of 2.98–3.42 ppm, one signal seems particularly important. The signal has chemical shift δ of 3.15 ppm. Based on 2D experiments (TOCSY and HSQC) of the urine sample and NMR measurements of the pure compound measured under exactly the same conditions as the urine, this signal was identified as the methyl protons (CH_3) in dimethyl sulfone (Fig. 7). Indeed, this symmetric compound has only one signal in the ^{1}H NMR spectrum, and no cross-peak in the TOCSY spectrum. Therefore the assignment of the signal is difficult and should be further verified. From the HSQC experiment, the chemical shift of the corresponding ^{13}C was found to be at 44 ppm, which also indicates assignment to dimethyl sulfone.

Dimethyl sulfone is an oxidation product of dimethyl sulfoxide (DMSO) and it is highly possible to find DMSO in urine as a result of an onion diet because onions contains many sulfoxides.[12] It has previously been shown that DMSO is metabolized to dimethyl sulfone in humans and rats.[46] DMSO is a universal solvent and has the characteristic property that it is able to penetrate the skin. DMSO is an industrial solvent

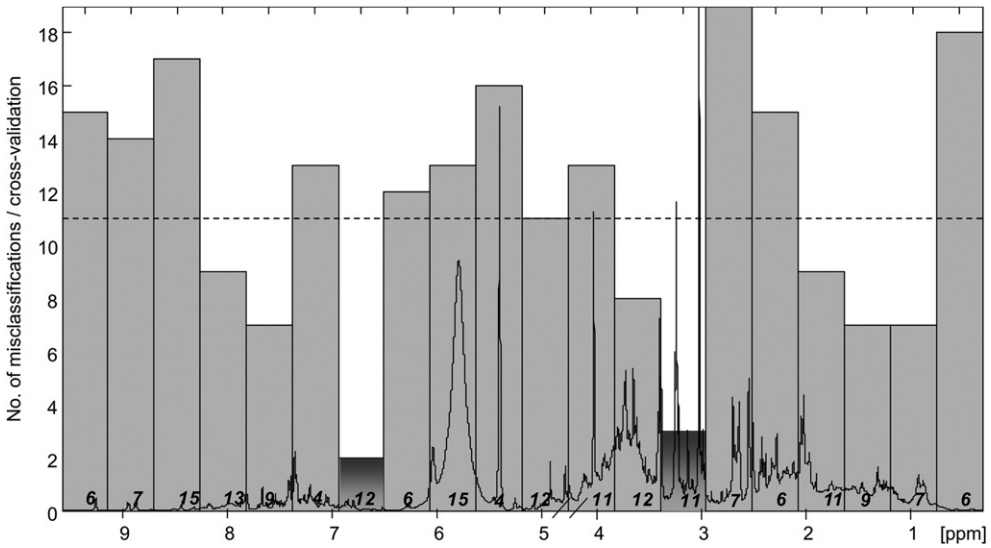


Fig. 4 iECVA plot ^{1}H NMR spectra of urine from onion-fed rats indicating the two best intervals for lowest number of misclassifications. The dotted line is number of misclassifications (11 for 11 LVs) for the global model and the italic numbers are optimal LVs in the interval model.

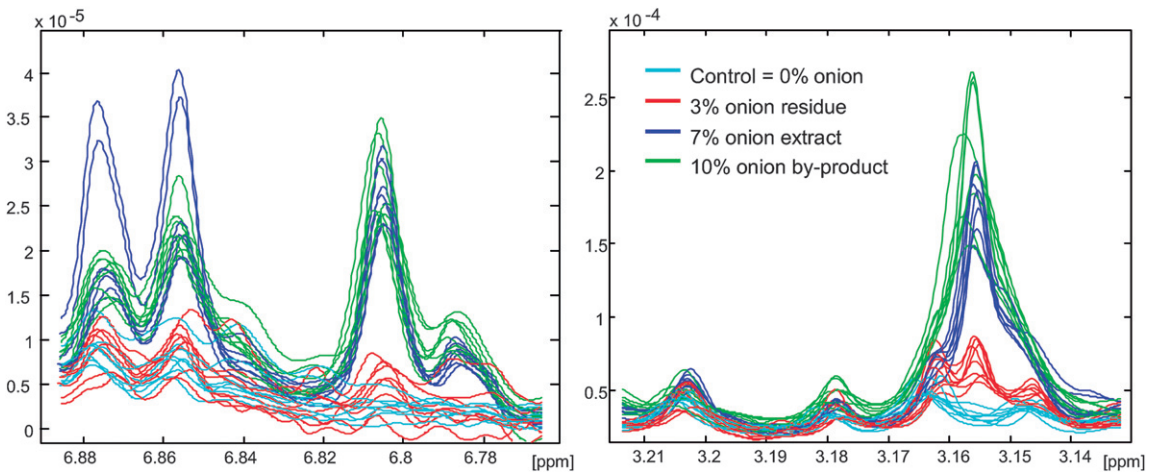


Fig. 5 Selected spectral regions from the iECVA model of the ^{1}H NMR spectra of urine from onion-fed rats, revealing difference in signal intensity for each onion fraction.

approved by the FDA for treatment of interstitial cystitis (bladder inflammation) and side effects include reports of onion odor breath. Several reports have suggested that DMSO may also be effective in inhibiting cholesterol-induced atherosclerosis in experimental animals.[47–49] Dimethyl sulfone has been reported in human sweat,[50] in urine following asparagus consumption[51,52] and in cow's milk from pasture-fed cows.[53] The compound therefore seems to originate from sulfur-rich herbs and plant foods but there may be a genetic element involved in its formation in humans. Dimethyl sulfone has recently been linked with the occurrence of skin cancer.[54,55] Gallagher and co-workers[55] found that skin cancer patients showed significantly higher levels of dimethyl sulfone in the skin measured by gas chromatography/mass spectrometry (GC-MS). An NMR study has also found detectable levels of dimethyl sulfone normally present in the blood and cerebrospinal fluid, suggesting that it derives from dietary sources, intestinal bacterial metabolism, and the body's endogenous methanethiol metabolism.[56]

The good correlation between onion dose and the NMR spectra of urine shows that the onion dietary biomarker is present in all fractions and is equally distributed in the fractions of the by-product. Apparently, the concentration of the dietary biomarker is proportional to the onion dose intake independent of the fed onion fraction. That dimethyl sulfone is present in all urine fractions may be due to the extraction procedure which was not able to eliminate the dietary biomarker from the by-product to the extract. The by-product consisted of intact cell walls which may be the reason why all dimethyl sulfone was not removed from the residue. Another explanation could be that the compound is only partly soluble in ethanol. A third explanation may be that dimethyl sulfone is a degradation product of more lipid-soluble organosulfur compounds from onion, and that these compounds were only weakly soluble in 60% ethanol.

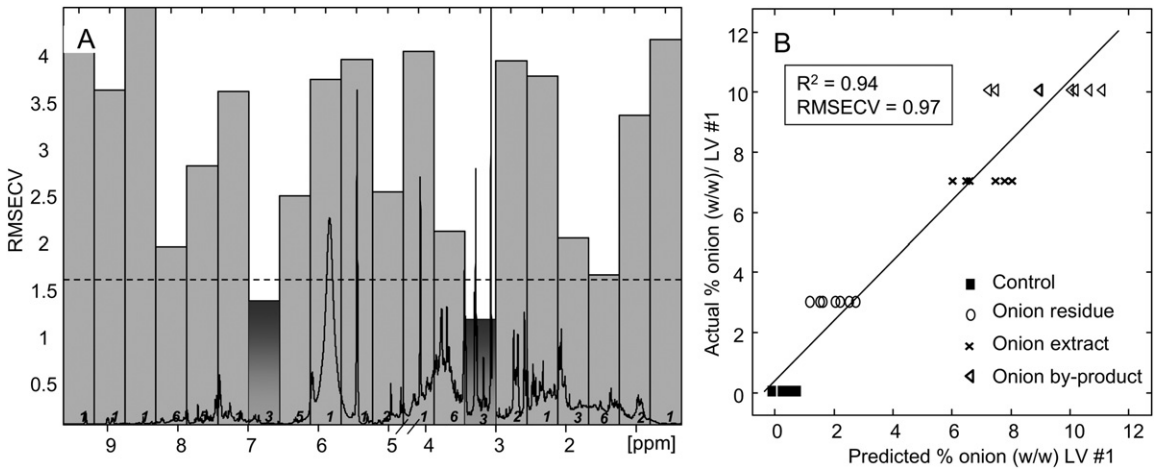


Fig. 6 (A) iPLS plot of the prediction of % (w/w) onion in feed obtained on ^{1}H NMR spectra of urine from onion-fed rats. Dashed line is RMSECV (7 LVs) for the global model. Italic numbers are optimal LVs in the interval model. The two best intervals are highlighted. (B) Actual *versus* predicted plot of PLS model of onion dose and urine from onion-fed rats, performed on the highlighted intervals (A).

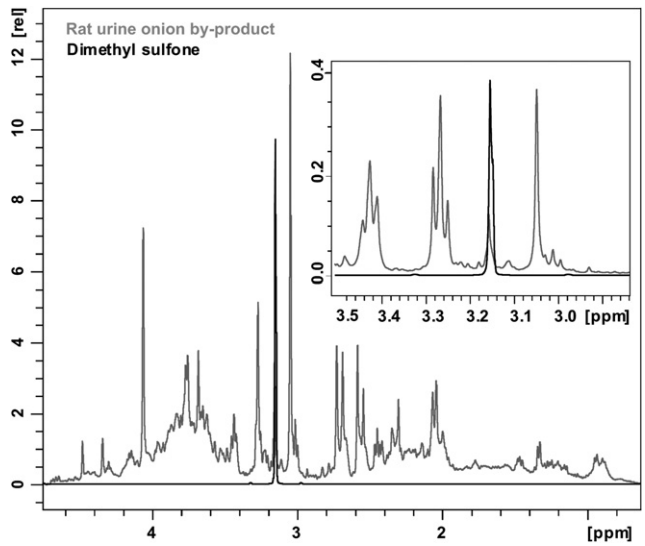


Fig. 7 ^{1}H NMR spectrum of selected urine from rat fed an onion by-product diet overlapped with a spectrum of pure dimethyl sulfone.

Conclusions

Two onion dietary biomarkers were identified as being dimethyl sulfone and 3-hydroxyphenylacetic acid. Quantitative PLS models showed that the onion dose responded the quantitative information in the urine ^{1}H NMR spectra primarily due to the dietary biomarker dimethyl sulfone. This indicated that urine from rats fed with the two fractions (extract and residue) of the onion by-product and rats fed with the onion by-product all contain the dietary biomarker and that the dietary biomarker is present in all fractions and in the same concentrations as the doses. Therefore, it was possible to identify an objective dietary biomarker for onion intake but not for the different onion products. Clearly, the dimethyl sulfone ends up in all fractions and is therefore a dietary biomarker for onion intake. Being able to quantify the dietary intake can be very beneficial as a control in diet intervention studies. The self-reported dietary intake in forms of food frequency questionnaires has been the dietary assessment method used most frequently in large-scale studies. This is primarily because it is easy to administer, it is less expensive than other dietary assessment methods, and it provides a rapid estimate of usual intake.[57] However, there exists a great problem in using food frequency questionnaires because self-reports of food intake are not accurate and sometimes misleading. McKeown *et al.* showed that correlations between 24 h urinary nitrogen excretion and dietary intake from the food frequency questionnaire were as low as 0.25.[58] Clearly, a potential exists in using the onion dietary biomarker in various nutrition studies. We are now in the process of carrying out a human study with an onion product in order to verify the usefulness of this marker and to determine whether genetic variability or variations in gut flora might affect its usefulness in humans.

Acknowledgements

This study was supported by funding from the Danish Ministry of Food, Agriculture and Fisheries (NuBI, 3304-FVFP-060696-01) and 'Urinary biomarkers for eating pattern using NMR spectroscopy and chemometrics' (3304-FVFP-060706-01). The authors acknowledge the financial support of the Spanish Ministry of Innovation and Science (AGL2003-09138-C04-01; 200670I081; Consolider-Ingenio 2010 Programme, FUN-C-FOOD CSD2007-00063; and AGL2008-04798-C02-01/ALI). E. R.-M. wishes to thank the Spanish Ministry of Science and Innovation for a Predoctoral Fellowship. Finally, Dr Manfred Spraul, Director NMR-Application and worldwide Coordinator Hyphenation, Bruker BioSpin, Germany is greatly acknowledged for help with assignment of the ^{1}H NMR spectra.

References

1 G. Griffiths, L. Trueman, T. Crowther, B. Thomas and B. Smith, *Phytother. Res.*, 2002, **16**(7), 603–615.
2 N. K. Gabler, E. Osrowska, M. Imsic, D. R. Eagling, M. Jois, B. G. Tatham and F. R. Dunshea, *Plant Foods Hum. Nutr.*, 2006, **61**(4), 179–185.

3 M. Corzo-Martínez, N. Corzo and M. Villamiel, *Trends Food Sci. Technol.*, 2007, **18**, 609–625.
4 E. Roldán-Marín, C. Sánchez-Moreno, R. Lloría, B. de Ancos and M. P. Cano, *LWT–Food Sci. Technol.*, 2009, **42**, 835–841.
5 K. R. Price and M. J. C. Rhodes, *J. Sci. Food Agric.*, 1997, **74**(3), 331–339.
6 S. P. Boyle, V. L. Dobson, S. J. Duthie, J. A. M. Kyle and A. R. Collins, *Eur. J. Nutrit.*, 2000, **39**(5), 213–223.
7 A. P. Femia, G. Caderni, M. Ianni, M. Salvadori, E. Schijlen, G. Collins, A. Bovy and P. Dolara, *Eur. J. Nutrit.*, 2003, **42**(6), 346–352.
8 S. Taché, A. Ladam and D. E. Corpet, *Eur. J. Cancer*, 2007, **43**(2), 454–458.
9 K. Nemeth and M. K. Piskula, *Crit. Rev. Food Sci. Nutr.*, 2007, **47**(4), 397–409.
10 V. Lanzotti, *J. Chromatogr., A*, 2006, **1112**(1–2), 3–22.
11 S. D. Stan, S. Kar, G. D. Stoner and S. V. Singh, *J. Cell. Biochem.*, 2008, **104**(1), 339–356.
12 K. Kumari and K. T. Augusti, *J. Ethnopharmacol.*, 2007, **109**(3), 367–371.
13 M. Ichikawa, I. Mizuno, J. Yoshida, N. Ide, M. Ushijima, Y. Kodera, M. Hayama and K. Ono, *J. Agric. Food Chem.*, 2006, **54**, 9811–9819.
14 Y. Yamamoto, S. Aoyama, N. Hamaguchi and G. S. Rhi, *Biosci., Biotechnol., Biochem.*, 2005, **69**(7), 1311–1317.
15 E. J. Saude, D. J. Adamko, B. H. Rowe, T. J. Marrie and B. D. Sykes, *Metabolomics*, 2007, **3**, 439–451.
16 J. C. Lindon, E. Holmes and J. K. Nicholson, *Prog. Nucl. Magn. Reson. Spectrosc.*, 2001, **39**(1), 1–40.
17 H. Hotelling, *J. Educ. Psychol.*, 1933, **24**, 417–441.
18 K. P. R. Gartland, C. R. Beddell, J. C. Lindon and J. K. Nicholson, *Mol. Pharmacol.*, 1991, **39**(5), 629–642.
19 J. K. Nicholson, J. C. Lindon and E. Holmes, *Xenobiotica*, 1999, **29**(11), 1181–1189.
20 J. C. Lindon, J. K. Nicholson, E. Holmes and J. R. Everett, *Concepts Magn. Reson.*, 2000, **12**(5), 289–320.
21 J. Forshed, I. Schuppe-Koistinen and S. P. Jacobsson, *Anal. Chim. Acta*, 2003, **487**(2), 189–199.
22 C. M. Slupsky, K. N. Rankin, J. Wagner, H. Fu, D. Chang, A. M. Weljie, E. J. Saude, B. Lix, D. J. Adamko, S. Shah, R. Greiner, B. D. Sykes and T. J. Marrie, *Anal. Chem.*, 2007, **79**(18), 6995–7004.
23 M. Harker, H. Coulson, I. Fairweather, D. Taylor and C. A. Daykin, *Metabolomics*, 2006, **2**(3), 105–112.
24 E. Roldán-Marín, B. N. Krath, M. Poulsen, M. L. Binderup, M. Hansen, S. Langkilde, M. P. Cano, C. Sánchez-Moreno and L. O. Dragsted, *Br. J. Nutr.*, 2009, DOI: 10.1017/S0007114509990870.
25 A. Bax, *J. Magn. Reson.*, 1985, **65**(1), 142–145.
26 L. Braunschweiler and R. R. Ernst, *J. Magn. Reson.*, 1983, **53**(3), 521–528.
27 G. Bodenhausen and D. J. Ruben, *Chem. Phys. Lett.*, 1980, **69**(1), 185–189.
28 S. Wold, H. Martens and H. Wold, *Lect. Notes .Math.*, 1983, **973**, 286–293.
29 L. Nørgaard, R. Bro, F. Westad and S. B. Engelsen, *J. Chemom.*, 2006, **20**(8–10), 425–435.
30 W. J. Krzanowski, *Principles of Multivariate Analysis: A User's Perspective*, Oxford University Press, New York, rev. edn, 2000.
31 C. R. Rao, *Advanced Statistical Methods in Biometric Research*, Wiley, New York, 1952.
32 L. Nørgaard, A. Saudland, J. Wagner, J. P. Nielsen, L. Munck and S. B. Engelsen, *Appl. Spectrosc.*, 2000, **54**(3), 413–419.
33 L. Eriksson, E. Johansson, N. Kettaneh-Wold and S. Wold, *Introduction to multi- and megavariate data analysis using projection methods (PCA & PLS)*, Umetrics, Umeå, Sweden, 1999, pp. 213–225.
34 H. A. Martens and P. Dardenne, *Chemom. Intell. Lab. Syst.*, 1998, **44**(1–2), 99–121.
35 M. L. Anthony, K. P. R. Gartland, C. R. Beddell, J. C. Lindon and J. K. Nicholson, *Arch. Toxicol.*, 1992, **66**(8), 525–537.
36 O. Beckonert, H. C. Keun, T. M. D. Ebbels, J. Bundy, E. Holmes, J. C. Lindon and J. K. Nicholson, *Nat. Protoc.*, 2007, **2**(11), 2692–2703.
37 M. A. Constantinou, E. Papakonstantinou, M. Spraul, S. Sevastiadou, C. Costalos, M. A. Koupparis, K. Shulpis, A. Tsantili-Kakoulidou and E. Mikros, *Anal. Chim. Acta*, 2005, **542**(2), 169–177.
38 T. M. D. Ebbels, E. Holmes, J. C. Lindon and J. K. Nicholson, *J. Pharm. Biomed. Anal.*, 2004, **36**(4), 823–833.
39 E. Holmes, J. K. Nicholson, A. W. Nicholls, J. C. Lindon, S. C. Connor, S. Polley and J. Connelly, *Chemom. Intell. Lab. Syst.*, 1998, **44**(1–2), 245–255.
40 W. S. Law, P. Y. Huang, E. S. Ong, C. N. Ong, S. F. Y. Li, K. K. Pasikanti and E. C. Y. Chan, *Rapid Commun. Mass Spectrom.*, 2008, **22**(16), 2436–2446.
41 L. Wei, P. Q. Liao, H. F. Wu, X. J. Li, F. K. Pei, W. S. Li and Y. J. Wu, *Toxicol. Appl. Pharmacol.*, 2008, **227**(3), 417–429.
42 E. Holmes, F. W. Bonner, B. C. Sweatman, J. C. Lindon, C. R. Beddell, E. Rahr and J. K. Nicholson, *Mol. Pharmacol.*, 1992, **42**(5), 922–930.
43 E. Holmes, P. J. D. Foxall, J. K. Nicholson, G. H. Neild, S. M. Brown, C. R. Beddell, B. C. Sweatman, E. Rahr, J. C. Lindon, M. Spraul and P. Neidig, *Anal. Biochem.*, 1994, **220**(2), 284–296.
44 E. G. Stanley, N. J. C. Bailey, M. E. Bollard, J. N. Haselden, C. J. Waterfield, E. Holmes and J. K. Nicholson, *Anal. Biochem.*, 2005, **343**(2), 195–202.
45 C. A. Andersson, *Chemom. Intell. Lab. Syst.*, 1999, **47**(1), 51–63.
46 H. B. Hucker, P. M. Ahmad, E. A. Miller and R. Brobyn, *Nature*, 1966, **209**(5023), 619.
47 A. F. Debons, K. Fani, F. A. Jimenez and M. L. Maayan, *J. Pharmacol. Exp. Therapeut.*, 1987, **243**(2), 745–757.
48 D. L. Layman, S. S. Alam and K. C. Newcomb, *Ann. N. Y. Acad. Sci.*, 1983, **411**(1 biological ac), 336–339.
49 I. Kedar and E. Sohar, *Isr. J. Med. Sci.*, 1981, **17**(4), 289–291.
50 A. Cork and K. C. Park, *Med. Vet. Entomol.*, 1996, **10**(3), 269–276.
51 R. H. Waring, S. C. Mitchell and G. R. Fenwick, *Xenobiotica*, 1987, **17**(11), 1363–1371.
52 S. C. Mitchell, R. H. Waring, D. Land and W. V. Thorpe, *Cell. Mol. Life Sci.*, 1987, **43**(4), 382–383.
53 B. Toso, G. Procida and B. Stefanon, *J. Dairy Res.*, 2002, **69**(4), 569–577.
54 M. Gallagher, J. Wysocki, J. J. Leyden, A. I. Spielman, X. Sun and G. Preti, *Br. J. Dermatol.*, 2008, **159**(4), 780–791.
55 M. Gallagher, G. Preti, S. Fakharzadeh, J. Wysocki, J. Kwak, C. J. Miller, C. D. Schmults, A. I. Spielman and X. Sun, *The 236th ACS National Meeting*, Philadelphia, PA, August 17–21, 2008, AGFD 227, ref. type: Abstract.
56 U. F. H. Engelke, A. Tangerman, M. A. A. P. Willemsen, D. Moskau, S. Loss, S. H. Mudd and R. A. Wevers, *NMR Biomed.*, 2005, **18**(5), 331–336.
57 W. Willett, *Nutritional epidemiology*, Oxford University Press, Oxford, UK, 2nd edn, 1998.
58 N. M. McKeown, N. E. Day, A. A. Welch, S. A. Runswick, R. N. Luben, A. A. Mulligan, A. McTaggart and S. A. Bingham, *Am. J. Clin. Nutrit.*, 2001, **74**(2), 188–196.

Chapter 8

Effects of an onion by-product on plasma lipids and platelet aggregation in healthy rats

Eduvigis Roldán-Marín, Runa I. Jensen, Britta N. Krath, Mette Kristensen, Morten Poulsen, M. Pilar Cano, Concepción Sánchez-Moreno, Lars O. Dragsted

Journal of Agricultural and Food Chemistry **(2009)**
Under review

Effects of an onion by-product on plasma lipids and platelet aggregation in healthy rats

Eduvigis Roldán-Marín[1,3,4], Runa I. Jensen[1], Britta N. Krath[1], Mette Kristensen[2], Morten Poulsen[3], M. Pilar Cano[4], Concepción Sánchez-Moreno[4], Lars O. Dragsted[1*]

[1]Institute of Human Nutrition, Faculty of Life Sciences, University of Copenhagen, 30-Rolighedsvej, DK-1958 Frederiksberg C, Denmark

[2]Department of Food Science, University of Copenhagen, 30-Rolighedsvej, DK-1958 Frederiksberg C, Denmark

[3]National Food Institute, Technical University of Denmark, 19 Mørkhøj Bygade, DK-2860 Søborg, Denmark

[4]Department of Plant Food Science and Technology, Instituto del Frío, Consejo Superior de Investigaciones Científicas (CSIC), C/ José Antonio Novais 10, Ciudad Universitaria, E-28040, Madrid, Spain

*To whom correspondence should be addressed. Professor Lars O. Dragsted. E-mail: ldra@life.ku.dk; tel.: +45 35 33 26 94, fax +45 35 33 24 69.

Abstract

Onion may contribute to the health effects associated with a high fruit and vegetable consumption. A considerable amount of onion production ends up as waste that might be used as by-products. Onion by-products have not yet been explored for potential health benefits. Our aim is to elucidate the safety and potential role of an onion by-product in affecting risk markers of cardiovascular disease (CVD). For that purpose, we have investigated the effects of an onion by-product (*Allium cepa* L. *cepa*, 'Recas') and two derived fractions, an extract and a residue, on the distribution of plasma lipids and on factors affecting cholesterol metabolism and platelet aggregation in healthy rats. The onion by-product and the onion extract were found to decrease leukocyte thromboxane A_2 synthase (*Txas*) gene expression. However, the onion by-product or its fractions did not seem to reduce cholesterol or down-regulate hepatic 3- hydroxy-3-methylglutaryl-Coenzyme A reductase (*Hmgcr*) gene expression. The onion residue even has the effect of increasing plasma triacylglycerides (TAG) and cholesterol in the very low density lipoprotein (VLDL-C). Neither total bile acids nor total primary or secondary bile acids were significantly affected by feeding rats the onion by-product or its fractions. The onion by-product extract may find use for isolation of inhibitors of platelet aggregation.

KEYWORDS: Onion by-product, CVD risk factors, bile acids, gene expression, plasma lipids, platelet aggregation.

INTRODUCTION

The reported health benefits of *Allium* vegetable constituents include cardiovascular effects, improvement of the immune function, lowering of blood glucose level, radioprotection, protection against microbial infections, and anti-cancer effects (*1*). Among *Allium* vegetables, most of the studies have focused on garlic (*Allium sativum* L.) and its constituents; particularly organosulfur compounds (OSCs) and their cardioprotective and anti-carcinogenic effects (*2-3*). Onion (*Allium cepa* L.) is among the most highly consumed vegetables worldwide and it has been a target of fewer studies despite the fact that this vegetable is one of the food items, which have been associated with better survival of heart attacks in observational studies (*4*).

Onion by-products have been characterised (*5*) and some biological responses of a particular onion by-product (*Allium cepa* L. *cepa*, 'Recas') and two derived onion fractions have been described in our previous investigations (*6*). Moreover, two dietary metabolic onion intake biomarkers have recently been identified (*7*). Taking into account that cardiovascular disease (CVD) is a prevalent disease worldwide; we consider it of importance to study the potential health benefits of an onion by-product in terms of CVD prevention (*8*).

CVD accounted for about 30% of the 58 million estimated deaths globally from all causes in 2005. Between 2006 and 2015, deaths due to non-communicable diseases are expected to increase by 17%, of which half will be due to CVD (*9*). The rise in CVD reflects a significant change in dietary habits, physical activity levels, and tobacco consumption worldwide. High blood pressure, high blood cholesterol, overweight, obesity, and type 2 diabetes are among the major biological risk factors. Unhealthy dietary practices include the high consumption of saturated fats, salt and refined carbohydrates, as well as low consumption of fruit and vegetables (*10*). Several early risk factors for CVD are known,

among them a high plasma level of triacylglycerides (TAG), total cholesterol (TC), cholesterol in the low density lipoprotein fraction (LDL-C), in the very low density lipoprotein fraction (VLDL-C), and a low level of cholesterol in the high density lipoprotein fraction (HDL-C). Moreover, platelet aggregation is also one of the major causes of thromboembolic disorders leading to the development of cardiovascular events such as myocardial infarction and ischemic stroke; platelet aggregation may therefore also represent a potential CVD risk factor. Prevention of CVD by reducing some of the main CVD risk factors represents one of the main targets of preventive nutrition.

To our knowledge no studies have focused on onion as an onion by-product and its potential role as a CVD preventive food ingredient. Therefore, we aimed to elucidate the potential protective role of an onion by-product in affecting CVD risk factors in a model study using healthy rats. We also aimed to study which major fractions of the onion by-product may be responsible for any effects in order to shed light on potential bioactive constituents.

MATERIALS AND METHODS

Chemicals

All chemical reagents used are analytical grade from Fluka (Steinheim, Germany), Merck (Darmstadt, Germany) and Sigma-Aldrich (Brøndby, Denmark). Ethanol (96%) was purchased from De Danske Spritfabrikker, Aalborg, Denmark. Water is MilliQ (Millipore, Bedford, MA) with >18Mohm resistivity. The bile acids: dehydrocholic acid (DHCA), 13C glycocholic acid (13C GA), ursodeoxycholic acid (UDOCA), chenodeoxycholic acid (CDOCA), litocholic acid (LCA) were purchased from Sigma-Aldrich chemicals (Brøndby, Denmark). The bile acids: tauroursodeoxycholic acid (TUDOCA), glycoursodeoxycholic acid (GUDOCA), taurocholic acid (TA), glycocholic acid (GA), taurochenodeoxycholic acid (TCDOCA), cholic acid (CA), taurodeoxycholic acid (TDOCA), glycodeoxycholic acid

(GDOCA), deoxycholic acid (DOCA) were purchased from Merck (Darmstadt, Germany). The bile acid standards, alpha-muricholic acid and beta-muricholic acid were obtained from Steraloids (Newport, Rhode Island, USA).

Onion by-product extraction and analysis.

The onion freeze-dried powder was obtained from an onion pasteurized paste by-product (*Allium cepa* L. *cepa*, 'Recas') at Instituto del Frío-CSIC, in Madrid (*5*). Fructans and fructooligosaccharides (FOS) were extracted from the onion by-product powder and two onion by-product derived fractions; an ethanolic onion extract (water/ethanol soluble) and an onion residue (dry residue), were produced as previously described (*6*). The extraction was carried out according to a modified Shiomi method as described by Jaime et al, 2001 (*11*). Sample extraction and preparation for each analysis performed is extensively explained in our previous paper (*6*). Briefly, soluble sugars were determined by standard methods (*12, 13, 14*). Starch was degraded to glucose units as previously described (*15*) and fructans were determined after fructanase treatment according to the protocol of the manufacturer (Megazyme Intl., Bray, Ireland). All assays were performed in microplates using a Spectra-Max 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA). High performance anion exchange chromatographic analysis of fructooligosaccharide size distribution was performed as described previously for glucans (*16*). For quercetin analysis, onion extracts were added with genistein as internal standard. Each onion product was analysed with and without preceding hydrolysis of glucosides in 1.2M HCl at 90°C for 2 hours. Separation was obtained on a 2.1 mm x 10 cm C_{18} BEH column (1.7 µm particle size) using a UPLC system (Waters, Milford, MA) coupled with a TQD operated in the multiple reaction mode (MRM) for quantitative analysis as described previously (*6*). CV% for all analyses were better than 5%.

Rat study design and sample collection.

Thirty two healthy male Fisher 344 rats obtained from Charles River (Sulzfeld, Germany) were fed during four weeks either a control diet; control diet added 10% of onion by-product powder, control diet added 7% of onion extract, or control diet added 3% onion residue. Every diet was based on a purified rodent diet produced at the National Food Institute, Technical University of Denmark and was nutritionally balanced as detailed previously (*6*). Animal experiments were carried out under the supervision of the Danish National Agency for the Protection of Experimental Animals. All animal study procedures have been approved by the Institutional Committee for Animal Experimentation and the National Food Institute has been approved for this type of experiment with rodents by the Danish Ministry of Justice. Faeces samples were collected two days before the termination of the rat study while the rats were housed singularly in metabolic steel cages with a device to separate urine from faeces. Total faeces from a 24-hour collection was stored frozen at -80 ºC until bile acid analysis. After 4 weeks on the experimental diets the animals were fasted overnight. The next day the rats were anesthetized in CO_2/O_2 and sacrificed by decapitation. Immediately after the decapitation, blood was collected into two different vials. One mL of blood was collected into a PAXgene blood RNA tube for purification of RNA from the white blood cells (WBC) (BD Denmark A/S, Brøndby, Denmark). The rest of the blood was collected in vacutainer^{TM} tubes containing heparin as an anticoagulant. After 10 min of incubation on ice the samples were centrifuged at 1500***g*** for 10 min, 4 °C. Plasma was removed for analyses of enzymes, triacylglycerides and lipoproteins. Rat liver was removed and grinded in liquid N_2 to a fine powder. Samples of 30 mg of liver were stored at -80 ºC for gene expression analysis.

Biochemical analysis. Markers of hepatic function and triacylglycerides

Alkaline phosphatase (AlP), alanine aminotransferase (ALAT), gamma glutamyl transferase (GGT), and triacylglycerides (TAG) concentrations were measured in rat plasma samples using an automated Roche/Hitachi 912 analyzer at 37 ºC in accordance with the instructions of the manufacturers (Roche Diagnostic GmbH Mannheim, Germany).

RNA isolation and quantitative real-time PCR

Liver samples were used for measurement of the expression of 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (*Hmgcr*) whereas blood samples were used for the thromboxane A_2 synthase (*Txas*) expression assay. Relative mRNA expression was quantified by Real-time PCR on an ABI 7900HT FAST System as described previously (*6*). Control group samples were pooled and used as a calibrant. TaqMan® Gene Expression Assays used were the following: Eukaryotic 18S rRNA Endogenous Control (catalog number 4352930E); rat *Hmgcr*, (catalog number Rn_00695772_g1), and rat *Txas* (*Tbxas*) (catalog number Rn_01456253_m1).

^{1}H NMR analysis and chemometric models for lipids quantification

Total cholesterol and cholesterol content in high, low and very low density lipoproteins (HDL-C, LDL-C, and VLDL-C) were analysed in rat plasma samples. For ^{1}H NMR analysis, plasma samples were thawed on ice and 100 µL plasma was transferred to a 5 mm NMR tube and 450 µL D_2O was added. NMR spectra were acquired on a Bruker Avance 400 MHz spectrometer (9.4 T) (Bruker Biospin Gmbh, Rheinstetten, Germany) at 311K, which corresponds to the body temperature of rats. Total cholesterol and cholesterol content in HDL, LDL and VLDL lipoproteins were then predicted by a previously developed chemometric models based on NMR data and interval Partial Least Square models from 60 Fisher 344 rats (17).

Bile acids analysis. LC/MS

The concentration of bile acids in faeces samples was measured by a novel LC/MS/MS method (Jensen *et al.*, in prep.). Briefly, total faces were weighed, homogenized with 6 volumes (w/v) of water to slurry, and 200 mg samples were aliquoted. The aliquots were diluted 14 times in water. 1 mL of this homogenate was added 13C glycocholic acid as internal standard and extracted three times with acetonitrile. The eluate was diluted with 0.1% formic acid and concentrated on an Oasis HLB 3cc column (Waters, Milford, MA). The acetonitrile eluate was evaporated to dryness and redissolved in 20% acetonitrile, 24% methanol, 0.1% formic acid (80% mobile phase A). Samples and standards were analysed on an Acquity UPLC with a TQ detector (Waters, operated in MRM mode with a gradient from phase A to B (100% acetonitrile) over 5 min. Between run CV% for the internal standard (n=48) was 13.5%. The individual compounds were quantified using QuanLynx version 4.1 (Waters) based on internal standards and external calibrants. Based on the analytical results for the individual primary and secondary bile acids these were summed for each rat.

Statistical analysis

The data were analyzed for normal distribution using the Shapiro-Wilcks W-test and for homogeneity of variance using Levenes test ($P>0.05$). Some data had to be log transformed in order to meet these criteria. The normally distributed and variance homogenous data were analysed by ANOVA. If significant differences were found between groups further comparisons were done using least square means. We used the SAS statistical package v. 9 (SAS Institute, Cary, NC, USA) and consider a *P*-value below 0.05 significant.

RESULTS

Composition of onion by-product and and fractions.

Fructose, glucose, sucrose and fructan content in the onion by-product and its two derived fractions are shown in **Table 1**. A semiquantitative size distribution analysis of the fructans in the extract indicated that more than 90% had 10 fructose residues or less and more than 60% had 5 residues or less. Very small amounts of longer-chain fructans were present. Starch was not found in any of the samples. Total quercetin content in the onion by-product, the extract, and the residue after hydrolysis of glycosides was found to be 3.37±0.52, 3.97±0.01, and 1.22±0.33 mg / g wet weight, respectively.

Markers of liver function. Gene expression

The effects of onion by-product feeding on hepatic enzyme activities of alanine aminotransferase (ALAT), alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT) are shown in **Table 2**. GGT activity was higher in rats fed with the onion by-product or the onion extract fraction compared to the control group. By contrast, ALAT and ALT activities were lower when rats were fed the onion by-product or its derived fractions compared to the control group.

Hepatic expression of the gene encoding the rate-limiting enzyme involved in cholesterol biosynthesis, the 3-hydroxyl-3-methylglutaryl-Coenzyme A reductase (*Hmgcr*), and leucocyte expression of the gene tromboxane A_2 synthase (*Txas*) are shown in **Table 3**. *Hmgcr* was not significantly altered (P=0.3) as a consequence of feeding with onion by-product or either of the fractions. *Txas* expression was significantly down-regulated (P=0.004) in rats fed the onion by-product and the onion extract.

Lipids

Triacylglycerides (TAG) concentrations, total cholesterol (TC) and cholesterol content in lipoproteins (HDL-C, LDL-C and VLDL-C) in rat plasma samples are shown in **Figure 1**.

The results show that rat plasma TAG and TC concentrations were not significantly altered in healthy, fasted rats fed with the onion by-product or an onion extract. By contrast, the onion residue fraction significantly increased TAG (p=0.001) and VLDL-C (p=0.016) cholesterol concentration values in the fasting state.

Bile acids

The concentration of bile acids in faeces of rats fed onion by-product, onion extract and onion residue are shown in **Table 4**. We determined a total of 15 different bile acids in the collected faecal samples, including the major primary rat bile acids, the alpha- and beta-muricholic acid. Neither total bile acids nor total primary or secondary bile acids were significantly affected by feeding the onion by-product or its fractions, although the excretion of primary bile acids were numerically doubled by feeding extract or residue fractions, but not by the whole onion by-product. This apparent increase was caused primarily by high excretion of alpha- and beta-muricholic acids following feeding of the rats with these fractions. The excretion of alpha-muricholic acid was significantly increased in the group of rats fed with onion extract (P=0.03). Other major bile acids or ratio between conjugated and unconjugated bile acids were not significantly affected by the treatments (data not shown). Faecal output differed considerably between rats from 0.22 - 2.49g/24h with no significant difference between the groups (data not shown). There was no relationship between faecal output and total bile acid excretion.

DISCUSSION

In the current study we aim to study the effects caused by an onion by-product intake on CVD risk factors, including total lipids and lipoproteins as well as platelet aggregation in a healthy rat model. The onion by-product (*Allium cepa* L. *cepa*, 'Recas') used to feed the rodents of our study offers the additional value of having a real possibility for being

developed as an antioxidant and antibrowning ingredient in foods (*5*). The safety of adding this onion product is therefore also an important aspect of the current study. A fructan and fructooligosacharide (FOS) extraction from the onion by-product target of our study was performed since one of our aims was to elucidate the role of onion soluble and insoluble fibre on some of the selected CVD risk factors. Afterwards, we analysed these compounds as well as free sugars, total starch, and quercetin in the fractions. It can be calculated that out of the total amount of these materials present in the feed containing onion by-product, approximately 85-90% is in the feed with extract and the remaining 9-15% is in the feed with residue (**Table 1**). Moreover, analysis of an excreted onion polysulphide metabolite in urine from rats fed with the by-product and its two fractions revealed that polysulphides appear to have the same concentration in all three tested products (7). Since the by-product fractions were fed in a dose corresponding to their presence in the by-product this means that polysulphides in this study were fed in relative amounts of 3:2:1 to the rat groups given by-product, extract and residue, respectively.

Since the liver is central for regulation of plasma lipids we also investigated liver function. Most of the rat studies reporting lipid-modulating effects of various forms of onion have used alloxan or streptozin-induced diabetic rats and report onion antihyperlipidemic and antyhyperglycemic effects (*18*). In the study by El-Dermedash et al**.** (*19*) diabetic rats fed with onion and garlic juices showed a reduction in plasma alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, alkaline and acid phosphatases, and the authors reported that these two *Allium* vegetables can inhibit the liver and renal damage caused by alloxan-induced diabetes. In our study, healthy rats were fed three 'Recas' onion products, an onion by-product, an onion extract, and an onion residue. Similarly to the above cited studies which used diabetic rodents, our results revealed a decreased leakage of hepatic

enzymes, except for GGT (**Table 2**). Overall, it could be inferred that in healthy rats these onion by-products did not cause any overt liver or renal damage that could affect cholesterol handling.

Concerning rat metabolic lipid regulation, our results show significantly increased TAG and VLDL-C concentrations in plasma of rats fed the onion residue (**Figure 1**). *Hmgcr* was not significantly down-regulated as a consequence of feeding rats with the onion by-product or either of the fractions (**Table 3**). In a study by Campos et al. (*20*) a decreased plasma TC, TAG, and LDL-C were seen in streptozotocin diabetic rats. Later on, Azuma et al. (*21*) found lower plasma TAG levels and no significant effects on cholesterol levels in diabetic rats fed an onion diet. Effects shown in other animal models such as pigs demonstrate that the consumption of onion modifies plasma lipid profiles in either healthy pigs or pigs consuming a high fat diet but that response was depending on variety of onion, feeding time and sampling time (*22, 23*). Our results are in agreement with these studies as far as plasma TAG, TC, and cholesterol concentrations in the LDL and HDL fractions were unaffected by feeding with the onion by-product and the onion extract. Onion fractions have not been tested previously so our observation of an increased level of cholesterol in the VLDL fraction and an increased level of plasma TAG after feeding this onion residue indicates that it stimulates up-regulation of hepatic lipid transport to peripheral tissues. This is often caused postprandially by fructose-rich or fatty foods. The result is unexpected here since the fructose level in the residue fraction is much lower than in the other fractions (*6*) and because the effect was observed after fasting. An altered regulation of hepatic cholesterol synthesis or loss of cholesterol metabolites in the form of bile acids could not explain the effect based on our data. We speculate that onion residue components may function as a slow-release formulation for fructose thereby keeping the animals for a longer time in a state where

fructose is released. Alternatively, the residue may have an effect on other aspects of VLDL-assembly that are suppressed by other constituents in the whole onion by-product. The bile acid synthetic pathway and the VLDL-C assembly/secretion pathway are regulated through sterol response element-binding protein (SREBP)-dependent transcription (*24, 25*). Therefore, additional gene expression studies would be required to assess this latter possibility.

Total and primary bile acid concentration in faeces as well as their total 24h excretion were not significantly increased (**Table 4**). However, there was a numerical doubling of faecal primary bile acid concentration after feeding the extract and the residue fractions, and faecal concentration of the major primary bile acid, alpha-muricholic acid, was significantly increased in rats fed with the onion extract. It cannot be ruled out that the large variations between rats in faecal bile acid concentrations and in 24h faecal output may have masked an effect, however the lack of effects on total cholesterol is in agreement with the observed null result also on bile acid excretion. The rat is not a good model for agents affecting reverse cholesterol transport since the transfer of cholesterol from LDL to HDL does not proceed in a similar manner in rodents and in humans. If onion affects this mechanism it has to be investigated in humans and would not be observed in the present study. The hypocholesterolemic effect of dietary fibre has been attributed to its ability to inhibit intestinal absorption of bile acids and neutral steroids, resulting in greater faecal bile acids and total steroid excretion (*26*). Several studies have reported the lipid lowering effect of fructans and onion dietary fibre components in rats (*27-29*) at dose levels of around10% in the diet, a level somewhat higher than ours. Also onion OSCs have been reported to have hypolipidemic effects (*30*), albeit at doses far above what could be obtained through foods. Particularly, water soluble OSCs had been reported to modulate lipid metabolism (*31*). Most

of the studies conducted in this area were focused on garlic OSCs (*32-33*). We have reported earlier that the major part of sugars and FOS in the onion by-product was recovered in the extract whereas the dose of OSC was twice as high in the extract as in the residue fed group of animals (*6, 7*). Neither the contrasts in OSCs nor those in FOS seem to have affected the plasma cholesterol distribution or bile acid excretion in the current study. The dose levels used in the studies with purified components by others were somewhat higher than achieved here with a whole food. Some of the results published by others are based on standard kits for measurement of lipoproteins; however we have observed that such kits do not work with rodent samples (17). Our chemometric method is based on modelling of lipoprotein data obtained by ultracentrifugation. It is therefore likely that previous reports may have overestimated the effects of FOS and OSCs on plasma lipoproteins in rats.

Onion has been previously studied in different animal models for its natural antithrombotic effects (*34-38*). Extracts from onions inhibit human platelet aggregation *in vitro*, *in vivo* and ex *vivo* (*39*). Thromboxane A_2 (TXA_2) is a potent inducer of platelet aggregation and a vasoconstrictor which is increased in thrombotic disorders. Inhibition of the platelet function including TXA_2 formation represents a promising approach for thrombosis prevention and therefore for CVD prevention. Inhibitory effect on arachidonic acid release and a combination of thromboxane A_2 synthase (TXAS) inhibition with TXA_2/PGH_2 receptor blockade might, at least partly, contribute to the antiplatelet effect of onion (*35*). Several human disorders have been related with an imbalance in the *TXAS* gene expression which is proposed to be involved in thrombopoiesis and lymphocyte differentiation (*40-41*). A number of diseases with a vascular component such as systemic sclerosis increases TXAS enzyme in activated WBC promoting platelet aggregation, endothelial dysfunction and

vascular damage (*42*). Moreover, *TXAS* gene up-regulation has been related with some cancer processes such as human colorectal carcinoma and invasive bladder cancer (*43, 44*).

Our results show that the *Txas* rat gene expression was decreased by the onion by-product and onion extract feeding (**Table 3**). We suggest that feeding rats these two onion products would trigger a lower TXA_2 release. It could be inferred that the onion by-product tested could have potential anti-clotting and related properties as previously reported by others (*39*).

Onion bulb sulphur content and genotype should be taken into account when assessing onion effects on platelets (*45-46*). It is generally accepted that the more pungent onions having high pyruvate content, and OSCs content indicator, also possess a high antithrombotic activity (*47*). By contrast, no significant correlation was found between onion quercetin content and antithrombotic activities (*48*). Since we have similar effects in the by-product and the extract fed groups and no effect in the residue fed group of rats this does not seem to reflect the relative exposures to total OSCs which would be close to 3:2:1 in the three groups, respectively (7). Special OSC-class constituents, especially the more water-soluble which are expected to be in the extract, or other compounds would therefore seem to be potentially more important for the response that we have observed here, and further analyses using LC-MS based metabolomic profiling will be carried out to search for metabolites which correlate with the biological response observed.

In conclusion, the onion by-product (*Allium cepa* L. var. *cepa,* 'Recas') or its fractions do not seem to reduce cholesterol or to affect hepatic *Hmgcr* rat gene expression. Feeding of rats with the onion residue increased cholesterol in the VLDL lipoprotein fraction and also increased plasma TAG, indicating an up-regulation of hepatic lipid transport to peripheral tissues. Neither total bile acids nor total primary or secondary bile acids were significantly

affected by feeding the onion by-product or its fractions. TXA_2 biosynthesis is decreased in leucocytes of rats fed the onion by-product and the onion extract, indicating that the onion by-product may exert anti-thrombotic effects. The main constituents that inhibit platelet aggregation are recovered in the onion extract but further studies are needed for their identification.

The effects differ between the onion extract and the onion residue indicating that specific bioactivities may be obtained by further refining the by-product. The effects of the onion by-product should be investigated in humans in order to assess the full safety of using the product as an alternative to synthetic additives, e.g. as an antioxidant and anti-browning agent.

ACKNOWLEDGEMENTS

ER-M, RIJ, BNK, MK, MOP, MPC, CS-M, and LOD no conflicts of interest. LOD and CS-M designed research together with ER-M. CS-M and MPC provided the onion by-product, ER-M produced the onion fractions. MOP provided the animal study protocol and diets. RIJ, BNK, MK, and ER-M conducted research. LOD analyzed data. ER-M wrote the paper under CS-M and LOD supervision. LOD had primary responsibility for the final content. We thank Vibeke Kegel for her excellent technical assistance.

Abbrevations

ALAT, alanine aminotransferase; ALP, alkaline phosphatase; CVD, cardiovascular disease; GGT, gamma glutamyl transferase; HDL-C, cholesterol in high density lipoprotein; *Hmgcr*, 3- hydroxy-3-methylglutaryl-Coenzyme A reductase rat gene; LDL-C, cholesterol in low density lipoprotein; OSC, organosulfur compounds; TAG, triacylglycerides; TXA_2, thromboxane $A_{2;}$ *Txas*, thromboxane A_2 synthase rat gene, TC, total cholesterol; VLDL-C, cholesterol in very low density lipoprotein.

LITERATURE CITED

1. Powolny, A.A.; Singh, S.V. Multitargeted prevention and therapy of cancer by diallyl trisulfide and related Allium vegetable-derived organosulfur compounds. *Cancer Lett*. **2008**, *269*, 305-314.
2. Benavides, G.A.; Squadrito, G.L.; Mills, R.W.; Patel, H.D.; Isbell, T.S.; Patel, R.P.; Darley-Usmar, V.M.; Doeller, J.E.; Kraus, D.W. Hydrogen sulfide mediates the vasoactivity of garlic. *Proc Natl Acad Sci USA*. **2007**, *104*, 17977-82.
3. Xiao, D.; Zeng, Y.; Hahm, E.R.; Kim, Y.A.; Ramalingam, S.; Singh, S.V. Diallyl trisulfide selectively causes Bax- and Bak-mediated apoptosis in human lung cancer cells. *Environ and Mol Mutagen*. **2009**, *50*, 201-212
4. Hertog, M.G.; Feskens, E.J.; Hollman, P.C.; Katan, M.B.; Kromhout, D. Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen Elderly Study. *Lancet*. **1993**; *342*, 1007–11.
5. Roldán, E.; Sánchez-Moreno, C.; de Ancos, B.; Cano, M.P. Characterisation of onion (*Allium cepa* L.) by-products as food ingredients with antioxidant and antibrowning properties. *Food Chem*. **2008**, *108*, 907-916.
6. Roldán-Marín, E.; Krath, B.N.; Poulsen, M.; Binderup, M-L.; Nielsen, T.H.; Hansen, M.; Barri, T.; Langkilde, S.; Cano, M.P.; Sánchez-Moreno, C.; Dragsted, L.O. Effects of an onion by-product on bioactivity and safety markers in healthy rats. *Br J Nut*. **2009**. In press (doi:10.1017/S0007114509990870)
7. Winning, H.; Roldán-Marín, E.; Dragsted, L.O.; Viereck, N.; Poulsen, M.; Sánchez-Moreno, C.; Cano, M.P.; Engelsen, S.B. An exploratory NMR nutri-metabonomic investigation reveals dimethylsulfone as a dietary biomarker for onion intake. *Analyst*. **2009**, *134*, 2344-2351.

8. The World Health Report 2008. Primary Health Care – Now More Than Ever. World Health Organization. http://www.who.int/whr/2008/en/index.html.
9. WHO World Health Organization Prevention of cardiovascular disease : Guidelines for assessment and management cardiovascular risk http://www.who.int/cardiovascular_diseases/guidelines/Full%20text.pdf
10. WHO World Health Organization Global Strategy on Diet, Physical Activity and Health. Cardiovascular disease: prevention and control.
11. Jaime, L.; Martínez, F.; Martín-Cabrejas, M.A.; Mollá, E.; López-Andréu, F.J.; Waldron, K.W.; Esteban, R.M. Study of total fructan and fructooligosaccharide content in different onion tissues. *J Sci Food Agric*. **2001**, *81*, 177-182.
12. Beutler, H.O. Monosaccharides and derivatives. D-Fructose. In *Methods of Enzymatic Analysis* (ed.H.U. Bergmeyer) Verlag Chemie, Weinheim, Germany, **1984**, Vol. 6, pp. 321-327.
13. Kunst, A.; Draeger, B; Ziegenhorn, J. Monosaccharides and derivatives. D-Glucose. In *Methods of Enzymatic Analysis* (ed. H.U. Bergmeyer) Verlag Chemie, Weinheim, Germany, **1984**, Vol. 6, pp. 163-172.
14. Outlaw, W.H.; Tarczynski, M.C. Poly-, Oligo- and Disaccarides. Sucrose. In *Methods of Enzymatic Analysis* (ed. H.U.Bergmeyer) Verlag Chemie, Weinheim, Germany, **1984**, Vol. 6, pp. 96-103.
15. Nielsen, T.H.; Skjærbæk, H.C.; Karlsen, P. Carbohydrate metabolism during fruit development in sweet pepper (*Capsicum annuum* L.) plants. *Physiologia Plantarum.* **1991**, *82*, 311-319.
16. Blennow, A.; Bay-Smidt, A.M.; Olsen, C.E.; Wischmann, B.; Møller, B.L. The degree of starch phosphorylation is related to the chain length distribution of the

neutral and the phosphorylated chains of amylopectin. *Carbohydr Res.* **1998**, *307*, 45-54.

17. Kristensen M, Savorani F, Ravn-Haren G et al. NMR and interval PLS as reliable methods for determination of cholesterol in rodent lipoprotein fractions. Analyst 2009; in press (DOI 10.1007/s11306-009-0181-3), 1-8.
18. Islam, M.S.; Choi, H.; Loots, D.T: Effects of dietary onion (*Allium cepa* L.) in a high-fat diet streptozotocin-induced diabetes rodent model. *Ann Nutr Metab*: **2008**, *53*, 6-12.
19. El-Demerdash, F.M.; Yousef, M.I.; El-Naga, N.I. Biochemical study on the effects of onion and garlic in alloxan-induced diabetic rats. *Food Chem Toxicol*. **2005**, *43*, 57-63.
20. Campos, K.E.; Diniz, Y.S.; Cataneo, A.C.; Faine, L.A.; Alves, M.J.Q.F.; Novelli, E.L.B. Hypoglycaemic and antioxidant effects of onion, *Allium cepa*: dietary onion addition, antioxidant activity and hypoglycaemic effects on diabetic rats. *Int J Food Sci Nutr*. **2003**, *54*, 241-246.
21. Azuma, K.; Minami, Y.; Ippoushi, K.; Terao, J. Lowering effects of onion intake on oxidative stress biomarkers in streptozotocin-induced diabetic rats. *J Clin Biochem Nutr*. **2007**, *40*, 131-140.
22. Ostrowska, E.; Gabler, N.K.; Sterling, S.J.; Tatham, B.G.; Jones, R.B.; Eagling, D.R.; Jois, M.; Dunshea, F.R. Consumption of brown onions (*Allium cepa* var. cavalier and var. destiny) moderately modulates blood lipids, haematological and haemostatic variables in healthy pigs. *Br J Nutr*. **2004**, *91*, 211-218.
23. Gabler, N.K.; Ostrowska, E.; Sterling, S.J.; Jones, R.B.; Tatham, B.J.; Eagling, D.R.; Jois, M.; Dunshea, F.R. Consumption of raw brown onions variably modulate plasma

lipid profile and lipoprotein oxidation in pigs fed a high-fat diet. *J Sci Food Agric*. **2005**, *85*, 154-160.

24. Ness, G.C.; Chambers, C.M. Feedback and hormonal regulation of hepatic 3-hydroxy-3-methylglutaryl Coenzyme A reductase: The concept of cholesterol buffering capacity. *Proc Soc Exp Biol Med*. **2000**, *224*, 8-19.
25. Kang, S.; Davis, R.A. Cholesterol and hepatic lipoprotein assembly and secretion. *Biochim Biophys Acta*. **2000**, *1529*, 223-30.
26. Han, K-H; Iijuka, M.; Shimada, K-i.; Sekikawa, M.; Kuramochi, K.; Ohba, K.; Ruvini, L.; Chiji, H.; Fukushima, M. Adzuki resistant starch lowered serum cholesterol and hepatic 3-hydroxy-3-methylglutaryl-CoA mRNA levels and increased hepatic LDL-receptor and cholesterol 7alpha-hydroxylase mRNA levels in rats fed a cholesterol diet. *Br J Nutr*. **2005**, *94*, 902-908.
27. Nishimura, N.; Taniguchi, Y.; Kiriyama, S. Plasma cholesterol-lowering effect on rats of dietary fiber extracted from immature plants. *Biosci Biotechnol Biochem*. **2000**, *64*, 2543-51.
28. Delzenne, N.M.; Kok, N. Effects of fructans-type prebiotics on lipid metabolism. *Am J Clin Nutr*. **2001**, *73*, 456S-458S.
29. Busserolles, J.; Gueux, E.; Rock, E.; Demigne, C.; Mazur, A.; Rayssiguier, Y. Oligofructose protects against the hypertriglyceridemic and pro-oxidative effects of a high fructose diet in rats. *J Nutr*. **2003**, *133*, 1903-1908.
30. Kumari, K.; Augusti, K.T. Lipid lowering effect of S-methyl cysteine sulfoxide from *Allium cepa* Linn in high cholesterol diet fed rats. *J Ethnopharmacol*. **2007**, *109*, 367-371.

31. Lin, C.C.; Yin, M.C. Effects of cysteine-containing compounds on biosynthesis of triacylglycerol and cholesterol and anti-oxidative protection in liver from mice consuming a high-fat diet. *Br J Nutr*. **2008**; *99*, 37-43.

32. Liu, L.; Yeh, Y.Y. Water-soluble organosulfur compounds of garlic inhibit fatty acid and triglyceride syntheses in cultured rat hepatocytes. Lipids. 2001, *36*, 395-400

33. Liu, L.; Yeh, Y.Y. S-Alk(en)yl cysteines of garlic inhibit cholesterol synthesis by deactivating HMG-CoA reductase in cultured rat hepatocytes. *J Nutr*. **2002**, *132*, 1129-34.

34. Bordia, T.; Mohammed, N.; Thomson, M.; Ali, M. An evaluation of garlic and onion as antithrombotic agents. *Prostaglandins Leukot Essent Fatty Acids*. **1996**, *54*, 183-186

35. Moon, C.H.; Jung, Y.S.; Kim, M.H.; Lee, S.H.; Baik, E.J.; Park, S.W. Mechanism for antiplatelet effect of onion: AA release inhibition, thromboxane A_2 synthase inhibition and TXA_2/PGH_2 receptor blockade. *Prostaglandins Leukot Essent Fatty Acids*. **2000**, *62*, 277-283.

36. Briggs, W.H.; Folts, J.D.; Osman, H.E.; Goldman, I.L. Administration of raw onion inhibits platelet-mediated thrombosis in dogs. *J Nutr*. **2001**, *131*, 2619-2622.

37. Jung, Y.S.; Kim, M.H.; Lee, S.H.; Baik, E.J.; Park, S.W.; Moon, C.H. Antithrombotic effect of onion in streptozotocin-induced diabetic rat. *Prostaglandins Leukot Essent Fatty Acids*. **2002**, *66*, 453-458.

38. Gabler, N.; Osrowska, E.; Imsic, M.; Eagling, D.; Jois, M.; Tatham, B.; Dunshea, F.R. Dietary Onion intake as part of a typical high fat diet improves indices of cardiovascular health using the mixed sex pig model. *Plant Foods Hum Nutr*. **2006**; *61*, 179-85.

39. Osmont, K.S.; Arnt, C.R.; Goldman, I.L. Temporal aspects of onion-induced antiplatelet activity. *Plant Foods Hum Nutr*. **2003**, *58*, 27-40.

40. Wang, L.H.; Kulmacz, R.J. Thromboxane synthase: structure and function of protein and gene. *Prostaglandins Other Lipid Mediat*. **2002**, *68-69*, 409-422.

41. Yu, I.S.; Lin, S-R.; Huang, C-C.; Tseng, H-Y.; Huang, P-H.; Shi, G-Y., Wu, H-L.; Tang, C-L.; Chu, P-H.; Wang, L-H.; Wu, K.K.; Lin, S-W. TXAS-deleted mice exhibit normal thrombopoiesis, defective hemostasis, and resistance to arachidonate-induced death. *Blood*. **2004**, *104*, 135-142.

42. Young, V.; Ho, M.; Vosper, H.; Belch, J.J.; Palmer, C.N. Elevated expression of the genes encoding TNF-alpha and thromboxane synthase in leucocytes from patients with systemic sclerosis. *Rheumatology*. **2002**, *41*, 869-75.

43. Sakai, H.; Suzuki, T.; Takahashi, Y.; Ukai, M.; Tauchi, K.; Fujii, T.; Horikawa, N.; Minamimura, T.; Tabuchi, Y.; Morii, M.; Tsukada, K.; Takeguchi, N. Upregulation of thromboxane synthase in human colorectal carcinoma and the cancer cell proliferation by thromboxane A_2. *FEBS Lett*. **2006**, *580*, 3368-3374.

44. Moussa, O.; Riker, J.M.; Klein, J.; Fraig, M.; Halushka, P.V.; Watson, D.K. Inhibition of thromboxane synthase activity modulates bladder cancer cell responses to chemotherapeutic agents. *Oncogene*. **2007**, *27*, 55-62.

45. Goldman, I.L.; Kopelberg, M.; Debaene, J.E.; Schwartz, B.S. Antiplatelet activity in onion (*Allium cepa*) is sulfur dependent. *Thromb Haemost*. **1996**, *76*; 450-452.

46. Perner, H.; Rohn, S.; Driemel, G.; Batt, N.; Schwarz, D.; Kroh, L.W.; George, E. Effect of nitrogen species supply and mycorrhizal colonization on organosulfur and phenolic compounds in onions. *J Agric Food Chem*. **2008**; *56*, 3538-3545.

47. Cavagnaro, P.F.; Sance, M.M.; Galmarini, C.R. Effect of heating on onion (*Allium cepa* L.) antiplatelet activity and pungency sensory perception. *Food Sci Technol Int*. **2007**, *13*, 447-453.

48. Yamada, K.; Naemura, A.; Sawashita, N.; Noguchi, Y.; Yamamoto, J. An onion variety has natural antithrombotic effect as assessed by thrombosis/thrombolysis models in rodents. *Thromb Res*. **2004**, *114*, 213-220.

This research was supported by funding from the Spanish Ministry of Science and Innovation (AGL2003-09138-C04-01; 200670I08; Consolider-Ingenio Programme 2010, FUN-C-FOOD CSD2007-00063; and AGL2008-04798-C02-01/ALI) and from the Danish Ministry of Food, Agriculture and Fisheries (NuBI, 3304-FVFP-060696-01). E.R.-M. wishes to thank the Spanish Ministry of Science and Innovation for a Predoctoral Fellowship.

Table 1. Glucose, fructose, sucrose and fructans content in the onion by-product and its two derived onion fractions.

	Glucose (mg/g)	Fructose (mg/g)	Sucrose (mg/g)	Fructans (mg/g)
Onion by-product	205.6±4.9[a]	189.4±9.2	96.9± 4.4	42.5± 4.8
Onion extract	215.8±4.8	199.3±5.6	85.7± 2.1	71.7± 8.8
Onion residue	102.8±8.6	95.5±8.6	53.2± 3.9	30.8± 2.9

[a] Numbers are means ± SD of three determinations based on wet weight. The humidity in each fraction was 17.3% 18.2% and 7.3%, respectively for the onion by-product, the extract and the residue.

Table 2. Onion by-product and two derived onion fractions effects on rat plasma alanine aminotransferase (ALAT), gamma glutamyl transferase (GGT) and alkaline phosphatase (ALP) activities.

RAT GROUP	ALAT (UI/L)	GGT (UI/L)	ALP (UI/L)
Control	91.14 ± 8.21	2.40 ± 0.59	753.57 ± 71.59
Onion by-product	85.0 ± 7.97*	3.40 ± 1.43*	693.87 ± 67.22*
Onion extract	76.87 ± 8.76*	3.19 ± 1.90*	673.50 ± 75.33*
Onion residue	76.37 ± 6.72*	2.67 ± 0.87	663.87 ± 31.10*

Results are expressed as mean ± SD; n=8. *$P < 0.05$ as compared with control.

Table 3. Effect of an onion by-product and two derived onion fractions on rat hepatic 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*Hmgcr*) and leucocyte tromboxane A_2 synthase (*Txas)* gene expression (*n*=5). Results are expressed as means ± standard deviations)

RAT GROUP	*Hmgcr*	*Txas*
Control	0.94 ± 0.69	0.98 ± 0.25
Onion by-product	0.53 ± 0.51	0.43 ±0.10*
Onion extract	0.46 ± 0.30	0.43 ±0.13*
Onion residue	0.54 ± 0.23	0.95 ± 0.35

Gene expression of target genes are given relative to the endogenous reference 18S rRNA and to a calibrant (RQ). Significantly different from control group *P<0.05.

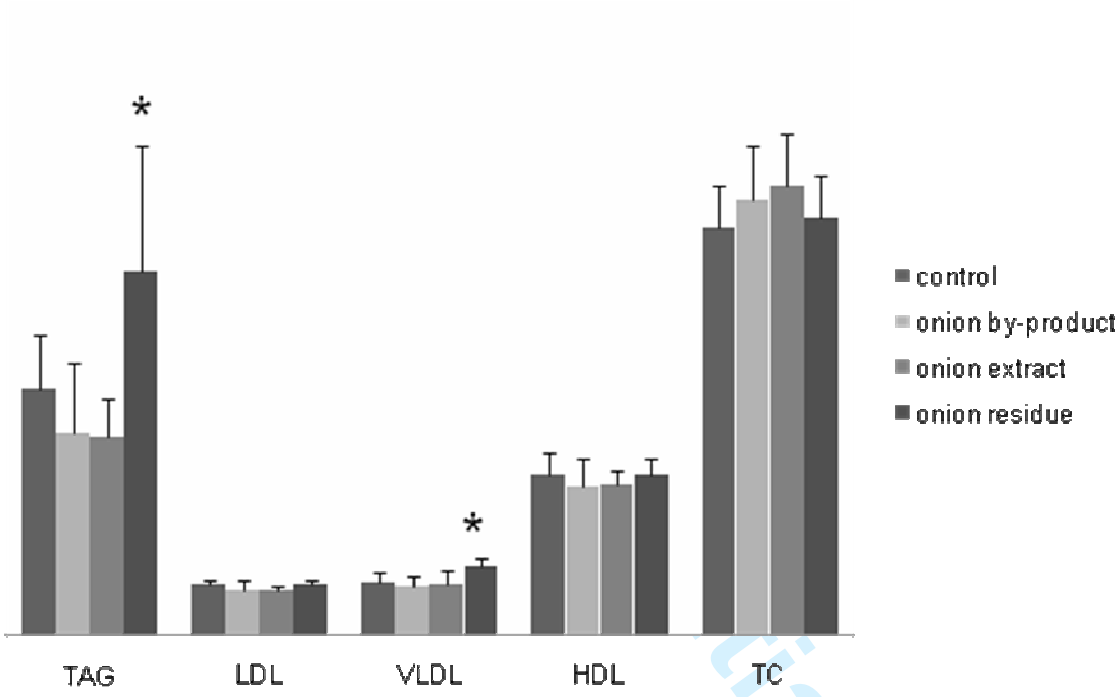
Table 4. Primary and secondary bile acid concentrations in faeces of rats fed with an onion by-product and two derived onion fractions.

RAT GROUP	Primary bile acids	Alpha-muricholic acid	Secondary bile acids
Control	18.4 ± 13.4	11.1 ± 6.7	12.6 ± 7.0
Onion by-product	20.8 ± 14.0	13.0 ± 8.3	13.8 ± 10.1
Onion extract	36.2 ± 20.8	23.1 ± 11.3*	13.7 ± 7.8

Onion residue	40.7 ± 43.9	21.2 ± 21.1	12.1 ± 10.1

Values are expressed as means ± standard deviations in units of ug/g faeces. * P<0.05.

Figure 1. Effects of feeding with onion by-product and its derived fractions on rat plasma triacylglycerides (TAG), total cholesterol (TC), and cholesterol in very low, low, and high lipoprotein fractions (VLDL-C, LDL-C and HDL-C).



Asterisk (*) on the bars indicate significant difference between the onion groups and the control group at *P*<0.05.

Chapter 9

General discussion

Chapter 9. General discussion

1. Effects of food processing technologies on onion nutritional and technological quality. Potential development of novel and innovative onion food ingredients

Nowadays, a research field for the food industry is the study of different vegetables food processing technologies in order to extend their shelf-life, whilst ensuring its safety and nutritional quality. Moreover, the consumer's interests stress more on the history of the food products daily eaten with special emphasis on their geographical origin and processes applied to food. These facts have also triggered to an increasing emergence of a new generation of natural food ingredients that can substitute the available synthetic additives.

Traditionally, thermal technologies have been used to preserve vegetables. However, certain deleterious effects have been shown when applying very low or high temperatures to vegetables in terms of possible freezing injuries, lack of microbiology safety, and loss of nutrients.

Novel nonthermal technologies have recently received considerable attention in response to consumer demands for more fresh and natural food products. These technologies represent an alternative for the conventionally food processing technologies. The importance and advantages of using nonthermal technologies are supported not only in food safety assurance but also in high sensory and nutritional quality. The long list of so-called nonthermal technologies contains promising alternatives to thermal treatments such as microfiltration (MF), ultrasonication, irradiation (ionizing radiation), high hydrostatic pressure (HHP), pulsed electric fields (PEF), ohmic, microwave or radio frequency heating among others.

Some of the most promising nonthermal processing technologies appear to be HHP, PEF and ultrasonication in combination with HHP. Gamma irradiation which also has great potential, suffers from an unfavourable public perception.

The first part of the present PhD Thesis focused on studying the impact of both, traditional food preservation technologies (thermal technologies such as freezing, pasteurization, and sterilization) (*Paper I*) and an emerging nonthermal technology (high hydrostatic pressure) (*Paper II*), on onion nutritional and technological quality.

We aimed to study from a nutritional and technological point of view the possible advantages and disadvantages of processing and preserving onion from different cultivars with conventional food thermal technologies and an emerging nonthermal technology (HHP). Subsequently, we discuss whether the different processed-onion

products obtained when processing onion with each technology chosen in our studies would be positively valuable in order to possibly develop novel and innovative onion ingredients.

1.1. Effects of food thermal processing technologies (freezing, pasteurization, and sterilization) on onion by-products nutritional and technological quality

The adequate processing and preservation of onions that came from all those onion not commercially available, onion surpluses or onion wastes would represent an interesting and sustainable possibility for the food industry nowadays. There is a global concern about sustainable vegetable production which requires a good waste management in order not to have great economic losses. Generally, onion wastes could be processed for obtaining useful onion by-products, afterwards and in order to obtain safe and good quality onion products, preservation or stabilization technologies are required.

One of our first aims was to study stabilized onion by-products functional properties that would be valuable for their potential development as natural onion food ingredients. To our knowledge no other studies have evaluate the effect caused by traditional thermal technologies on onion by-products properties.

Therefore, in our first work we studied the impact of conventional thermal technologies including freezing (-18 ºC), pasteurization (100 ºC, 11-17 min) and sterilization (115 ºC, 17-31 min) on the bioactive compound content, antioxidant and antibrowning properties of onion by-products (juice, paste, and bagasse) elaborated from two onion cultivars ‘Recas’ and ‘Figueres’ (*Allium cepa* L. var. *cepa*, ‘Recas’ and *Allium cepa* L. var. *cepa*, ‘Figueres’) (*Paper I*).

Firstly, it is important to take into account that a proper harvesting and post-harvest handling methods, as well as proper storage of vegetables not immediately eaten, will help maintain the flavour, texture, and nutritive value. An onion proper storage requires good air circulation, relative dryness and cool temperatures. Onions are not sensitive to chilling and can be stored at -2 to -3 °C, since the highest freezing point is -0.8 °C. However, Storage at < -4 °C may cause freezing injuries (Onion-USDA, 2009).

Onions subject of our study were onion wastes, including onion surplus and onion not commercially available, these onions were processed into onion by-products. Immediately after, thermal technologies were held to stabilize and preserve these onion by-products.

Regarding onion by-products bioactive compound content, it was shown that all stabilized onion by-products from 'Recas' cultivar had a significantly higher bioactive compound content than those by-products from 'Figueres' cultivar (Tables 1, 2, 3-*Paper I*). Frozen and sterilized 'Recas' pastes together with pasteurized 'Recas' bagasse showed the highest total phenol content. Frozen 'Recas' paste had the highest quercetin content among all the stabilized pastes analyzed. Pasteurized and sterilized 'Recas' bagasses showed the highest total quercetin content among the high temperature-preserved by-products. Moreover, pasteurized and sterilized 'Recas' pastes were significantly different to frozen 'Recas' paste.

Onion antioxidant activity had been widely studied and in general it has been related to its phenol content and particularly to its quercetin content (Kim & Kim, 2006; Prakash *et al*., 2007). In terms of antioxidant activity, the pastes from the two onion cultivars assayed in our study showed generally high antiradical efficiency values (Figures 1a, 1b, 1c-*Paper I*). Concerning pasteurizacion technology applied to preserve 'Recas' onion by-products it has to be noticed that pasteurized 'Recas' paste offered better antioxidant characteristics than pasteurized 'Recas' bagasse or juice. Therefore this onion pasteurized 'Recas' paste would be better valuable as an antioxidant food ingredient than those bagasses or juices due to the lower concentration (EC_{50}) needed to scavenge the stable radical $DPPH^{\bullet}$ (Table 2-*Paper I*).

Concerning onion antibrowning properties, we demonstrated that the polyphenol oxidase (PPO) activity from avocado fruit were significantly reduced by all the onion by-products analyzed. Sterilized 'Recas' and 'Figueres' pastes showed more accused inhibitory PPO effect than pasteurized or frozen ones. It was shown the same behaviour by the two onion cultivar pastes either when pasteurization or sterilization technologies were applied. However, sterilization was significantly different from pasteurization or freezing technologies applied to those pastes (Table 4-*Paper I*).

One of our first observations was that when onion by-products were frozen at -18 ºC they maintained roughly intact their bioactive compound content. However, it seems that this technology could compromise onion safety as it is shown in Table 1 of the current chapter section. Several remarks can be done into this aspect such as that a freezing technology sometimes does not completely destroy bacteria, molds, and yeasts although it does retard their growth. Moreover, onion freezing injuries were early described by Palta *et al.* (1977). It was described that freezing injury in onion bulb tissues causes enhanced K^+ (potassium) efflux accompanied by a small but significant

loss of Ca^{2+} (calcium) following incipient freezing injury and swelling of protoplasm during the post-thaw secondary injury. The protoplasmic swelling of the cell is thought to be caused by the passive influx of extracellular K^+ into the cell followed by water uptake (Arora & Palta, 1986). Later on, these authors reported that the recovery of onion freeze-injured tissue depends on the functional activity of plasma membrane ATPase (Arora & Palta, 1991).

Thus, the temperature of the freezing technology in our study would be not recommended to preserve onion by-products since these onion products might be microbiology unsafe and could present freezing injuries which might be irreversible and modify the onion by-product primary texture.

By contrast, the thermal technologies of sterilization (115 ºC, 17-31 min) and pasteurization (100 ºC, 11-17 min) held to preserve the onion by-products of our study assure their safety (Table 1). Moreover, these technologies presented the additional advantages of enhancing onion antibrowning properties and mainly maintain their bioactive compound content and antioxidant properties compared to the freezing technology.

Onion antibrowning properties have been ascribed principally to their organosulfur compounds (OSCs) content and the Maillard products formed during the thermal treatment, these two compounds have been described to act synergistically (Billaud *et al*., 2004; Gruber *et al*., 2004; Kim *et al*., 2005). Our results are in agreement with other previous studies that found heated onion extracts to inhibit other fruits and vegetables PPO activities and therefore preventing them from the undesirable browning reactions which causes their loss of organoleptic quality (Ding *et al*., 2002; Kim *et al*., 2005; Lee, 2007).

Sterilization (115 ºC, 17-31 min) has the additional disadvantage of being a more aggressive technology due to the higher temperatures applied compared to pasteurization. Moreover, high temperatures might caramelize the onion by-products carbohydrates and particularly onion fructose which generally starts to caramelize at 110 ºC. Thus, sterilizing the onion by-products of our study would also be not recommended due not only to the onion nutritional quality loss, but also to the sensorial quality loss and to the possible undesirable brown colors and sweet flavors development in these by-products.

Therefore, after analyzing the advantages and disadvantages of freezing and sterilization food technologies held to stabilize the onion by-products, we demonstrated

that pasteurization (100 ºC, 11-17 min) as a thermal technology did not compromise onion by-products safety and nutritional quality, enhancing their technological antibrowning properties. Taking into account that the adoption of mild preservation technologies under European legislation is an ongoing process, as shown by the EC Novel Foods Regulation (Nº 258/97), pasteurizing onion by-products would be a good challenge for the food industry nowadays.

Table 1. Onion by-products (*Allium cepa* L. var. *cepa*, 'Recas') microbiological data

Onion by-product (*Allium cepa* L. var. *cepa,* 'Recas')	**Preservation technology**	**Aerobic mesophiles**	**SRC (Sulphite-reducing *Clostridium*)**	***E. coli***	***Salmonella***	***Listeria***
Juice	**Without treatment**	$2.6*10^6$	$1.1*10^3$	$1.0*10^1$	Absence	Absence
	Freezing	$3.9*10^4$	<10	<10	Absence	Absence
	Pasteurization	<10	<10	<10	Absence	Absence
	Sterilization	<10	<10	<10	Absence	Absence
Paste	**Without treatment**	$1.4*10^6$	$1.1*10^2$	$1.0*10^1$	Absence	Absence
	Freezing	$3.3*10^4$	<10	<10	Absence	Absence
	Pasteurization	<10	<10	<10	Absence	Absence
	Sterilization	<10	<10	<10	Absence	Absence
Bagasse	**Without treatment**	$1.2*10^6$	$2.8*10^2$	$1.0*10^1$	Absence	Absence
	Freezing	$4.0*10^4$	<10	<10	Absence	Absence
	Pasteurization	<10	<10	<10	Absence	Absence
	Sterilization	<10	<10	<10	Absence	Absence

1.2. Evaluation of processing onion at high-pressure combined with low temperature to obtain onion products with a high nutritional quality

Considering all the advantages and marketing opportunities offered by the high-pressure processing (HPP) as nonthermal technology, in our second investigation we experimentally designed a study comprising a response surface methodology according to a central composite face-centered design in which the variable ranges of pressure were 100-400 MPa and temperature 5-50 ºC, setting a constant time to 5 min.

We decided to choose onion from one of the Spanish varieties more commonly consumed, traded and extended up to date (*Allium cepa* L. var *cepa*, 'Grano de Oro'). This fresh onion was processed under ten chosen treatments that combine high-pressures (HP) and temperatures. Our aim was to determine and study the variation of the bioactive compound content and the antioxidant activity on these HP-processed

onion products to evaluate the effects caused by the HPP technology on fresh onion (*Paper II*).

Concerning onion bioactive compound content, it was shown that the extraction of onion total phenols and flavonols were significantly affected by the pressure and temperature applied in the different HPP treatments chosen. High pressure (400 MPa) and low temperature (5 ºC) treatment increased the extractability of total phenol from 'Grano de Oro' onion (Figure 1-*Paper II*). Similarly, this low temperature (5 ºC) with pressures of 100 and 400 MPa significantly increased the onion extractability of quercetin-4'-glucoside (Q_{MG}) (Figure 3-*Paper II*), total quercetin (TQ) (Figure 2-*Paper II*), and quercetin-3,4'-diglucoside (Q_{DG}) (Figure 4-*Paper II*), yielding an increase in their contents of 33, 26, and 17%, respectively, compared with untreated onion.

The total phenol and flavonol content rise found in 400 MPa/5 ºC-processed onion could be due to the disruption of the onion vegetative vacuoles where these phenolic compounds are confined and that the low temperature applied did not cause an important bioactive compound content loss. Another hypothesis could be that high pressure combined with low temperature treatments could help the extraction of cell wall membrane fixed polyphenols. Several authors have described similar effects with other bioactive compounds such as carotenoids in pressurized orange juice, persimmon puree and tomato-based products (De Ancos *et al.*, 2000, 2002; Krebbers *et al*., 2003; Sánchez-Moreno *et al.*, 2004, 2006; Plaza *et al.*, 2006).

Referring to the antioxidant activities analyzed in our study, it was shown a clear trend towards an increase in the antioxidant activity of pressurized onion from 100 to 400 MPa (Figure 5-*Paper II*). Processing onion at low (5 ºC) and medium (27.5 ºC) temperatures with the high-pressure of 400 MPa maintained the antioxidant activity of fresh onion. Moreover, we showed that total phenol content played an important role exerting influence on onion antioxidant activity at 5 ºC.

Disadvantages of thermal processing technologies have been described previously.In general, the use of high temperatures is commonly known to cause detrimental changes by affecting nutritional quality of processed products. Several studies reported that thermal treatments triggered to an onion flavonol content loss (Price *et al.*, 1997; Makris & Rossiter, 2001; Rohn *et al.*, 2007). Moreover, loss of onion organoleptic attributes with high temperatures have also been described, outer layer tissue of pre-peeled onions exposed to heat treatment at 80 ºC had been shown to have irreversible membrane damage (Hyun-Hee *et al*., 2003). Kim & Kim (2006) reported that the firmness of onion

decreased gradually with increasing heating time at a relatively high temperature of 90 ºC, and 100 ºC. Similarly, previous studies reported the antioxidant activity loss when heating onion compared to fresh onion (Fu, 2004; Kawamoto *et al.*, 2004).

In our study, we found higher levels of bioactive compounds in processed onion compared to fresh onion although antioxidant activity generally did not differ in fresh or processed onion. Our results are also partly in agreement with those by McInerney *et al.* (2007) who reported that antioxidant activity and levels of other bioactive compounds such as carotenoids of some other vegetables (carrots, green beans, and broccoli) before and after exposure to high pressures (up to 600 MPa for 2 min) were essentially no different.

We demonstrated that processing fresh onion with high-pressures (400 MPa) and low temperatures (5 ºC) could positively enhance onion bioactive compounds extractabilities leading to HP-processed onion products that have higher bioactive compound content respect to fresh onion. In the same way, this onion processing at 400 MPa/5 ºC maintained the antioxidant activity of fresh onion. Therefore, HP-processed onion products offer an interesting nutritional added value that maintains fresh onion antioxidant capacity.

Processing fresh onion with the nonthermal technology high hydrostatic pressure (HHP) combined with low temperatures might have additional advantages than processing fresh onion with conventional thermal technologies that apply high temperatures. Therefore, the high-pressure processing (HPP) technology could represent an alternative to some of these traditionally used thermal technologies.

Taking into account these *in vitro* onion studies carried out, we postulated that the pasteurized onion pastes (*Allium cepa* L. var. *cepa*, 'Recas'), which was demonstrated to have excellent antibrowning properties as a technological added value, and 400 MPa/5 ºC-processed onion products (*Allium cepa* L. var *cepa*, 'Grano de Oro'), with an enhanced bioactive compound content (quercetin-4'-glucoside (Q_{MG}); total quercetin (TQ), and quercetin-3,4'-diglucoside (Q_{DG})) as a nutritional added value, could be develop as novel and innovative foods. These onion products could be included into one of the classified categories described by the EC Regulation (Nº 258/97). One of the categories included is food and food ingredients to which a production process not currently used has been applied where that process gives rise to significant changes in the composition or structure of the foods or food ingredients which affect their

nutritional value, metabolism or level of undesirable substances (e.g. enzymatic conversion methods) (Europa-Food Safety-Biotechnology-Novel Foods. Novel Foods-Review of Regulation (EC) 258/97).

2. Onion bioactivity and metabolism

Apart from the onion freshness and nutritional quality aspects, additional values such as health claims become of great interest for consumers. In this sense, we postulate that some of the onion products studied could be added to different food dishes as substitutes of other synthetic food ingredients giving an attractive natural value to those dishes and subsequently satisfy consumer's demands. In addition, it was also of our interest to study different *in vivo* biological responses evolved by these onion products and carefully establish whether if their use as food ingredients would exert potential health benefits, and furthermore, if their development would represent a worthwhile new challenge for the food industry nowadays.

Therefore, the second part of this PhD Thesis focused on studying the *in vivo* bioactivity and metabolism pathways of onion products both in healthy rats and in overweight humans. Firstly, a rat study was conducted with one of the onion by-products (*Allium cepa* L. var. *cepa*, 'Recas'). Secondly, a human study was conducted with an onion product elaborated from fresh onion from the same 'Recas' onion cultivar.

Principal aspects and results on the rat study fed onion by-products are discussed in the current Chapter 9 while some important remarks on the human intervention study are explained in Chapter 10 (future prospects) due to the fact that some of the analyses are currently taking place and some papers are currently in preparation.

The bioactivity of onion by-products was the first key topic into this section; it was of our interest studying their *in vivo* antioxidant properties and safety as well as their effects on different aspects related with gut health environment (*Paper III*).

The second key topic was to study onion in order to provide a general incipient idea of how onion is metabolized. Therefore, it was of our interest searching for novel dietary biomarkers for onion intake (*Paper IV*) and describing onion involvement in platelet and lipid metabolism pathways (*Paper V*).

These *in vivo* studies (*Paper III, IV, and V*) discussed below would represent a firstly approach for understanding onion by-products (*Allium cepa* L. var. *cepa,* 'Recas')

biological responses in healthy rats. Taking into account the onion *in vitro* study carried out and previously discussed (*Paper I*) and these *in vivo* studies, it would be possible to consider these onion by-products appropriate for their potential commercial development as novel and innovative food ingredients.

As the main flavonoids present in onions (quercetin and its glucosides) have been target of many investigations, our research aims were mainly focused on onion fructooligosaccharides (FOS) and organosulfur compounds (OSCs), other interesting bioactive compounds with proven health benefits that have been target of a lesser amount of studies.

2.1. Onion by-products: *in vivo* safety, antioxidant and prebiotic properties

The development of onion food ingredients from onion wastes would hold a sustainable management of these onions adding an extra profitable value to these onion products. To our knowledge, no rat studies have evaluated the biological responses of onion by-products elaborated from onion wastes processing and preservation. Therefore, it was of our interest to evaluate the *in vivo* properties of onion by-products.

In the current *in vivo* rat study three principal steps were carried out before starting all the analyses performance. Firstly, a freeze-drying process was held in order to elaborate an onion powder from the pasteurized onion paste previously obtained from onion wastes (*Allium cepa* L. var. *cepa,* 'Recas') (*Paper I*). Interestingly, it has been described onion products in form of onion powders to be used chiefly as a constituent in various food products, for example; they are sold to manufacturing concerns as an industrial raw material, hotels, restaurants, caterers, and domestic consumption. Thus, we considered that freeze-drying the onion by-product chosen to obtain an onion powder was a good manner to preserve, storage, and possibly market this onion product.

Secondly, an extraction of the soluble dietary soluble fibre of this onion powder was carried out (Jaime *et al.*, 2001) and subsequently three onion by-products were tested *in vivo*, the onion by-product, an onion extract (rich in fructans and FOS), and an onion residue). Thirdly, the rat study was conducted with thirty-two rats allocated into four groups of eight rats and fed during four weeks with a control diet added a 10% of onion by-product, a diet added a 7% of onion extract, and a diet added 3% onion residue (*Paper III*).

One of our first aims was to prove the *in vivo* safety of these onion by-products. Results from the comet assay performed (Tice *et al.*, 2000) in rat liver and white blood

cells (WBC) assessed that onion by-products were not genotoxic (data not shown). Therefore, they did not involve any risk that could compromise human health and consequently these onion products became appropriate for their use as food ingredients when added to foods. However, additional studies would have to be done to study their potential anti-genotoxicity as a health protective effect. Into this research area, several studies have reported the chemopreventive effects of certain OSCs (Arranz *et al.*, 2006).

Interestingly, our results showed a reduction on the haemoglobin concentration in rats fed onion (Figure 1-*Paper III*). This effect is in agreement with other studies that have described a reduction in erythrocytes counts and haemoglobin levels involving oxidative damage to erythrocytes and consequent haemolytic anemia and Heinz body formation (Ostrowska *et al.*, 2004). Onion OSCs have been proposed to be responsible of this toxic effect in rats due to its ability to generate reactive oxidative species in presence of glutathione (GSH) (Yamamoto *et al.*, 1999; Munday *et al.*, 2003). Therefore, we decided to additionally measure the gene encoding the enzyme responsible of the heme synthesis, the 5-sminolevulinate synthase 1 (*Alas1*), and try to elucidate whether if this effect was regulated genetically or not. Looking at our results, it was shown that this gene was not significantly down or upregulated by onion (Table 4-*Paper III*). Therefore, it could be inferred that the haeme synthesis might be affected at the translational level or at another step.

As a second aim, we attempted to confirm the onion *in vivo* antioxidant properties described recently in the literature (Park *et al.*, 2007; Son *et al.*, 2008). For that purpose, the antioxidant enzymes catalase (CAT), glutathione peroxidase (GPx1), and glutathione reductase (GR) were measured in erythrocytes and liver (Table 3-*Paper III*). The hepatic expression of genes encoding the γ-glutamate-cysteine ligase catalytic subunit (*Gclc*) and NAD(P)H:quinone oxidoreductase (*Nqo1*) were also measured (Table 4-*Paper III*). The phase II enzymes convert carcinogens to inactive metabolites readily excreted from the body. Nuclear factor E2-related factor-2 (Nrf2) is an indispensable positive regulator of many antioxidant and phase II detoxifying enzymes. On activation by oxidative or electrophilic stress, Nrf2 protein stabilizes, translocates to the nucleus, heterodimerizes with small Maf proteins, and binds to the so-called antioxidant response element, a common regulatory element found in the 5'-flanking regions of antioxidant and detoxification enzymes. There is a large number of genes regulated by antioxidant response element, including enzymes involved in GSH metabolism, such as the subunits of the rate-limiting enzyme of glutathione synthesis,

glutamate-cysteine ligase catalytic (GCLC) and modifier (GCLM) subunit genes. NAD(P)H:quinone oxidoreductase-1 (NQO1) is also a Nrf2 target gene which detoxifies xenobiotic quinones (Levolen *et al*., 2007). Therefore, the induction of the phase II antioxidant enzymes and some of these described Nrf2 target genes by chemopreventive agents present in vegetables would be an effective strategy to protect cells against multistage carcinogenesis in experimental animals as well as in clinical trials.

Our results showed that generally the onion by-products tested exerted antioxidant properties as shown by some of the enhanced antioxidant enzymes activities (GR and GPx1 in erythrocytes of rats fed the onion extract) and the upregulation of the *Gclc* gene (in liver of rats fed the onion residue) (Table 3, 4-*Paper III*). However, some of our results are still uncertain as manifested by the decreased activity shown in hepatic GPx1 activity and the absence of hepatic regulation on *Gclc* gene when rats were fed the onion by-product and the onion extract. In this sense, it also has to be taken into account that the antioxidant enzyme studied could trigger different activities depending on the specific tissue analyzed.

Antioxidant effects shown in onions can be ascribed to their onion OSCs and flavonol (quercetin and its glysosides) content as other studies previously reported (Higuchi *et al.*, 2003; Nishimura *et al.*, 2006; Son *et al.*, 2008). Particularly onion OSCs have been proposed to be antioxidant and chemopreventive agents due to their ability to increase phase II enzyme activities. Furthermore, alk(en)yl substituents and the number of sulfur atoms in OSCs compounds are important moeties to take into account when reporting onion chemopreventive effects. It was shown that the onion diprop-1-enyl substituent and the compounds with two or three sulfur atoms are highly effective in exerting these effects (Guyonnet *et al.*, 2001; Teysser *et al.*, 2001; Munday *et al.*, 2004; Munday *et al.*, 2005).

As a third aim, we were interested in study the role of onion on rat gut health environment. For that purpose, several analysis were performed to determinate pH, ß-glucosidase (BGL), and ß-glucuronidase (GUS) enzymes activities, and short chain fatty acids (SCFA) concentrations in caecal content as well as the transit time measurement.

Our results showed that onion by-products tested exerted prebiotic properties. A significant lowering effect of the caecal pH in rats fed the onion by-products without effect on transit time was found (Table 5-*Paper III*). BGL and GUS activities were enhanced in rats fed onion by-products (Figure 2-*Paper III*) and increased levels of the

SCFA propionate and butyrate were found in the caecal content of rats fed onion by-products (Figure 3-*Paper III*). In terms of physiological responses valuables for a proper colon cancer prevention, decreased pH is often seen as a consequence of caecal fermentation and it has been reported to be a predictor of lower risk of colon cancer in rats fed sugars, oligofructose and inuline (Hansen *et al.*, 2008). Moreover SCFA and particularly butyrate acid has been reported to be a colon cancer protective agent (Pool-Zobel & Sauer, 2007). It seems that not only the onion FOS but also the onion insoluble dietary fibre are fermented in the caecum of rats fed onion by-products and that this fermentation affected the functionality and possibly the composition of gut microbiota leading to a healthier phenotype. Thus, it could be inferred that onion dietary fibre might be involved in the *in vivo* prebiotic effects of onion by-products.

2.2. A nutri-metabonomic study in urine from rats fed onion by-products using ^{1}H NMR and chemometrics

Significant progress have been made in developing technologies to measure the responses of living systems to xenobiotics either at the genetic level or at the level of the expression of cellular proteins, using genomic and proteomic methods, respectively. Even in combination, genomic and proteomic methods still not provide the range of information needed for understanding integrated cellular function in living system, since both ignore the dynamic metabolic status of the whole organism (Lindon *et al.*, 2000). Thus, a metabonomic approach that aims to augment and complement the information provided by genetic and proteomic responses to xenobiotic exposure is necessary.

To date and to our knowledge no *in vivo* metabonomic studies have been done focusing on onion by-products. Therefore, it was of our interest performing a nutri-metabonomic study in order to investigate the effects of onion intake on rat metabolism. For that purpose, we aimed to evaluate the *in vivo* metabolome of healthy rats following the intake of three onion diets (onion by-product, onion extract, and onion residue). Briefly, the urine from the thirty two rats of the study described (*Paper III*) was collected in a period of 24 h before rats were sacrificed and ^{1}H NMR spectra were recorded for these urine samples, afterwards, chemometric analysis were performed (*Paper IV*).

Metabonomics is defined as ‘the quantitative measurement of the multi-parametric metabolic responses of living systems to pathophysiological stimuli or genetic modification’ (Nicholson *et al.*, 1999, 2002). One major strength of metabonomics is

the possibility that metabolic biomarkers will be more easily used across species than transcriptomics and proteomic biomarkers. Through the combination of transcriptomics, proteomics, and metabonomics an improved understanding of an organism´s total biology will result, the ultimate goal of system's biology is the integration of data acquired from living organism at the gene, protein, and metabolite levels.

Metabonomics is applicable to a wide range of biomedical research areas including pharmaceutical areas. In terms of diseases studies, metabonomics plays a role in improved, differential diagnosis, and prognosis of human diseases, particularly chronic and degenerative diseases. Other application where major expansion is expected are in food science and nutritional studies, sports medicine, and lifestyle studies, including the effects of diet, exercise, and stress, and the evaluation of the effects of interactions among drugs and between drugs and diet (Lindon *et al.*, 2004; Lindon & Nicholson, 2008; Wishart, 2008).

A wide range of spectroscopic techniques are used in metabonomics studies, they are often used in a so-called 'hyphenated' mode (e.g. LC–NMR–MS) (Lindon & Nicholson, 2008). Particularly, NMR-based metabonomics has proven to be particularly apposite for the rapid analysis of complex biological samples. Nuclear magnetic resonance (NMR) spectroscopy provides detailed information on nuclear structure, both for pure compounds and in complex mixtures, but it also can be used to probe metabolite molecular dynamics and mobility through the interpretation of NMR spin relaxation times and by the determination of molecular diffusion coefficients (Liu *et al.*, 1996; Lindon & Nicholson, 2008). The ^{1}H NMR spectra results generate a unique metabolic fingerprint for each complex biological mixture. If the status of a given organism changes, such as in a disease state or following exposure to a drug, the unique metabolic fingerprint or signature reflects this change.

The word metabonomics has been coined to describe the combined application of spectroscopy and multivariate statistical approaches to studies of the multicomponent composition of biofluids, cells, and tissues. It is distinct from the related concept of metabolomics, which is much broader in concept and much less well defined (Coen & Kuchel, 2004). Chemometrics is the science of relating measurements made on a chemical system or process to the state of the system via application of mathematical or statistical methods. It has become an essential part in the modern chemical and biomedical industries and particularly it is a relevant and important tool for the analysis

of ^{1}H NMR spectra data generated in metabonomic studies (Alam & Alam, 2004; Winning *et al.*, 2008).

In our metabonomic study, ^{1}H NMR spectra from the three onion by-products used to feed rats (onion by-product, onion extract, and onion residue) were obtained (Figure 1-*Paper IV*). With these preliminary results it was difficult to assure that the onion by-product spectrum equals the extract plus the residue spectrum; however it was shown many similarities in the spectra of these three onion products, differing the onion extract from the others by more intense signals in the aromatic region and an ethanol signal due to the extraction methodology carried out.

Besides, ^{1}H NMR spectra data were obtained from the urine samples of the thirty-two rats fed onion. Several remarks have to be taken into account when using rat urine as a biological fluid for metabonomic studies. Particularly, it is important to be careful in urine collection, storage, and sample preparation for NMR spectroscopy. It is not recommendable to freeze-dry this biofluid. Reconstituting into D_2O phosphate buffer solution (H_2O, D_2O, TSP-d4 (perdeuterated 3-trimethylsilyl propionate sodium salt), NaN_3, pH 7.4) a freeze-dried urine sample or adding a substantial amount of D_2O to it to provide a NMR field lock, could cause that certain ^{1}H NMR resonances will be lost by H D exchange. Moreover, freeze-dried urine samples might also causes the loss of volatile components. Urine is a biofluid very prone to the microbiological contamination. Thus, when experiments involve collection from laboratory animals housed into metabolic cages, urine samples should be collected into receptacles that are either cooled with dry ice or have a small amount of the bacteriocide sodium azide. Immediately after, urine should be stored deep frozen. However, other considerations when collecting and storing urine have to be taken into account for the assessment of kidney tubular integrity in toxicological experiments. Normalizing the urine pH to a range of about 6.7-7.6 it is also important in order to obtain stable urine without pH-dependent chemical shifts. Adding 100-200 mM phosphate buffer in the D_2O for the lock signal followed by centrifugation removes precipitated salts and maintains urine into this range of pH for many hours during which NMR measurement can be made (Lindon *et al.*, 2000). In our study, we took into account all these considerations for the rat urine samples collection, storage, and preparation for NMR spectroscopy.

The composition of urine is complex and highly variable both between species and within species according to life style and age. A wide range of organic acids and bases, simple sugars, and polysaccharides, heterocycles, polyols, low molecular weight

proteins, and polypeptides are present together with inorganic species such as Na^+, K^+, Ca^{2+}, Mg^{2+}, HCO^{3-}, SO_4^{2-}, and phosphates. Rats and other rodents have much higher levels of taurine, citrate, succinate, 2-oxo-glutarate, and allantoin than humans and this is clearly apparent in the ^{1}H NMR spectra. Rat and other rodent urine are generally more concentrated than human urine. Moreover species, strains, genetics, age, hormone concentrations, diurnal cycles, diet, temperature, stress, and gut microflora all contribute to the metabolic profile of rat urine (Slupsky *et al.*, 2007).

Prior to the chemometric analysis, the urinary metabolic data were normalized; this data transformation minimizes inter-sample variation due to differences in gross urinary concentration between samples caused by volume and dry matter differences. The average of ^{1}H NMR rat urine spectra of each feed group is shown in Figure 2-*Paper IV*. These spectra appeared very similar despite the different feeding schemes followed.

Chemometric analysis was carried out in order to obtain optimal quantitative and qualitative information from the spectra. Pareto-scaling method, a scaling or pre-transformation NMR data method, was also used in order to assure that all signals are influencing the model. Multivariate data analysis in the form of principal component analysis (PCA) model was performed. PCA was not able to distinguish the four feeding groups or to group the samples into an onion and a control group (Figure 3-*Paper IV*). Therefore, extended canonical variates analysis (ECVA) model was applied for classification of feed groups. In order to improve the calibration models and to investigate the influential areas of the spectra, interval ECVA (iECVA) (Figure 4-*Paper IV*) was performed and two spectral intervals were selected at signal chemical shift of 6.50-6.95 ppm and 2.98-3.42 ppm (Figure 5-*Paper IV*). The high correlation found between the signal at 3.25 and the 3-hydroxiphenilacetic at 6.80 indicates that this compound is involved in onion metabolism.

Furthermore, in order to investigate quantitative information regarding onion dose, interval partial least squares (iPLS) regression was also employed (Noogaard *et al.*, 2000) (Figure 6-*Paper IV*). This model related ^{1}H NMR spectra and the onion dose (0, 3, 7, 10). The two optimal intervals selected, exactly the same at those found when iECVA were performed (6.50-6.95 ppm and 2.98-3.42 ppm), reveal that NMR urine spectra contain robust quantitave information about onion dose. Moreover, although the same quantitative information was extracted from iECVA and iPLS further orthogonalization analysis would be required in order to describe the dose response from these data.

Taking into account that the ^{1}H NMR spectra of urine show thousands of sharp peaks from predominantly small-molecules metabolites and that two dimensional (2D) NMR spectroscopy is demonstrated to be useful for increasing signal dispersion and for elucidating the connectivities between signals and helps to identify metabolites (Pedersen *et al.*, 2006; Noda, 2007; Lindon & Nicholson, 2008), two of these 2D NMR experiments, total correlation spectroscopy (TOCSY) and heteronuclear single quantum coherence (HSQC), spectra were acquired on urine from a rat fed onion by-product. A signal seemed particular important at the chemical shift of 3.15 ppm. Based on the data from these 2D experiments performed on this rat urine sample and in the NMR measurement of the pure compound, this signal was identified as dimethyl sulfone.

2.2.1. Dietary biomarkers for onion intake

In metabolomic studies with human volunteers, it has been noted that volunteers frequently do not report all their medication or food supplements. Therefore, it would be highly desirable to gain knowledge about what test-persons really eat under a diet intervention. *In vivo* investigations using animals as models make it possible to study biomarkers after different specific food intakes.

In the current study, two onion biomarkers, 3-hydroxyphenylacetic and dimethyl sulfone, were identified. Dimethyl sulfone is present in the urine of rats fed the three onion products in the same concentration and doses and thus it is a dietary onion biomarker. However, it was only possible to identify a biomarker for onion intake but not for onion by-products.

Dimethyl sulfone (Figure 1) is an oxidation product of the dimethyl sulfoxide (DMSO). DMSO was reported to be metabolized in humans and rats to dimethyl sulfone (Hucker *et al*., 1966). This metabolite has been found in human sweet (Cork & Park, 1996) and urine (Waring *et al.*, 1987) after asparagus consumption and it seemed to be originated from sulfur rich herbs such as onion. Ichikawa *et al.* (2006) described the pharmacokinetics of another organosulfur compound, cycloalliin, found in onion and garlic. These authors reporting that this compound was recovered in the high percentage of 97.8% into the 48 h rat urine. Earlier, Mullen *et al*. (2004) identified flavonoid metabolites in urine of healthy volunteers after the consumption of red onions.

To our knowledge, few studies have been done using biofluid samples of humans such as blood or urine and focusing on the search of sulfur metabolites derived from

onion OSCs. Several studies found into this research area are related to the current biomarker identified. Engelke *et al.* (2005) that found dimethyl sulfone in human cerebrospinal and blood fluids and it was suggested that it derives from dietary sources, intestinal bacterial metabolism, and the body's methanethiol metabolism. Recently, this metabolite has also been linked with the occurrence of skin cancer (Gallagher *et al.*, 2008).

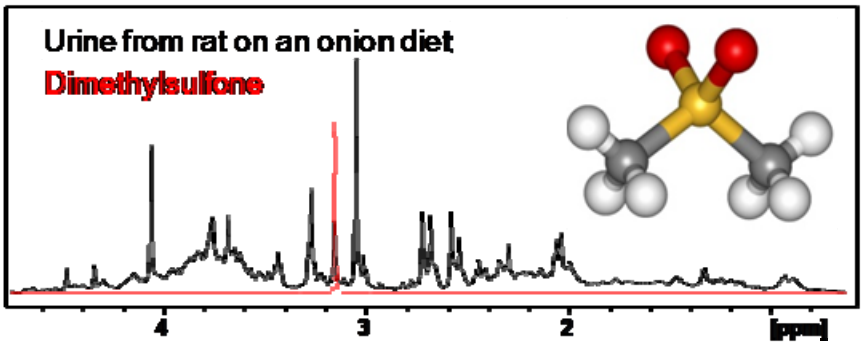


Figure 1. An exploratory NMR nutri-metabonomic investigation reveals dimethyl sulfone as a dietary biomarker for onion intake. The present research paper presents the finest original results from this work that combines the use of high resolution NMR spectroscopy with advanced multivariate data techniques to discover two exclusive biomarkers in urine followed by onion feed

Metabonomics studies have been shown to have a wide range of applications in nutrition studies. The potentialities of nutritional metabonomics for the discovery of new biomarkers and the characterization of metabolic phenotypes and their possible utilizations for personalized nutrition to provide health maintenance at the individual level is an emerging nutritional field that is becoming important in practice nowadays (Gibney *et al.*, 2005; German *et al.*, 2005; Rezzi *et al.*, 2007; Kim *et al.*, 2008).

Overall, using dimethyl sulfone as a biomarker for onion intake could be potentially a good tool in dietary intervention studies in which it could be possible to quantify the dietary intake of this vegetable. The use and quantification of the current biomarker and other biomarkers from other foods in dietary intervention studies could be an alternative dietary assessment method that would solve the problem of the not acute and sometimes misleading self reports of food intake in the food frequency questionnaires traditionally used in many nutritional large-scale studies.

2.3. Onion and cardiovascular disease (CVD)

The rise in CVD reflects a significant change in diet habits, physical activity levels, and tobacco consumption worldwide. Between 2006 and 2015, deaths due to non

communicable diseases (half of which will be due to CVD) are expected to increase by 17%. Cardiovascular effects, improvement of the immune function, lowering of blood glucose level, radioprotection, protection against microbial infections, and anticarcinogenic effects are some of the known health benefits of *Allium* vegetables (Powolny & Singh, 2008). Several OSCs have been reported to exert cardioprotective and anticarcinogenic effects (Benavides *et al*., 2007; Xiao *et al.*, 2009).

To date, most of the *in vivo* investigations studying *Allium* vegetables and CVD or cancer prevention have focused either on garlic (*Allium sativum* L.) or on its OSCs. However, to our knowledge few *in vivo* studies have been done focusing on onion (*Allium cepa* L.) and CVD prevention. Onion had been previously studied in animal models for its lipid lowering, inmunomodulatory, and natural antithrombotic effects (Bordia *et al*., 1996; Moon *et al*., 2000; Gabler *et al*., 2006).

To our knowledge there is no study focusing on onion in form of onion by-product and their potential CVD protective effects. Therefore, our study aimed to elucidate the potential role of onion (*Allium cepa* L. var. *cepa*, 'Recas') by-products either in delaying or in preventing CVD onset. Based on the rat study performed in our first study (*Paper III*), we investigated the *in vivo* effects of onion by-products on lipid metabolism and platelet aggregation pathways in healthy rats (*Paper V*). We aimed to elucidate if onion by-products have potential CVD preventive properties in terms of hypolipidemic and antiaggregatory effects.

2.3.1. Onion and lipid metabolism

Firstly, in our study several rat hepatic enzymes activities were measured in order to evaluate if these onion by-products caused any liver or renal damage that could compromise liver integrity (Table 2-*Paper V*). It could be inferred that apparently onion by-products did not cause any liver or renal damage in healthy rats, however, it could not be ruled out that onion by-products have hepatoprotector effects as long as other studies use onion by-products to feed hepatic and renal damaged animals.

In order to study different lipid metabolism aspects, triacylglycerides (TAG), total cholesterol (TC), cholesterol in high density lipoprotein (HDL-C) cholesterol in low density lipoprotein (LDL-C), cholesterol in very low density lipoprotein (VLDL-C) were measured in plasma of healthy rats fed onion by-products (Figure 1-*Paper V*). Cholesterol distribution in the different lipoproteins fractions were measured by the NMR and interval PLS methodology described by Kristensen *et al*., 2009. The

expression of the target gene involved in the biosynthesis of cholesterol in liver, the 3-hidroxy-3-methylglutaryl-CoA reductase (*Hmgcr*), was also measured (Table 3-*Paper V*). Moreover, fourteen bile acids were quantified in faeces samples of these rats fed onion by-products (Table 4-*Paper V*).

Our results show that feeding rats the onion by-product and the onion extract slightly lowered plasma TAG and TC concentrations; by contrast, onion residue significantly enhanced plasma TAG and VLDL-C concentrations. The onion by-products hypocholesterolemic effect could be a consequence of either the inhibition of hepatic cholesterol biosynthesis by downregulating the *Hmgcr* gene expression or by an enhanced turnover to bile acids which would cause an increase in faecal bile acids concentrations excreted through gastrointestinal tract.

HMG-CoA reductase enzyme is generally regarded as catalyzing the rate-limiting step in the biosynthesis of cholesterol. Quantitatively, the major products derived from cholesterol are bile acids. *Hmgcr* gene expression in animal liver is regulated by a wide variety of physiological agents including bile acids and cholesterol. It appears that bile acids act transcriptionally to regulate hepatic *Hmgcr* gene expression (Ness & Chambers, 2000). *Hmgcr* gene was not significantly affected by any of the onion products tested (Table 3-*Paper V*). Focusing on plasma lipids measured, our results showed a significantly increased TAG and VLDL-C concentrations in plasma of rats fed the onion residue (Figure 1-*Paper V*). By contrast, the onion by-product and onion extract did not show the same behaviour. Onion fractions have not been tested previously, consequently our observation after feeding this onion residue indicates that it stimulates upregulation of hepatic lipid transport to peripheral tissues. This is often caused postprandially by fructose-rich or fatty foods. Moreover, it was shown that neither total bile acids nor total primary or secondary bile acids concentration in faeces, as well as their total 24 h excretion, were significantly increased by feeding the onion by-product or its fractions (Table 4-*Paper V*).

Onion FOS and water soluble OSCs found in these extracts might be responsible of some of these shown effects. The hypocholesterolemic effect of dietary fibre has been attributed to its ability to inhibit intestinal absorption of bile acids and neutral steroids, resulting in greater faecal bile acids and total steroid excretion (Busserolles *et al*., 2003; Han *et al.*, 2005). Onion OSCs have been also described to have hypolipidemic effects (Kumari & Augusti, 2007). Particularly, water soluble OSCs had been reported to modulate lipid metabolism. Most of the studies conducted into this area had focused on

garlic and some of its OSCs content (Liu & Yeh, 2001; Liu & Yeh, 2002). A recent study in mice consuming high fat diet supported that hydrophilic cysteine-containing compounds naturally formed in onion are potent agents for affecting TAG and cholesterol hepatic biosynthesis and protect liver against high saturated fat associated oxidative damage (Lin &Yin, 2008).

2.3.2. Onion and platelet aggregation

Platelet aggregation is the major cause of thromboembolic events leading to CVD. Prevention of arterial thrombotic diseases has a high priority in developed countries. As inappropriate diet has been shown to be an important risk factor for thrombotic events, regular antithrombotic diet may offer a convenient and effective way of prevention. Platelet adhesion to subendothelial components, such as collagen, activates metabolic pathways of arachidonic acid that lead to thromboxane A_2 (TXA_2) formation through cyclooxygenase (COX) and TXA_2 synthase (TXAS) pathway. TXA_2 is a potent inducer of platelet aggregation and a vasoconstrictor which is increased in thrombotic disorders. Inhibition of the platelet function including TXA_2 formation represents a promising approach for the thrombosis prevention and therefore for CVD prevention.

A number of diseases with a vascular component such as systemic sclerosis increases platelet aggregation and activates WBC. Increased TXAS in these activated WBC will promote platelet aggregation, endotelial dysfuction and vascular damage (Young *et al.*, 2002). Recently, Sakai *et al*. (2006) reported that TXAS is upregulated in the tissue of human colorectal carcinoma, and that TXA_2 stimulates the cancer cell proliferation.

Extracts from onions have been shown to inhibit *in vitro*, *in vivo*, and *ex vivo* human and rat platelet aggregation (Osmont *et al.*, 2003; Yamada *et al.*, 2004). It has been reported that the inhibitory effect on arachidonic acid (AA) release and the combination of TXAS inhibition with TXA_2/PGH_2 receptor blockade without effect on COX activity might, at least partly, contribute to the antiplatelet effect of onion (Moon *et al*., 2000). Several studies have described antiplatelet effect in different animal models fed onion (Briggs *et al*., 2001, Jung *et al.*, 2002).

In our study, it was shown that tromboxane A_2 synthase (*Txas*) gene expression was significantly downregulated in white blood cells (WBC) samples of rats fed either the onion by-product or the onion extract (Table 3-*Paper V*). Therefore, it could be inferred that the onion by-products tested have antiplatelet properties. Furthermore, it seems that water soluble OSCs content of the onion by-products tested might be partly responsible

of the *Txas* downregulation in rat WBC. Likely, it is generally accepted that pungent onions have higher pyruvate content and possess higher antithrombotic activity (Cavagnaro *et al*., 2007). Pyruvic acid had been used as an indicator for OSCs content due to the fact that S-Alkenyl–L-cysteine sulfoxides are activated by the enzyme allinase to produce pyruvic acid, ammonia, and sulfenic acids when *Allium* tissue is damaged (Perner *et al.*, 2008). Moreover, not only bulb sulfur content but also onion genotype should be taken into account in studies assessing onion antiplatelet effects (Goldman *et al*., 1996).

Chapter 10

Conclusions

Future prospects

Chapter 10. Conclusions. Future prospects

1. Conclusions

Based on the *in vitro* and *in vivo* experiments carried out with onion (*Allium cepa* L.) in the present PhD Thesis it was concluded:

Conclusion I

Traditional thermal technologies used to preserve and stabilize onion by-products from two onion cultivars wastes affect their nutritional and technological quality. Pasteurization seems to be a good technology that did not compromise onion safety and nutritional quality of onion by-products compared with sterilization and freezing technologies. Pasteurized 'Recas' onion paste (*Allium cepa* L. var. *cepa*, 'Recas') is a safe onion by-product with a good nutritional and technological quality that was chosen among all the onion by-products analyzed to be the most appropriate onion product for the potential design and development of novel functional onion ingredients.

Conclusion II

Onion (*Allium cepa* L. var *cepa*, 'Grano de Oro') bioactive compound content and antioxidant activity is affected by high hydrostaic pressure processing. Processing fresh onion at high pressures and low temperatures maintains the antioxidant activity and increases the extractabilities of the flavonols found in onion (quercetin and its glucosides) compared with the unprocessed fresh onion. The introduction of a nonthermal food processing technology like high-pressure processing to process fresh onion would improve onion functional properties.

Conclusion III

A rat study in which healthy rats were four-week fed a pasteurized onion paste (*Allium cepa* L. var. *cepa*, 'Recas') and two derived onion fractions (an extract rich in fructans and fructoligosaccharides (FOS) and a residue) shows that these onion by-products are not genotoxic. They support *in vivo* antioxidant properties, decrease caecal pH, increase short chain fatty acids (SCFA) production, and alter the gut environment functionality of healthy rats. Therefore, from this rat study it was concluded that the onion by-product analyzed is a safe onion product that exerts interesting antioxidant and prebiotic properties.

Conclusion IV

A nutri-metabonomic investigation using nuclear magnetic resonance (^{1}H NMR) spectra in combination with chemometrics is an excellent tool for diverting onion by-products (*Allium cepa* L. var. *cepa*, 'Recas') fed rat groups by their urine profile. The organosulfur compound dimethyl sulfone was identified as a dietary biomarker for onion intake. Being able to detect and quantify specific onion intake biomarkers is highly beneficial in control of nutritionally enhanced functional foods and in human intervention studies where it is often a problem to verify objectively that volunteers are compliant.

Conclusion V

The onion by-product and its derived fractions (*Allium cepa* L. var. *cepa*, 'Recas') used to feed the healthy rats do not seem to reduce cholesterol significantly. This onion by-product may exert antithrombotic effects as evidenced by the rat leucocite thromboxane A_2 synthase downregulation; the main constituents that causes the inhibition of platelet aggregation are recovered into the onion extract. This onion by-product extract may find use for isolation of inhibitors of platelet aggregation and therefore may exert an additional positive health effect in terms of cardiovascular disease prevention.

2. Future prospects

2.1. Onion *in vitro* studies

Further *in vitro* studies analyzing the effect of processing onion (*Allium cepa* L.) by high hydrostatic pressure (HHP) at different pressures and temperatures are required. Moreover, these possible further studies could be compared with the two studies presented in the current PhD Thesis. Thus, we would be able to compare the effect of a nonthermal technology such as high-pressure processing (HPP) with other traditional processing technologies (thermal technologies) and nonthermal processing technologies.

In vitro studies on organoleptic attributes, texture, cell viability, pungency, OSCs content, and antibrowning properties of nonthermally-processed onion products might also be of our interest.

Therefore, with these studies we could also be able to compare different nutritional and technological properties between different onion cultivars and onion including fresh onion, onion by-products, and nonthermal-processed onion. It would be possible to confirm an enhanced onion bioactive compound content and antibrowning properties when onion is processed by nonthermal technologies and therefore to plan a possible food ingredient development.

Currently, more *in vitro* studies and analysis with 'Recas' and other cultivars onion are taking place in our Department of Plant Food Science and Technology and other analysis have been planned by our research group including microestructure studies to relate the tissue damage produced by high-pressure processing with the enhancement of the antioxidant and functional properties of onion.

2.1. Onion *in vivo* studies

Through the development of the current PhD Thesis we were aware that *in vivo* studies were required in order to potentially develop functional onion ingredients. For that reason, we decided to test *in vivo* the biological activity of an onion product obtained from 'Recas' onion wastes and characterized in one of our *in vitro* studies. A preliminary step for demonstrating onion bioactivity, safety, metabolism, and possibly protective health effects have been shown in this PhD Thesis using healthy rats as animal models and 'Recas' onion in form of a freeze-dried onion powder (a pasteurized onion paste by-product). Furthermore, we were able to describe part of the effects

shown in rat lipids and platelet aggregation when they are fed this onion by-product and even identified an interesting dietary onion biomarker.

However, further analysis are still lacking in the rat study performed. For example, it would have been interesting to determine glutathion (GSH) in order to relate the antioxidant enzymes activities shown with the effects on GSH homeostasis. In order to deeply study the lipid metabolism in rats fed onion the expression of some target genes involved in lipid metabolism would be required. Thus, it would be interesting to study the expression of hepatic genes encoding enzymes such as cholesterol 7-alfa-hydroxylase (CYP7A1) (involved in hepatic bile acid synthesis), sterol response element-binding protein 1c (SREBP-1c) (involved in fatty acid synthesis), SREBP-2 (involved in cholesterol synthesis), and LDL receptor gene expression. Furthermore, a deeply study including thromboxane-prostanoid (TP) receptor determination would be also of our interest for further research. *Allium* species as onion could have *in vivo* thromboxane A_2 synthase/thromboxane-prostanoid receptor (TXAS/TP) dual inhibitory effects acting as CVD protective agents and possibly helping in a cancer preventive diet. Other measurements of *in vivo* coagulation parameters would also be required.

It would also have been interesting to have added another rat group feed a known onion organosulfur compound and to determine other bioactive compounds such as onion saponins.

As a further step an intervention human study was conducted, for that purpose a collaboration was established between our Department of Plant Food Science and Technology at Instituto del Frío, CSIC, in Madrid, Spain and the Department of Human Nutrition in the Faculty of Life Sciences in the University of Copenhagen, in Frederisberg, Denmark. In this sense, it is worth to take into account that to date few studies *in vivo* had focus on the *Allium* vegetable onion (*Allium cepa* L.) added as a food ingredient into precooked dishes and on the possible relation between some of the onion bioactive compounds found in this ingredient and their potential health benefits in overweight humans. Details of the study design are explained in Chapter 2 of the current PhD Thesis. Currently, this paper is under preparation and its aim is the study of the role of an onion ingredient on antioxidant, antiaggregatory, antiinflammatory, and obesity parameters and some others parameters related with CVD prevention.

Furthermore, a MSc Thesis was carried out by Birgitte Borg on the study of onion and its effect on haemostasis and red blood cells (RBC) and further metabonomic studies using samples of plasma and urine from these volunteers have been planned.

Capítulo 10

Conclusiones

1. Conclusiones

Basado en los experimentos *in vitro* e *in vivo* llevados a cabo con cebolla (*Allium cepa* L.) en la presente Tesis Doctoral concluimos:

Conclusión I

Las tecnologías térmicas tradicionales para la conservación y estabilización de subproductos de cebolla de dos cultivares de cebolla afectan a la calidad nutricional y tecnológica de estos subproductos. La pasteurización mostró ser una buena tecnología que no comprometió la seguridad y la calidad nutricional de subproductos de cebolla en comparación con la esterilización y la congelación. La pasta de cebolla 'Recas' pasteurizada (*Allium cepa* L. var. *cepa,* 'Recas') es un subproducto de cebolla seguro con una buena calidad nutricional y tecnológica que fue elegido de entre todos los subproductos de cebolla analizados como el producto de cebolla más apropiado para el potencial diseño y desarrollo de ingredientes funcionales de cebolla.

Conclusión II

El contenido en compuestos bioactivos y actividad antioxidante de la cebolla (*Allium cepa* L. var *cepa*, 'Grano de Oro') es afectado por el procesado con alta presión hidrostática. El procesado de cebolla 'Grano de Oro' fresca a altas presiones y bajas temperaturas mantiene la actividad antioxidante e incrementa la extractibilidad de los flavonoles presentes en la cebolla (quercetina y sus glucósidos) respecto a la cebolla no procesada. La introducción de una tecnología no térmica de procesado de alimentos como la alta presion hidrostática para el procesado de cebolla fresca incrementaría las propiedades funcionales de la cebolla.

Conclusión III

El estudio en ratas sanas llevado a cabo durante cuatro semanas en el que las ratas fueron alimentadas con la pasta pasteurizada de cebolla (*Allium cepa* L. var. *cepa*, 'Recas') y dos fracciones de cebolla derivadas (un extracto rico en fructanos y fructooligosacáridos y un residuo) muestra que estos subproductos de cebolla no son genotóxicos. Además, estos subproductos tienen propiedades antioxidantes *in vivo*, disminuyen el pH intestinal, aumentan la producción de ácidos grasos de cadena corta y alteran la funcionalidad del ambiente intestinal en ratas sanas. Por tanto, de este estudio

en ratas se concluye que el subproducto de cebolla analizado es un producto de cebolla seguro que muestra interesantes propiedades antioxidantes y prebióticas.

Conclusión IV

Una investigación nutri-metabonómica usando espectrofotometría de resonancia magnética nuclear (^{1}H RMN) en combinación con quimiometría es una excelente herramienta para discernir entre grupos de ratas alimentadas con subproductos de cebolla (*Allium cepa* L. var. *cepa*, 'Recas') mediante su perfil de orina. El compuesto organosulfurado dimetil sulfona se identificó como biomarcador dietético para la ingesta de cebolla. Ser capaces de detectar y cuantificar biomarcadores específicos de la ingesta de cebolla es altamente beneficioso en el control de alimentos funcionales nutricionalmente suplementados y en estudios de intervención en humanos en los cuales verificar objetivamente si los voluntarios cumplen los objetivos del estudio es frecuentemente un problema.

Conclusión V

El subproducto de cebolla y sus fracciones derivadas (*Allium cepa* L. var. *cepa*, 'Recas') utilizados para alimentar las ratas sanas de nuestro estudio no reducen el colesterol significativamente. Este subproducto podría tener efectos antitrombóticos como evidencia la disminución de la expresión de la tromboxano A_2 sintasa en leucocitos; los principales constituyentes que causan la inhibición de la agregación plaquetaria se encuentran en el extracto del subproducto de cebolla. Este extrato podría tener uso para el aislamiento de inhibidores de agregación plaquetaria y por tanto podría tener un efecto positivo para la salud en términos de prevención de enfermedad cardiovascular.

References

Aherne SA, O'Brien NM (2002) Dietary flavonols: chemistry, food content, and metabolism. Nutrition, 18:75-81.

Alam TM, Alam MK (2004). Chemometric analysis of NMR spectroscopy data: A review. Annual Reports on NMR Spectroscopy, 54:41-80.

Ali M, Bordia T, Mustafa T (1999) Effect of raw *versus* boiled aqueous extract of garlic and onion on platelet aggregation. Prostaglandins Leukotrienes and Essential Fatty Acids, 60:43-47.

Ali M, Thomson M, Afzal M (2000) Garlic and onions: their effect on eicosanoid metabolism and its clinical relevance. Prostaglandins Leukotrienes and Essential Fatty Acids, 62:55-73.

Arora R, Palta JP (1986) Protoplasmic swelling as a symptom of freezing-injury in onion bulb cells - its simulation in extracellular KCl and prevention by calcium. Plant Physiology, 82:625-629.

Arora R, Palta JP (1991) A loss in the plasma-membrane ATPase activity and its recovery coincides with incipient freeze-thaw injury and post thaw recovery in onion bulb scale tissue. Plant Physiology, 95:846-852.

Arranz N, Haza AI, García A, Möller L, Rafter J, Morales P (2007) Protective effects of organosulfur compounds towards N-nitrosamine-induced DNA damage in the single-cell gel electrophoresis (SCGE)/HepG2 assay. Food and Chemical Toxicology, 45:1662-1669.

Azuma K, Minami Y, Ippoushi K, Terao J (2007) Lowering effects of onion intake on oxidative stress biomarkers in streptozotocin-induced diabetic rats. Journal of Clinical Biochemistry and Nutrition, 40:131-140.

Babu PS, Srinivasan K (1999) Renal lesions in streptozotocin-induced diabetic rats maintained on onion and capsaicin containing diets. Journal of Nutritional Biochemistry, 10:477-483.

Benavides GA, Squadrito GL, Mills RW, Patel HD, Isbell TS, Patel RP, Darley-Usmar VM, Doeller JE, Kraus DW (2007) Hydrogen sulfide mediates the vasoactivity of garlic. Proceedings of the National Academy of Sciences of the United States of America, 104:17977-82.

Benkeblia N, Onodera S, Shiomi N (2005) Variation in 1-fructo-exohydrolase (1-FEH) and 1-kestose-hydrolysing (1-KH) activities and fructo-oligosaccharide (FOS) status in onion bulbs. Influence of temperature and storage time. Journal of the Science of Food and Agriculture, 85:227-234.

Benkeblia N, Varoquaux P (2003). Effect of nitrous oxide (NO) on respiration rate, soluble sugars and quality attributes of onion bulbs *Allium cepa* cv. *Rouge Amposta* during storage. Postharvest Biology and Technology, 30:161-168.

Benkeblia N (2005) Free-radical scavenging capacity and antioxidant properties of some selected onions *(Allium cepa* L.) and garlic (*Allium sativum* L.) extracts. Brazilian Archives of Biology and Technology, 48:753-759.

Bianchini F, Vainio H (2001) *Allium* vegetables and organosulfur compounds: Do they help prevent cancer? Environmental Health Perspectives, 109:893-902.

Billaud C, Brun-Mérimée S, Louarme L, Nicolas J (2004) Effect of glutathione and Maillard reaction products prepared from glucose or fructose with glutathione on polyphenol oxidase from apple - I: Enzymatic browning and enzyme activity inhibition. Food Chemistry, 84:223-233.

Block E, Gulati H, Putman D, Sha DY, You NN, Zhao SH (1997) *Allium* chemistry: Synthesis of 1-[alk(en)ylsulfinyl]propyl alk(en)yl disulfides (cepaenes), antithrombotic flavorants from homogenates of onion (*Allium cepa*). Journal of Agricultural and Food Chemistry, 45:4414-4422.

Block E, Naganathan S, Putman D, Zhao SH (1993) Organosulfur chemistry of garlic and onion: Recent results. Pure and Applied Chemistry, 65:625-632

Boeing H, Jedrychowski W, Wahrendorf J, Popiela T, Tobiaszadamczyk B, Kulig A (1991) Dietary risk-factors in intestinal and diffuse types of stomach-cancer - a multicenter case-control study in Poland. Cancer Causes Control, 2:227-233.

Bonaccorsi P, Caristi C, Gargiulli C, Leuzzi U (2008) Flavonol glucosides in *Allium* species: A comparative study by means of HPLC-DAD-ESI-MS-MS. Food Chemistry, 107:1668-167.

Boots AW, Haenen GRMM, Bast A (2008) Health effects of quercetin: From antioxidant to nutraceutical. European Journal of Pharmacology, 585:325-337.

Bordia T, Mohammed N, Thomson M, Ali M. (1996) An evaluation of garlic and onion as antithrombotic agents. Prostaglandins, Leukotrienes and Essential Fatty Acids, 54:183-186.

Boyle SP, Dobson VL, Duthie SJ, Kyle JAM, Collins AR (2000) Absorption and DNA protective effects of flavonoid glycosides from an onion meal. European. Journal of Nutrition, 39:213-223.

Briggs WH, Folts JD, Osman HE, Goldman IL (2001) Administration of raw onion inhibits platelet-mediated thrombosis in dogs. Journal of Nutrition, 131:2619-2622.

Busserolles J, Gueux E, Rock E, Demigne C, Mazur A, Rayssiguier Y (2003) Oligofructose protects against the hypertriglyceridemic and pro-oxidative effects of a high fructose diet in rats. Journal of Nutrition, 133:1903-1908.

Caridi D, Trenerry VC, Rochfort S, Duong S, Laugher D, Jones R (2007) Profiling and quantifying quercetin glucosides in onion (*Allium cepa* L.) varieties using capillary zone electrophoresis and high performance liquid chromatography. Food Chemistry, 105:691-699.

Cavagnaro PF, Sance MM, Galmarini CR (2007) Effect of heating on onion (*Allium cepa* L.) antiplatelet activity and pungency sensory perception. Food Science and Technology International, 13:447-453.

Choi SI, Hong EY, Lee JH, Lee YS, Kim GH (2008) Antioxidant and antimicrobial activities of the ethanol extract of *Allium victoriallis* L. var. *platyphyllum*. Food Science and Biotechnology, 17:313-318.

Coen M, Kuchel PW (2004) Metabonomics based on NMR spectroscopy. Chemistry in Australia, 71:13-17.

Corea G, Fattorusso E, Lanzotti V, Capasso R, Izzo AA (2005) Antispasmodic saponins from bulbs of red onion, *Allium* cepa L. var. Tropea. Journal of Agricultural and Food Chemistry, 53:935-940.

Cork A, Park KC (1996) Identification of electrophysiologically-active compounds for the malaria mosquito, Anopheles gambiae, in human sweat extracts. Medical and Veterinary Entomology, 10:269-276.

Davis JM, Murphy EA, Carmichael MD, Davis B (2009) Quercetin increases brain and muscle mitochondrial biogenesis and exercise tolerance. American Journal of Physiology-Regulatory Integrative and Comparative Physiology, 296:R1071-R1077.

Day AJ, Mellon F, Barron D, Sarrazin G, Morgan MRA, Williamson G (2001) Human metabolism of dietary flavonoids: Identification of plasma metabolites of quercetin. Free Radical Research, 35:941-952.

De Ancos B, González E, Cano MP (2000) Effect of high-pressure treatment on the carotenoid composition and the radical scavenging activity of persimmon fruit purees. Journal of Agricultural and Food Chemistry, 48:3542-3548.

De Ancos B, Sgroppo S, Plaza L, Cano MP (2002) Possible nutritional and health-related value promotion in orange juice preserved by high-pressure treatment. Journal of the Science of Food and Agriculture, 82:790-796.

Ding C-K, Chachin K, Ueda Y, Wang CY (2002) Inhibition of loquat enzymatic browning by sulfhydryl compounds. Food Chemistry, 76:213-218.

Dorant E, Van Den Brandt P, Goldbohm RA (1996) A prospective cohort study on the relationship between onion and leek consumption, garlic supplement use and the risk of colorectal carcinoma in The Netherlands. Carcinogenesis, 17:477-484.

Dorant E, Van Den Brandt PA, Goldbohm RA, Sturmans F (1996) Consumption of onions and a reduced risk of stomach carcinoma. Gastroenterology, 110:12-20.

El-Demerdash FM, Yousef MI, Abou El-Naga NI (2005) Biochemical study on the hypoglycemic effects of onion and garlic in alloxan-induced diabetic rats. Food and Chemical Toxicology, 43:57-63.

Engelke UFH, Tangerman A, Willemsen M, Moskau D, Loss S, Mudd SH, Wevers RA (2005) Dimethyl sulfone in human cerebrospinal fluid and blood plasma confirmed by one-dimensional H-1 and two-dimensional H-1-C-13 NMR. NMR in Biomedicine, 18:331-336.

Ernst M, Feldheim W (2000) Fructans in higher plants and in human nutrition. Journal of Applied Botany–Angewandte Botanik, 74:5-9.

Europa-Food Safety-Biotechnology-Novel Foods. Novel Foods-Review of Regulation (EC) 258/97.
(http://ec.europa.eu/food/food/biotechnology/novelfood/initiatives_en.htm. Accesed: 24 October, 2009).

Ewald C, Fjelkner-Modig S, Johansson K, Sjoholm I, Akesson B (1999) Effect of processing on major flavonoids in processed onions, green beans, and peas. Food Chemistry, 64:231-235.

FAO (Food and Agriculture Organization of the United Nations). Chapter XXVI Onions: Post-Harvest Operation. (http://www.fao.org/inpho/content/compend/text/ch26_01.htm#1_2. Accesed: 24 October, 2009).

FAO (Food and Agriculture Organization of the United Nations). Technical manual on small-scale processing of fruits and vegetables. (http://www.fao.org/docrep/X0209E/x0209e00.HTM. Accesed: 24 October, 2009).

Fu HY (2004) Free radical scavenging and leukemia cell growth inhibitory properties of onion powders treated by different heating processes. Journal of Food Science, 69:50-54.

Gabler NK, Osrowska E, Imsic M, Eagling DR, Jois M, Tatham BG, Dunshea FR (2006) Dietary onion intake as part of a typical high fat diet improves indices of cardiovascular health using the mixed sex pig model. Plant Foods for Human Nutrition, 61:179-185.

Galeone C, Pelucchi C, Levi F, Negri E, Franceschi S, Talamini R, Giacosa A, La Vecchia C (2006) Onion and garlic use and human cancer. American Journal of Clinical Nutrition, 84:1027-1032.

Galeone C, Tavani A, Pelucchi C, Negri E, La Vecchia C (2009) *Allium* vegetable intake and risk of acute myocardial infarction in Italy. European Journal of Nutrition, 48:120-123.

Gallagher M, Wysocki J, Leyden JJ, Spielman AI, Sun X, Preti G (2008) Analyses of volatile organic compounds from human skin. British Journal of Dermatology, 159:780-791.

Galluzzo P, Martini C, Bulzomi P, Leone S, Bolli A, Pallottini V, Marino M (2009) Quercetin-induced apoptotic cascade in cancer cells: Antioxidant versus estrogen receptor alpha-dependent mechanisms. Molecular Nutrition & Food Research, 53:699-708.

Gao CM, Takezaki T, Ding JH, Li MS, Tajima K (1999) Protective effect of allium vegetables against both esophageal and stomach cancer: A simultaneous case-referent study of a high-epidemic area in Jiangsu province, China. Japanese Journal of Cancer Research, 90:614-621.

German JB, Watkins SM, Fay LB (2005) Metabolomics in practice: Emerging knowledge to guide future dietetic advice toward individualized health. Journal of the American Dietetic Association, 105:1425-1432.

Gibney MJ, Walsh M, Brennan L, Roche HM, German B, van Ommen B (2005) Metabolomics in human nutrition: opportunities and challenges. American Journal of Clinical Nutrition, 82:497-503.

Glasser G, Graefe EU, Struck F, Veit M, Gebhardt R (2002) Comparison of antioxidative capacities and inhibitory effects on cholesterol biosynthesis of quercetin and potential metabolites. Phytomedicine, 9:33-40.

Goldberg DM, Spooner RJ (1983) Assay of glutathione reductase. In: Bergmayer HU, ed. Methods in Enzymology, 3:258-265.

Goldman IL, Kopelberg M, Debaene JEP, Schwartz BS (1996) Antiplatelet activity in onion (*Allium* cepa) is sulfur dependent. Thrombosis and Haemostasis, 76:450-452.

Gonzalez CA, Pera G, Agudo A, Bueno-De-Mesquita HB, Ceroti M, Boeing H, Schulz M, Del Giudice G, Plebani M, Carneiro F, Berrino F, Sacerdotde C, Tumino R, Panico S, Berglund G, Siman H, Hallmans G, Stenling R, Martinez C, Dorronsoro M, Barricarte A, Navarro C, Quiros JR, Allen N, Key TJ, Bingham S, Day NE, Linseisen J, Nagel G, Overvad K, Jensen MK, Olsen A, Tjonneland A, Buchner FL,

Peeters PH, Numans ME, Clavel-Chapelon F, Boutron-Ruault MC, Roukos D, Trichopolou A, Psaltopoulou T, Lund E, Casagrande C, Slimani N, Jenab M, Riboli E (2006) Fruit and vegetable intake and the risk of stomach and oesophagus adenocarcinoma in the European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST). International Journal of Cancer, 118:2559-2566.

Gonzalez CA, Sanz JM, Marcos G, Pita S, Brullet E, Saigi E, Badia A, Riboli E (1991) Dietary factors and stomach-cancer in Spain - a multicenter case-control study. International Journal of Cancer, 49:513-519.

Gonzalez-Saiz JM, Esteban-Diez I, Rodriguez-Tecedor S, Pizarro C (2008) Valorization of onion waste and by-Products: MCR-ALS applied to reveal the compositional profiles of alcoholic fermentations of onion juice monitored by near-infrared spectroscopy. Biotechnology and Bioengineering, 101:776-787.

Griffiths G, Trueman L, Crowther T, Thomas B, Smith B (2002) Onions-A global benefit to health. Phytotherapy Research, 16: 603-615.

Gruber P, Vieths S, Wangorsch A, Nerkamp J, Hofmann T (2004) Maillard reaction and enzymatic browning affect the allergenicity of Pru av 1, the major allergen from cherry (*Prunus avium*). Journal of Agricultural and Food Chemistry, 52:4002-4007.

Guyonnet D, Belloir C, Suschetet M, Siess M-H, Le Bon A-M (2001) Antimutagenic activity of organosulfur compounds from Allium is associated with phase II enzyme induction. Mutation Research: Genetic Toxicology and Environmental Mutagenesis, 495:35-145.

Han K-H, Iijuka M, Shimada K-i, Sekikawa M, Kuramochi K, Ohba K, Ruvini L, Chiji H, Fukushima M (2005) Adzuki resistant starch lowered serum cholesterol and hepatic 3-hydroxy-3-methylglutaryl-CoA mRNA levels and increased hepatic LDL-receptor and cholesterol 7α-hydroxylase mRNA levels in rats fed a cholesterol diet. British Journal of Nutrition, 94:902-908.

Hansen M, Baunsgaard D, Autrup H, Vogel UB, Møller P, Lindecrona R, Wallin H, Poulsen HE, Loft S, Dragsted LO (2008) Sucrose, glucose and fructose have similar genotoxicity in the rat colon and affect the metabolome. Food and Chemical Toxicology, 46: 752-760.

Hansson LE, Nyren O, Bergstrom R, Wolk A, Lindgren A, Baron J, Adami HO (1993) Diet and risk of gastric-cancer - a population-based case-control study in Sweden. International Journal of Cancer, 55:181-189.

Helen A, Krishnakumar K, Vijayammal PL, Augusti KT (2000) Antioxidant effect of onion oil (*Allium cepa* Linn) on the damages induced by nicotine in rats as compared to alpha-tocopherol. Toxicology Letters, 116:61-68.

Hertog MGL, Hollman PCH, Venema DP (1992) Optimization of a quantitative HPLC determination of potentially anticarcinogenic flavonoids in vegetables and fruits. Journal of Agricultural and Food Chemistry, 40:1591-1598.

Higuchi O, Tateshita K, Nishimura H (2003) Antioxidative activity of sulfur-containing compounds in *Allium* species for human low-density lipoprotein (LDL) oxidation in vitro. Journal of Agricultural and Food Chemistry, 51:7208-7214.

Hollman PC, de Vries JH, van Leeuwen SD, Mengelers MJ, Katan MB (1995) Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. American Journal of Clinical Nutrition, 62:1276-1282.

Horiuchi J, Tada K, Kobayashi M, Kanno T, Ebie K (2004) Biological approach for effective utilization of worthless onions - vinegar production and composting. Resource, Conservation and Recycling, 40:97-109.

Hotelling H (1933) Analysis of a complex of statistical variables into principal components. Journal of Educational Psychology, 24:417-441.

Hu JF, La Vecchia C, Negri E, Chatenoud L, Bosetti C, Jia XY, Liu RZ, Huang GR, Bi DZ, Wang CX (1999) Diet and brain cancer in adults: A case-control study in northeast China. International Journal of Cancer, 81:20-23.

Hubbard GP, Wolffram S, de Vos R, Bovy A, Gibbins JM, Lovegrove JA (2006) Ingestion of onion soup high in quercetin inhibits platelet aggregation and essential components of the collagen-stimulated platelet activation pathway in man: a pilot study. British. Journal of Nutrition, 96:482-488.

Hucker HB, Ahmad PM, Miller EA, Brobyn R (1966) Metabolism of dimethyl sulphoxide to dimethyl sulphone in the rat and man. Nature, 209: 619-620.

Hyun-Hee L, Seok-In H, Young-Sook H, Dongman K (2003) Effect of hot water treatment on biochemical changes in minimally processed onion. Food Science and Biotechnology, 12:445-450.

Ichikawa M, Mizuno I, Yoshida J, Ide N, Ushijima M, Kodera Y, Hayama M, Ono K (2006) Pharmacokinetics of cycloalliin, an organosulfur compound found in garlic and onion, in rats. Journal of Agricultural and Food Chemistry, 54: 9811-9819.

Irkin R, Korukluoglu M (2007) Control of *Aspergillus niger* with garlic, onion and leek extracts. African Journal of Biotechnology, 6:384-387.

Iscovich JM, Labbe KA, Castelleto R, Calzona A, Bernedo A, Chopita NA, Jmelnitzsky AC, Kaldor J (1992) Colon cancer in Argentina I. Risk from intake of dietary items. International Journal of Cancer, 51:851-857.

Iyengar R, McEvily AJ (1992) Anti-browning agents: alternatives to the use of ulfites in foods. Trends in Food Science & Technology, 3:60-64.

Jacobsen H, Poulsen M, Dragsted LO, Ravn-Haren G, Meyer O, Lindecrona, RH (2006) Carbohydrate digestibility predicts colon carcinogenesis in azoxymethane treated rats. Nutrition and Cancer, 55:163-170.

Jaime L, Martín-Cabrejas MA, Mollá E, López-Andréu FJ, Esteban RM (2001) Effect of storage on fructan and fructooligosaccharide of onion (*Allium cepa* L.). Journal of Agricultural and Food Chemistry, 49:982-988.

Jaime L, Martínez F, Martín-Cabrejas MA, Mollá E, López-Andréu FJ, Waldron KW, Esteban RM (2001) Study of total fructan and fructooligosaccharide content in different onion tissues. Journal of the Science of Food and Agriculture, 81:177-182.

Jaime L, Mollá E, Fernandez A, Martín-Cabrejas MA, López-Andréu FJ, Esteban RM (2002) Structural carbohydrate differences and potential source of dietary fiber of onion (*Allium cepa* L.) tissues. Journal of Agricultural and Food Chemistry, 50:122-128.

Jang MS, Sanada A, Ushio H, Tanaka M, Ohshima T (2002) Inhibitory effects of `Enokitake' mushroom extracts on Polyphenol Oxidase and prevention of apple browning. Lebensmittel-Wissenschaft und-Technologie, 35:697-702.

Johansson LH, Borg HLA (1988) A spectrophotometric method for determination of catalase activity in small tissue samples. Analytical Biochemistry, 174:331-336.

Jung YS, Kim MH, Lee SH, Baik EJ, Park SW, Moon CH (2002) Antithrombotic effect of onion in streptozotocin-induced diabetic rat. Prostaglandins, Leukotrienes and Essential Fatty Acids, 66:453-458.

Kahane R, Vialle-Guérin E, Boukema I, Tzanoudakis D, Bellamy C, Chamaux C, Kik C (2001) Changes in non-structural carbohydrate composition during bulbing in sweet and high-solid onions in field experiments. Environmental and Experimental Botany, 45:73-83.

Kannel WB (2005) Overview of hemostatic factors involved in atherosclerotic cardiovascular disease. Lipids, 40:1215-1220.

Kawamoto E, Sakai Y, Okamura Y, Yamamoto Y (2004) Effects of boiling on the antihypertensive and antioxidant activities of onion. Journal of Nutritional Science and Vitaminology, 50:171-176.

Kim JW, Huh JE, Kyung SH, Kyung KH (2004a) Antimicrobial activity of alk(en)yl sulfides found in essential oils of garlic and onion. Food Science and Biotechnology, 13:235-239.

Kim JW, Kim YS, Kyung KH (2004b) Inhibitory activity of essential oils of garlic and onion against bacteria and yeasts. Journal of Food Protection, 67:499-504.

Kim KB, Chung MW, Um SY, Oh JS, Kim SH, Na MA, Oh HY, Cho WS, Choi KH (2008) Metabolomics and biomarker discovery: NMR spectral data of urine and hepatotoxicity by carbon tetrachloride, acetaminophen, and D-galactosamine in rats. Metabolomics, 4:377-392.

Kim M-J, Kim CY, Park I (2005) Prevention of enzymatic browning of pear by onion extract. Food Chemistry, 89:181-184.

Kim SJ, Kim GH (2006) Quantification of quercetin in different parts of onion and its DPPH radical scavenging and antibacterial activity. Food Science and Biotechnology, 15:39-43.

Krebbers B, Matser AM, Hoogerwerf SW, Moezelaar R, Tomassen MMM, & van den Berg RW (2003) Combined high-pressure and thermal treatments for processing of tomato puree: evaluation of microbial inactivation and quality parameters. Innovative Food Science and Emerging Technologies, 4:377-385.

Kristensen M, Savorani F, Ravn-Haren G, Poulsen M, Markowski J, Larsen F, Dragsted L, Engelsen S (2009) NMR and interval PLS as reliable methods for determination of cholesterol in rodent lipoprotein fractions. Metabolomics, doi: 10.1007/s11306-009-0181-3

Kumari K, Augusti KT (2007) Lipid lowering effect of S-methyl cysteine sulfoxide from *Allium cepa* Linn in high cholesterol diet fed rats. Journal of Ethnopharmacology, 109:367-371.

Kyung KH, Lee YC (2001) Antimicrobial activities of sulfur compounds derived from S-alk(en)yl-L-cysteine sulfoxides in *Allium* and *Brassica*. Food Reviews International, 17:183-198.

Langos M, Hofstetter W, Dolder S, Felix R, Muhlbauer RC, Brenneisen R (2007) A gamma-glutamyl peptide from onion inhibits the development and activity of osteoclasts *in vitro.* Calcified Tissue International, 80:91-92.

Lanzotti V (2006) The analysis of onion and garlic. Journal of Chromatography A, 1112:3-22.

Lara-Villoslada F, de Haro O, Camuesco D, Comalada M, Velasco J, Zarzuelo A, Xaus J, Galvez J (2006) Short-chain fructooligosaccharides, in spite of being fermented in the upper part of the large intestine, have anti-inflammatory activity in the TNBS model of colitis. European Journal of Nutrition, 45:418-425.

Lee M-K (2007) Inhibitory effect of banana polyphenol oxidase during ripening of banana by onion extract and Maillard reaction products. Food Chemistry, 102:146-149.

Lee SK, Hwang JY, Kang MJ, Kim YM, Jung SH, Lee JH, Kim JI (2008) Hypoglycemic effect of onion skin extract in animal models of diabetes mellitus. Food Science and Biotechnology, 17:130-134.

Lee SU, Lee JH, Choi SH, Lee JS, Ohnisi-Kameyama M, Kozukue N, Levin CE, Friedman M (2008) Flavonoid content in fresh, home-processed, and light-exposed onions and in dehydrated commercial onion products. Journal of Agricultural and Food Chemistry, 56:8541-8548.

Levonen A-L, Inkala M, Heikura T, Jauhiainen S, Jyrkkanen H-K, Kansanen E, Maatta K, Romppanen E, Turunen P, Rutanen J, Yla-Herttuala S (2007) Nrf2 gene transfer induces antioxidant enzymes and suppresses smooth muscle cell growth *in vitro* and reduces oxidative stress in rabbit aorta *in vivo*. Arteriosclerosis, Thrombosis and Vascular Biology, 27:741-747.

Lin C-c, Yin M-c (2008) Effects of cysteine-containing compounds on biosynthesis of triacylglycerol and cholesterol and anti-oxidative protection in liver from mice consuming a high-fat diet. British Journal of Nutrition, 99:37-43.

Lindon JC, Holmes E, Bollard ME, Stanley EG, Nicholson JK (2004). Metabonomics technologies and their applications in physiological monitoring, drug safety assessment and disease diagnosis. Biomarkers, 9:1-31.

Lindon JC, Nicholson JK (2008) Analytical technologies for metabonomics and metabolomics, and multi-omic information recovery. Trends in Analytical Chemistry, 27:194-204.

Lindon JC, Nicholson JK (2008) Spectroscopic and statistical techniques for information recovery in metabonomics and metabolomics. Annual Review of Analytical Chemistry, 1:45-69.

Lindon JC, Nicholson JK, Holmes E, Everett JR (2000) Metabonomics: Metabolic processes studied by NMR spectroscopy of biofluids. Concepts in Magnetic Resonance 12:289-320.

Liu L, Yeh YY (2001) Water-soluble organosulfur compounds of garlic inhibit fatty acid and triglyceride syntheses in cultured rat hepatocytes. Lipids, 36:395-400.

Liu L, Yeh YY (2002) S-Alk(en)yl cysteines of garlic inhibit cholesterol synthesis by deactivating HMG-CoA reductase in cultured rat hepatocytes. Journal of Nutrition, 132:1129-1134.

Liu ML, Nicholson JK, Lindon JC (1996) High-resolution diffusion and relaxation edited one- and two-dimensional ^{1}HNMR spectroscopy of biological fluids. Analytical Chemistry, 68:3370-3376.

Ly TN, Hazama C, Shimoyamada M, Ando H, Kato K, Yamauchi R (2005) Antioxidative compounds from the outer scales of onion. Journal of Agricultural and Food Chemistry, 53:8183-8189.

Makris DP, Rossiter JT (2001) Domestic processing of onion bulbs (*Allium cepa*) and asparagus spears (*Asparagus officinalis*): effect on flavonol content and antioxidant status. Journal of Agricultural and Food Chemistry, 49:3216-3222.

MAPA (2008) Anuario de estadística agroalimentaria Chapter 11.49 and 11.5 (http://www.mapa.es/es/estadistica/pags/anuario/2007/indice.asp. Accesed: 24 October, 2009).

Martínez MV, Whitaker JR (1995) The biochemistry and control of enzymatic browning. Trends in Food Science & Technology, 6:195-200.

Matheson EM, MAinous AG 3rd, Carnemolla MA (2009) The association between onion consumption and bone density in perimenopausal and postmenopausal non-Hispanic white women 50 years and older. Menopause, 16(4):756-9.

McAnlis GT, McEneny J, Pearce J, Young IS (1999) Absorption and antioxidant effects of quercetin from onions, in man. European Journal of Clinical Nutrition, 53:92-96.

McInerney JK, Seccafien CA, Stewart CM, Bird AR (2007). Effects of high-pressure processing on antioxidant activity, and total carotenoid content and availability, in vegetables. Innovative Food Science and Emerging Technologies, 8:543-548.

Millen AE, Subar AF, Graubard BI, Peters U, Hayes RB, Weissfeld JL, Yokochi LA, Ziegler RG, for the PLCO Cancer Screening Project Team (2007) Fruit and vegetable intake and prevalence of colorectal adenoma in a cancer screening trial. American Journal of Clinical Nutrition, 86:1754-1764.

Mogren LM, Caspersen S, Olsson ME, Gertsson U (2008) Organically fertilized onions (*Allium cepa* L.): Effects of the fertilizer placement method on quercetin content and soil nitrogen dynamics. Journal of Agricultural and Food Chemistry, 56:361-367.

Mogren LM, Olsson ME, Gertsson UE (2007) Quercetin content in stored onions (*Allium cepa* L.): effects ot storage conditions, cultivar, lifting time and nitrogen fertiliser level. Journal of the Science of Food and Agriculture, 87:1595-1602.

Moon CH, Jung, YS, Kim MH, Lee SH, Baik EJ, Park SW (2000) Mechanism for antiplatelet effect of onion: AA release inhibition, thromboxane A_2 synthase inhibition and TXA_2/PGH_2 receptor blockade. Prostaglandins, Leukotrienes and Essential Fatty Acids, 62: 277-283.

Moon JH, Nakata R, Oshima S, Inakuma T, Terao J (2000) Accumulation of quercetin conjugates in blood plasma after the short-term ingestion of onion by women. American Journal of Physiology - Regulatory, Integrative, and Comparative Physiology, 279:461-467.

Moreno FJ, Corzo-Martinez M, del Castillo MD, Villamiel M (2006) Changes in antioxidant activity of dehydrated onion and garlic during storage. Food Research International, 39:891-897.

Moriarty RM, Naithani R, Surve B (2007) Organosulfur compounds in cancer chemoprevention. Mini Reviews in Medicinal Chemistry, 7:827-38.

Muhlbauer RC, Lozano A, Reinli A (2002) Onion and a mixture of vegetables, salads, and herbs affect bone resorption in the rat by a mechanism independent of their base excess. Journal of Bone and Mineral Research, 17:1230-1236.

Mullen W, Boitier A, Stewart AJ, Crozier A (2004) Flavonoid metabolites in human plasma and urine after the consumption of red onions: analysis by liquid chromatography with photodiode array and full scan tandem mass spectrometric detection. Journal of Chromatography A, 1058:163-168.

Mullen W, Edwards CA, Crozier A (2006) Absorption, excretion and metabolite profiling of methyl-, glucuronyl-, glucosyl- and sulpho-conjugates of quercetin in human plasma and urine after ingestion of onions. British Journal of Nutrition, 96:107-116.

Munday R, Munday CM (2001) Relative activities of organosulfur compounds derived from onions and garlic in increasing tissue activities of quinone reductase and glutathione transferase in rat tissues. Nutrition and Cancer-an International Journal, 40:205-210.

Munday R, Munday CM (2004) Induction of phase II enzymes by aliphatic sulfides derived from garlic and onions: An overview. In: Quinones and Quinone Enzymes, Pt B. Academic Press Inc, San Diego, p 449-456.

Munday R, Munday CM (2004) Induction of phase II enzymes by aliphatic sulfides derived from garlic and onions: An Overview. Methods in Enzymology, 382:449-456

Munday R, Munday CM, Munday JS (2005) Hemolytic anemia and induction of phase II detoxification enzymes by diprop-1-enyl sulfide in rats: Dose-response study. Journal of Agricultural and Food Chemistry, 53:9695-9700.

Munday R, Munday JS, Munday CM (2003) Comparative effects of mono-, di-, tri-, and tetrasulfides derived from plants of the Allium family: redox cycling in vitro and hemolytic activity and Phase II enzyme induction in vivo. Free Radical Biology and Medicine, 34:1200-1211.

Negishi O, Ozawa T (2000) Inhibition of enzymatic browning and protection of sulfhydryl enzymes by thiol compounds. Phytochemistry, 54:481-487.

Nemeth K, Piskula MK (2007) Food content, processing, absorption and metabolism of onion flavonoids. Critical Reviews in Food Science and Nutrition, 47:397-409.

Ness GC, Chambers CM (2000) Feedback and hormonal regulation of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase: the concept of cholesterol buffering capacity. Proceedings of the Society for Experimental Biology and Medicine, 224:8-19.

Nicholson JK, Connelly J, Lindon JC, Holmes E (2002) Metabonomics: a platform for studying drug toxicity and gene function. Nature Reviews Drug Discovery, 1:153-161.

Nicholson JK, Lindon JC, Holmes E (1999). 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica, 29:1181-1189.

Nishimura H, Higuchi O, Tateshita K, Tomobe K, Okuma Y, Nomura Y (2006) Antioxidative activity and ameliorative effects of memory impairment of sulfur-containing compounds in *Allium* species. Biofactors, 26:135-146.

Noda I (2007) Recent advancement in the field of two-dimensional correlation spectroscopy. Journal of Molecular Structure, 883:2-26.

Norgaard L, Saudland A, Wagner J, Nielsen JP, Munck L, Engelsen SB (2000) Interval partial least-squares regression (iPLS): A comparative chemometric study with an example from near-infrared spectroscopy. Applied Spectroscopy, 54:413-419.

Norton T, Sun DW (2008) Recent advances in the use of high-pressure as an effective processing technique in the food industry. Food and Bioprocess Technology, 1:2-34.

Nuutila AM, Kammiovirta K, Oskman- Caldentey KM (2002) Comparison of methods for the hydrolysis of flavonoids and phenolic acids from onion and spinach for HPLC analysis. Food Chemistry, 76: 519-525.

Nuutila AM, Puupponen-Pimiä R, Aarni M, Oksman-Caldentey KM (2003) Comparision of antioxidant activities of onion and garlic extracts by inhibition of lipid peroxidation and radical scavenging activity. Food Chemistry, 81: 485-493.

Olthof MR, Hollman PCH, Vree TB, Katan MB (1998) Bioavailabilities of quercetin-3-glucoside and quercetin-4 '-glucoside do not differ in humans. In: 19th International Conference on Polyphenois. American Institute of Nutrition, Lille, France, p 1200-1203.

Onion-USDA (2009) Dr. Franciszek Adamicki Department of Vegetable Storage, Research Institute of Vegetable Crops. Skiemiewice, Poland. (http://www.ba.ars.usda.gov/hb66/099onion.pdf. Accesed: 24 October, 2009).

Onions, Vegetables, NASS, USDA, April 2008. Agriculture Market Resource Center. Profile:onion (http://www.agmrc.org/commodities__products/vegetables/onion_profile.cfm. Accessed: 24 October, 2009).

Osmont KS, Arnt CR, Goldman IL (2003) Temporal aspects of onion-induced antiplatelet activity. Plant Foods for Human Nutrition 58:27-40.

Ostrowska E, Gabler NK, Sterling SJ, Tatham BG, Jones RB, Eagling DR, Jois M, Dunshea FR (2004) Consumption of brown onions (*Allium cepa* var. cavalier and var. density) moderately modulates blood lipids, haematological and haemostatic variables in healthy pigs. British Journal of Nutrition, 91:211-218.

Paganga G, Miller N, Rice- Evans CA (1999) The polyphenolic content of fruit and vegetables and their antioxidant activities. What does a service constitute? Free Radical Research, 30:153-162.

Paglia DE, Valentine WN (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. Journal of Laboratory and Clinical Medicine, 70:158-169.

Palta JP, Levitt J, Stadelmann EJ (1977) Freezing injury in onion bulb cells .1. Evaluation of conductivity method and analysis of ion and sugar efflux from injured cells. Plant Physiology, 60:393-397.

Palta JP, Levitt J, Stadelmann EJ (1977) Freezing injury in onion bulb cells .II. Post-thawing injury or recovery. Plant Physiology, 60:398-401.

Park J, Kim J, Kim MK (2007) Onion flesh and onion peel enhance antioxidant status in aged rats. Journal of Nutritional Science and Vitaminology, 53:21-29.

Park SY, Yoo SS, Shim JH, Chin KB (2008) Physicochemical properties, and antioxidant and antimicrobial effects of garlic and onion powder in fresh pork belly and loin during refrigerated storage. Journal of Food Science, 73: 577-584.

Pedersen HT, Dyrby M, Engelsen SB, Bro R (2006) Application of multi-way analysis to 2D NMR data. Annual Reports on NMR Spectroscopy, 59:207-233.

Perner H, Rohn S, Driemel G, Batt N, Schwarz D, Kroh LW, George E (2008) Effect of nitrogen species supply and mycorrhizal colonization on organosulfur and phenolic compounds in onions. Journal of Agricultural and Food Chemistry, 56:3538-3545.

Plaza L, Sánchez-Moreno C, De Ancos B, Cano MP (2006a) Carotenoid content and antioxidant capacity of Mediterranean vegetable soup (gazpacho) treated by high-pressure/temperature during refrigerated storage. European Food Research and Technology, 223:210-215.

Plaza L, Sánchez-Moreno C, Elez-Martínez P, De Ancos B, Martín-Belloso O, Cano, MP (2006b) Effect of refrigerated storage on vitamin C and antioxidante activity of orange juice processed by high-pressure or pulsed electric fields with regard to low pasteurization. European Food Research and Technology, 223:487-493

Pool-Zobel BL, Sauer J (2007) Overview of experimental data on reduction of colorectal cancer risk by inulin-type fructans. Journal of Nutrition, 137:S2580-S2584.

Powolny AA, Singh SV (2008) Multitargeted prevention and therapy of cancer by diallyl trisulfide and related Allium vegetable-derived organosulfur compounds. Cancer Letters, 269:305-314.

Prakash D, Singh BN, Upadhyay G (2007) Antioxidant and free radical scavenging activities of phenols from onion (*Allium* cepa). Food Chemistry, 102:1389-1393.

Price KR, Bacon JR, Rhodes MJC (1997). Effect of storage and domestic processing on the content and composition of flavonol glucosides in onion (*Allium cepa*). Journal of Agricultural and Food Chemistry, 45:938-942.

Rezzi S, Ramadan Z, Fay LB, Kochhar S (2007) Nutritional metabonomics: Applications and perspectives. Journal of Proteome Research, 6:513-525.

Roberfroid MB (2007) Inulin-Type Fructans: Functional Food Ingredients. Journal of Nutrition, 137:2493-2502.

Rohn S, Buchner N, Driemel G, Rauser M, Kroh LW (2007) Thermal degradation of onion quercetin glucosides under roasting conditions. Journal of Agricultural and Food Chemistry, 55:1568-1573.

Roldán E, Sánchez-Moreno C, de Ancos B, Cano MP (2008) Characterisation of onion (*Allium cepa* L.) by-products as food ingredients with antioxidant and antibrowning properties. Food Chemistry, 108:907-916.

Roldán-Marín E, Sánchez-Moreno C, Lloría R, de Ancos B, Cano MP (2009) Onion high-pressure processing: Flavonol content and antioxidant activity. LWT - Food Science and Technology, 42:835-841.

Roldán-Marín E, Krath BN, Poulsen M, Binderup M-L, Nielsen TH, Hansen M, Barri T, Langkilde S, Cano MP, Sánchez-Moreno C, Dragsted LO (2009) Effects of an

onion by-product on bioactivity and safety markers in healthy rats. British Journal of Nutrition, DOI:10.1017/S0007114509990870.

Roldán-Marín E, Jensen RI, Kristensen M, Krath BN, Poulsen M, Cano MP, Sánchez-Moreno C, Dragsted LO (2009) Effects of an onion by-product on plasma lipids and platelet aggregation in healthy rats. Journal of Agricultural and Food Chemistry (under review)

Rose P, Whiteman M, Moore PK, Zhu YZ (2005) Bioactive S-alk(en)yl cysteine sulfoxide metabolites in the genus *Allium*: the chemistry of potential therapeutic agents. Natural Product Reports, 22:351-368.

Sakai H, Suzuki T, Takahashi Y, Ukai M, Tauchi K, Fujii T, Horikawa N, Minamimura T, Tabuchi Y, Morii M, Tsukada K, Takeguchi N (2006) Upregulation of thromboxane synthase in human colorectal carcinoma and the cancer cell proliferation by thromboxane A_2. FEBS Letters, 580:3368-3374.

Sakakibara H, Yoshino S, Kawai Y, Terao J (2008) Antidepressant-Like Effect of Onion (*Allium cepa* L.) powder in a rat behavioral model of depression. Bioscience, Biotechnology, and Biochemistry, 72:94-100.

San Martín MF, Barbosa Cánovas GV, Swanson BG (2002) Food processing by high hydrostatic pressure. Critical Reviews in Food Science and Nutrition, 42:627-645.

Sance MM, Gonzalez RE, Soto VC, Galmarini CR (2008) Relationships between antiplatelet activity, dry matter content and flavor in onion cultivars. Journal of Food Agriculture & Environment, 6:41-46.

Sánchez-Moreno C, Larrauri, JA, Saura-Calixto F (1998) A procedure to measure the antiradical efficiency of polyphenols. Journal of the Science of Food and Agriculture, 76:270-276.

Sánchez-Moreno C, Plaza L, De Ancos B, Cano MP (2004) Effect of combined treatments of high-pressure and natural additives on carotenoid extractability and antioxidant activity of tomato puree (*Lycopersicum esculentum* Mill.). European Food Research and Technology, 219:151-160.

Sánchez-Moreno C, Plaza L, de Ancos B, Cano MP (2006) Impact of high-pressure and traditional thermal processing of tomato pure´e on carotenoids, vitamin C and antioxidant activity. Journal of the Science of Food and Agriculture, 86:171-179.

Sankaranarayanan R, Varghese C, Duffy SW, Padmakumary G, Day NE, Nair MK (1994) A case-control study of diet and lung-cancer in Kerala, South-India. International Journal of Cancer, 58:644-649.

Santas J, Carbó R, Gordon MH, Almajano MP (2008) Comparison of the antioxidant activity of two Spanish onion varieties. Food Chemistry, 107:1210-1216.

Schieber A, Stintzing FC, Carle R (2001) By-products of plant food processing as a source of functional compounds-Recent developments. Trends in Food Science and Technology, 12:401-413.

Slimestad R, Fossen T, Vagen IM (2007) Onions: A source of unique dietary flavonoids. Journal of Agricultural and Food Chemistry, 55:10067-10080.

Slobodianik N, Insani M, Feliu MS, Galmarini CR (2007) Antioxidant effects from onion (*Allium cepa* L.) on oxidative stress of rats cardiac muscle. In: Experimental

Biology 2007 Annual Meeting. Federation of American Societies for Experimental Biology, Washington, DC, p A726-A726.

Slupsky CM, Rankin KN, Wagner J, Fu H, Chang D, Weljie AM, Saude EJ, Lix B, Adamko DJ, Shah S, Greiner R, Sykes BD, Marrie TJ (2007) Investigations of the effects of gender, diurnal variation, and age in human urinary metabolomic profiles. Analytical Chemistry, 79: 6995-7004.

Son Y, Jung W-K, Jeon Y-J, Kim S, Lee C (2008) Protective effects of fermented onion juice containing higher amount of querectin aglycone against oxidative stress by 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) treatment in Sprague–Dawley rats. European Food Research and Technology, 226:473-482.

Srinivasan K (2005) Plant foods in the management of diabetes mellitus: Spices as beneficial antidiabetic food adjuncts. International Journal of Food Sciences and Nutrition, 56:399-414.

Stajner D, Milic N, Canadanovic-Brunet J, Kapor A, Stajner M, Popovic BM (2006) Exploring *Allium* species as a source of potential medicinal agents. Phytotherapy Research, 20:581-584.

Steinmetz KA, Potter JD (1993) Food-group consumption and colon cancer in the adelaide case-control study I Vegetables and fruit. International Journal of Cancer, 53:711-719.

Takahama U, Hirota S (2000) Deglucosidation of quercetin glucosides to the aglycone and formation of antifungal agents by peroxidase dependent oxidation of quercetin on browning of onion scales. Plant Cell Physiolology, 41:1021-1029.

Teyssier C, Amiot MJ, Mondy N, Auger J, Kahane R, Siess MH (2001) Effect of onion consumption by rats on hepatic drug-metabolizing enzymes. Food and Chemical Toxicology, 39:981-987.

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF (2000) Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. Environmental and Molecular Mutagenesis, 35:206-221.

Tuyns AJ, Kaaks R, Haelterman M (1988) Colorectal-cancer and the consumption of foods - a case-control study in Belgium. Nutrition and Cancer-an International Journal, 11:189-204.

Tuyns AJ, Kaaks R, Haelterman M, Riboli E (1992) Diet and gastric-cancer - A case-control study in Belgium. International Journal of Cancer, 51:1-6.

USDA National Nutrient Database for Standard Reference, Release 21 (2008). Food Group: 11: Vegetables and Vegetable Products. Raw onion (*Allium cepa* L.) Refuse: 10% Stem ends, sprouts and defects (http://www.nal.usda.gov/fnic/foodcomp/Data/; http://www.nal.usda.gov/fnic/foodcomp/Data/SR21/reports/sr21fg11.pdf. Accesed: 24 October, 2009).

Vagen IM, Slimestad R (2008) Amount of characteristic compounds in 15 cultivars of onion (*Allium cepa* L.) in controlled field trials. Journal of the Science of Food and Agriculture, 88:404-411.

Vinson JA, Hao Y, Su XH, Zubik L (1998) Phenol antioxidant quantity and quality in foods: vegetables. Journal of Agricultural and Food Chemistry, 46:3630-3634.

Waldron, K (1999) Finding useful ingredients in onion waste. Emerging Food R&D Report.

Waldron, K (2001) Useful ingredients from onion waste. Food Science and Technology, 15:38-41.

Wang HX, Ng TB (2004) Isolation of allicepin, a novel antifungal peptide from onion (*Allium cepa*) bulbs. Journal of Peptide Science, 10:173-177.

Wang TJ, Gona P, Larson MG, Tofler GH, Levy D, Newton-Cheh C, Jacques PF, Rifai N, Selhub J, Robins SJ, Benjamin EJ, D'Agostino RB, Vasan RS (2006) Multiple biomarkers for the prediction of first major cardiovascular events and death. New England Journal of Medicine, 355:2631-2639.

Waring RH, Mitchell SC, Fenwick GR (1987) The chemical nature of the urinary odor produced by man after asparagus ingestion. Xenobiotica, 17:1363-1371.

Wensing M, Ludt S, Campbell S, van Lieshout J, Volbracht E, Grol R, on behalf of the EPACPG (2009) European practice Aassessment of cardiovascular risk management (EPA Cardio): protocol of an international observational study in primary care. Implementation Science, 4:3.

Wetli HA, Brenneisen R, Tschudi I, Bigler P, Sprang T, Schurch S, Muhlbauer RC (2004) Gamma-glutamyl-peptide isolated from onion by bioassay guided fractionation inhibits resorption activity of osteoclasts. In: 26th Annual Meeting of the American Society for Bone and Mineral Research, Seattle, WA, p S314-S314.

WHO (World Health Organization) Cardiovascular disease (http://www.who.int/cardiovascular_diseases/en/. Accesed: 24 October, 2009).

WHO (World Health Organization) Prevention of cardiovascular disease: Guidelines for assessment and management cardiovascular risk http://www.who.int/cardiovascular_diseases/guidelines/Full%20text.pdf. Accesed: 24 October, 2009).

Winning H, Larsen FH, Bro R, Engelsen SB (2008) Quantitative analysis of NMR spectra with chemometrics. Journal of Magnetic Resonance, 190:26-32.

Wishart DS (2008) Metabolomics: applications to food science and nutrition research. Trends in Food Science & Technology, 19:482-493.

Winning H, Roldán-Marín E, Dragsted LO; Viereck N, Poulsen M, Sánchez-Moreno, Cano MP, Engelsen SB (2009) An exploratory nuri-metabonomic investigation reveals dimethyl sulfone as a dietary biomarker for onion intake. Analyst, 134: 2344-2351.

Wold S, Martens H, Wold H (1983) The Multivariate Calibration-Problem in Chemistry Solved by the PLS Method: Lecture Notes in Mathematics, 973:286-293.

Woo KS, Hwang IG, Kim TM, Kim DJ, Hong AT, Jeong HS (2007) Changes in the antioxidant activity of onion (*Allium cepa*) extracts with heat treatment. Food Science and Biotechnology, 16:828-831.

Xiao D, Zeng Y, Hahm ER, Kim YA, Ramalingam S, Singh SV (2009) Diallyl trisulfide selectively causes Bax- and Bak-mediated apoptosis in human lung cancer cells. Environmental and Molecular Mutagenesis, 50:201-212.

Xiao H, Parkin KL (2007) Isolation and identification of potential cancer chemopreventive agents from methanolic extracts of green onion (*Allium cepa*). Phytochemistry, 68:1059-1067.

Yamada K, Naemura A, Sawashita N, Noguchi Y, Yamamoto J (2004) An onion variety has natural antithrombotic effect as assessed by thrombosis/thrombolysis models in rodents. Thrombosis Research, 114:213-220.

Yamato O, Hayashi M, Kasai E, Tajima M, Yamasaki M, Maede Y (1999) Reduced glutathione accelerates the oxidative damage produced by sodium n-propylthiosulfate, one of the causative agents of onion-induced hemolytic anemia in dogs. Biochimica et Biophysica Acta-General Subjects, 1427:175-182.

Yang J, Meyers KJ, Van der Heide J, Liu RH (2004) Varietal differences in phenolic content and antioxidant and antiproliferative activities of onions. Journal of Agricultural and Food Chemistry, 52:6787-6793.

You WC, Blot WJ, Chang YS, Ershow A, Yang ZT, An Q, Henderson BE, Fraumeni JF, Wang TG (1989) *Allium* vegetables and reduced risk of stomach-cancer. Journal of the National Cancer Institute, 81:162-164.

Young V, Ho M, Vosper H, Belch JJF, Palmer CAN (2002) Elevated expression of the genes encoding TNF-alpha and thromboxane synthase in leucocytes from patients with systemic sclerosis. Rheumatology, 41:869-875.

Yuan L, Ji TF, Wang AG, Yang JB, Su YL (2008) Two new furostanol saponins from the seeds of *Allium cepa* L. Chinese Chemical Letters, 19:461-464.

Zohri AN, Abdel- Gaward K, Saber S. (1995) Antibacterial, antidermatophytic and antioxigenic activities of onion (*Allium cepa* L.) oil. Microbiology Research, 150: 167-172.

Agradecimientos-Acknowledgements

Esta Tesis Doctoral es el resultado de 4 años de mi vida en los que he tenido la oportunidad de aprender y crecer cada día, tanto a nivel profesional como personal. Ha sido pues un largo camino en el que tenido la gran suerte de conocer y compartir muchos buenos momentos con inumerables compañeros, algunos ahora muy buenos amigos.

Así, en primer lugar me gustaría agradecer a mis directoras de Tesis, Conchy y Pilar, sin las cuales no habría sido posible llevar a cabo este a veces tan ardúo camino. Gracias por vuestro apoyo, sobre todo en esos momentos en los que se hace todo cuesta arriba. Muchas gracias Conchy, por tus buenos consejos, por saber escuchar, y por haber escuchado todas mis dudas y preguntas, muchas veces tontas. Gracias por todo lo que he aprendido y compartido contigo, por haberme ayudado a mantener la calma en esos momentos difíciles, y por haber intentado que todo saliera bien, por haberme apoyado y darme ánimos. Gracias Pilar, por ser para mi esa 'voz de la experiencia' en todo momento.

Muy especialmente me gustaría expresar mi más sincero agradecimiento a Begoña por todo su apoyo y conocimiento, por su ayuda incondicional. Y sobre todo Begoña, gracias por ser la persona tan excepcional que eres cada día. Querría agradecer también a Lucía (Luciluz!!) todo su apoyo y su amistad, todos esos momentos que hemos pasado juntas en el 'labo' al compás nuestro 'Sergio' y por nuestros laaargos cafés…Están plasmados en cada una de estas líneas de alguna manera, ya lo sabes.

En cuanto a la mis 'compis', han sido muchos los que han compartido conmigo buenos y a veces no tan buenos momentos. Muchas gracias a todos, tanto a los que iniciaron este camino junto conmigo como los que llegaron un poco después al *marvellous* 'Frío´s world'. Con todos ellos he aprendido algo nuevo y diferente, y a todos ellos guardaré un cariño muy especial. Muchas gracias muy muy especial a Rosana, y a Roberto. A Marina, Inés, Cristina, Joaquín y a Delia. Mil gracias por tu sonrisa y alegría cada mañana a la pequeña-Gran Sarixx! A mis chicas de la sala de becarios-Lácteos: Pilar Luna, Valle, Mariví, Paquita y Maricarmen. Muchísimas gracias Pedro, por tu amistad, ya sabes que siempre me queda Lleida pasando por Barcelona…Y mil gracias a mi Patri Patri Patriló, y a mi Itziar-e castellonense ahora…Siempre nos quedará Granada. Y a mis maravillosos y mejores siempre! Charlix y Asier, a mi Meriló, a Clarita y a como no, a Evita 'de rosita'. Muchas gracias a todos los demás miembros del departamento de Vegetales así como a los técnicos y demás compañeros de los demás departamentos del Instituto, y en especial a Maricarmen, por sus años pasados y experiencia en el Frío y sobre todo por sus ánimos en los comienzos.

Así dar las muchas gracias también a Ramón y a Felipe, que seguirán siendo para mí la alegría de todas las mañanas al entrar al Frío y a Feli, las tardes en el Frío no serían igual sin tu presencia y nuestras charlitas por medio de los labos y los despachos...

Y como no, unas enormes gracias con apartado punto y aparte muuy especial a mi 'Spanish Connection' Copenaguense. Muchas gracias a mi Manolín, Manolín, a mi Elia (vs xicona), al Josele (the king of Linares), a Erkuden, y a Manuel. Sin todos ellos, y sobre todo sin nuestros cociditos y comiditas a lo Spanish no hubiera sido posible sobrevivir a esos fríos inviernos daneses.

I would like to express my sincere gratitude to Lars Ove Dragsted. Thanks Lars, for your useful advice and comments not only about work but also about general 'life'…the more important thing that sometimes we forgot when we are so focused on something…and not to talk only about onions all the time! Thanks a lot for everything there…words could´t express aaall my gratitude…

Thanks to all the people I had the opportunity to work with and meet at Mørkhøj (*that name that I can´t still not pronounce, not even write) and at Life…I would have spent there ages talking to all of you everyday.*

A very special thanks to Gitte, first one I met at CPH airport...and without suitcases!! Thanks Gitte, for all those long winter times at Denmark sharing a morning smile with me…thanks for your friendship and for being that especial person that you are. Thanks a lot Vibeke, you were almost like my Danish 'mum', I will always remember you…and thanks a lot Britt, you know that this onion nightmare wouldn´t be possible without you, sharing that work breaks with you was so nice. Thanks Mona-Lisa, Margit, Morten, Max, Lea, and Lars at Mørkhøj and Mette, Sussane, Sanne, Birgitte, Runa and the always helpful Life Secretary Suzzane and all the technicians at Life, in special to Hanne. You were really really nice with me all the time and really helpful.

Thanks a lot Wu (Annie) for your friendship and for sharing CPH with me the fisrt time, living with you was so nice...that Chinese-Spanish meals were great!!! Tak-gracias to Ulrich (Ulito) and Sonia for their warmly welcome everytime I come back to Denmark, lots of 'embrazos' to you two!! And thanks a lot Jytte, for being so nice with me and for showing me a bit of your Danish world there…Now it´s my turn to show you our Spanish world here…

Tusind tak til jer allesammen for at give mig et stykke af Danmark i Spanien. I husker mig måske som "løg-pigen" i København, men jeg håber, at jeg ikke får jer til at græde som løg men derimod til at grine!! *(Thanks all of you for making me have a bit of Denmark in Spain, you might remember me as the 'onion' girl in CPH…I hope that I don´t make people cry like onions but smile instead!!).*

Finalmente y como los postres en una gran comilona, los más dulces, y para quien bien me conoce, los que más me gustan y me importan, y sin los que no podría sobrevivir. Quisiera así darle las gracias a toda mi familia y en especial a mi madre, a mi padre y a mi hermano Ramón, por estar siempre apoyándome y dándome ánimos, gracias por vuestro gran cariño. Unas enormes gracias muy especialmente a mi tita Regina, gracias por ser esa persona tan especial para mi y por haber compartido y estar conmigo en esta aventura día a día...

Así gracias a todos mis amigos, ya sabeis quienes sois cada uno de vosotros, sería imposible nombraros a todos uno a uno y alguno seguro se me olvida con este cabezón que tengo (Ana, Carmentxu, Beti, Sole, Raquel, Pol, Pachi, Belen, Xavi….y muuuchos más que no se me olvidan..es que ya no me caben!..Gracias Alberto, ese 'peazo' de artista con esa megaportada de última hora). Gracias a todos vosotros por haber estado y seguir estando a mi lado en todo momento, gracias por haber sabido bajarme a la vida real cuando todo se me hacía cuesta arriba…

I com no, al meu xiquet Josep, moltes gràcies per estar al meu costat, saps que sense tu no haguera sigut el mateix…

☺Quka