Models of the fate of glucosinolates in *Brassicaceae* from processing to digestion

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This research was conducted under the auspices of the Graduate school VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

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Thesis

submitted in fulfilment of the requirements for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus

Prof. Dr M. J. Kropff,

in the presence of the

Thesis Committee appointed by the Academic Board

on Friday 22 May 2015

at 11 a.m. in the Aula.

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PhD thesis Wageningen University, Wageningen, NL (2015)

With references, with summary in English and Dutch

ISBN

To Anand

Table of contents

Chapter 1

General Introduction

1.1 Introduction

Epidemiological studies, prospective cohort studies as well as case control studies, have been carried out on the correlation between a diet high in *Brassica* vegetables and the formation and development of cancer, such as colorectal, lung, breast, bladder, stomach and prostate cancer (Steinbrecher, Nimptsch, Hüsing, Rohrmann, & Linseisen, 2009; Traka & Mithen, 2008; Verhoeven, Goldbohm, van Poppel, Verhagen, & van den Brandt, 1996; Verkerk et al., 2009; Voorrips et al., 2000). Even if the metabolism is not entirely understood it has been shown in animal and cell studies that degradation products of glucosinolates (GSs), especially isothiocyanates, play an important role in the risk reduction of various cancers (Traka & Mithen, 2008; Verkerk et al., 2009). In this introduction the health effects of glucosinolates will first be briefly reviewed, after which the main focus of the research is described in this thesis: the effects of processing and digestion on the fate of GSs and the use of modelling tools to study this.

1.2 Effects of processing and digestion on phytochemicals

In our diet we ingest not only essential macronutrients, vitamins and minerals, but also non-nutrient health-beneficial components. One group of non-nutrient, bioactive food components are phytochemicals. Phytochemicals can have various effects on the human body, such as acting as antioxidants, preventing against cancer, supporting of the immune system and the digestion, be antimicrobial, anti-thrombotic, anti-inflammatory, decreasing cholesterol levels and regulating blood pressure and blood glucose levels (Watzl & Leitzmann, 1999). In high concentrations, however, they can also be toxic.

Individual concentrations of phytochemicals in vegetables can be substantially altered on the way from farm to fork by storage, packaging, and industrial as well as domestic processing. As an example, the effects of cultivation and different processing steps on GSs in cabbage were estimated by Dekker et al (2000) and lead to variation in concentration up to a factor of 100 in the same vegetable at the moment of consumption. During storage, the main factors influencing the GSs concentration are time, temperature, humidity and the gas composition above the vegetable. The GSs

concentration of *Brassica* vegetables during packaging is mainly affected by the gas composition and humidity. If, by incidence during storage and/or packaging, or intentionally during processing, intact cell structures of *Brassica* vegetables are damaged, the GSs content can alter as well. On the one hand, GSs can be hydrolyzed by the endogenous enzyme myrosinase or leach out of cells and washed away, e.g., after a washing step. On the other hand, it was shown that the concentration of especially indole GSs can be increased after cutting and several days of storage (Verkerk, Dekker, & Jongen, 2001). Besides physical damage, time and temperature are important factors for GSs degradation. Often, *Brassica* vegetables are thermally treated before consumption such as cooking, steaming, frying or microwaving to make them more palatable or easier digestible, these processes may, in addition, lead to losses of GSs by degradation and/or leaching.

After ingestion, the phytochemicals need to be released from the food matrix during digestion, to get absorbed by the gut epithelium. The extent to which this release occurs is called bioaccessibility and can be affected by i) the physicochemical characteristics of the compound of interest, ii) the food matrix, iii) the storage and processing of the food and iv) the composition of the meal to which the food belongs. The characteristics of the phytochemical that affect bioaccessibility include molecular weight, solubility, hydrophobicity and redox potential (Duchateau & Klaffke, 2008). Food preparation steps such as cooking or processing change the food matrix and might therewith change the bioaccessibility as well (Dekker et al., 2000), for instance, more carotenoids are released from cooked carrots into micelles than from raw carrots (Hornero-Méndez & Mínguez-Mosquera, 2007). The composition of the meal can have an effect on bioaccessibility, for instance, epigallocatechins from tea can bind noncovalently to caseins if milk is added to the tea (Jobstl, Howse, Fairclough, & Williamson, 2006).

The bioaccessibility of GSs from *Brassica* vegetable and their lipophilic bioactive breakdown products isothiocyanates might be influenced by processing conditions and additional food components. In an *in vivo* study with microwaved broccoli, ingested with and without the addition of meat, no differences in bioaccessibility could be found (Rungapamestry, Duncan, Fuller, & Ratcliffe, 2007a). They hypothesized that the isothiocyanates might interact with compounds present in the meal if they are already formed before ingestion and not when they are formed in the intestine by the human microflora.

1.3 Glucosinolates and their health effect

GSs are secondary plant metabolites especially prevalent in plants of the family *Brassicaceae* like broccoli, cauliflower, radish, Brussels sprouts, mustard, turnip and cabbage. The GS content and pattern differs between crops as well as between varieties, but depends also on cultivation location and conditions (Verkerk et al., 2009). GSs are β-thioglucoside-N-hydroxy sulphates, which are sulfur-linked to a β-D-glucopyranose moiety, and with an indolic, aliphatic or aromatic side chain characterizing the molecule. GSs are found in all plant organs, but their concentration and profile can differ within the plant and varies depending on the stage of plant development. They are stored in the intact plant in cell vacuoles. Besides these GS storing cells, *Brassica* vegetables also contain myrosin cells, containing the glucosinolate-hydrolyzing enzyme complex myrosinase (β-thioglucosidase, EC 3.2.1.147) (Kissen, Rossiter, & Bones, 2008). If the cell structure is ruptured, e.g. due to attack by insects, cutting or chewing, myrosinase can get into contact with its substrate and hydrolyze GSs. Myrosinase will detach the glucose-group from the parental molecule. The ensuing intermediate product is unstable and will be chemically transformed to either isothiocyanates, nitriles, thiocyanates or epithionitriles, depending on the chemical structure of the GSs and the environment of the reaction (Angelino & Jeffery, 2014). If the endogenous thermolabile epithiospecifier protein (ESP) and iron ions for its activation are present, the production of nitriles and epithionitriles is favoured (Bones & Rossiter, 2006; Wang, Farnham, & Jeffery, 2012). At neutral pH and without the presence of iron ions and ESP, isothiocyanates will be formed (Angelino & Jeffery, 2014; Hayes, Kelleher, & Eggleston, 2008), which have been shown in animal and cell studies to reduce the risk of several cancers (Traka & Mithen, 2008; Verkerk et al., 2009). The most studied isothiocyanate is sulforaphane (SF), the breakdown product of the glucosinolate glucoraphanin (GR). GR-rich vegetables in the human diet are, e.g., broccoli and red cabbage. SF is believed to alter carcinogen metabolism, induce tumor cell cycle arrest and apoptosis, inhibit angiogenesis and metastasis, change the histone acetylation status, and induce antioxidant, anti-inflammatory, and immunomodulatory activities (reviewed by Dinkova-Kostova & Kostov, 2012).

If GSs are ingested without the presence of active myrosinase, e.g., after heat treatment, they can be hydrolyzed to a lesser extent by enzymes of the gut flora

compared to the hydrolysis by the endogenous myrosinase (Getahun & Chung, 1999; Matusheski et al., 2006; Shapiro et al., 1998).

Many studies have been performed on the fate of GSs in *Brassicaceae* (Hanschen, Platz, et al., 2012; Oerlemans, Barrett, Suades, Verkerk, & Dekker, 2006; Wang et al., 2012) or in model solutions (Hanschen, Brüggemann, et al., 2012; Wu, Mao, You, & Liu, 2014) during various thermal processes, while some studies are done on the digestion of *Brassica* vegetable in humans (Conaway et al., 2000; Oliviero, Verkerk, Vermeulen, & Dekker, 2014; Rungapamestry et al., 2007a). However, a study with a global approach, integrating food science, food processing and nutrition, has not been described before, to the best of our knowledge.

1.4 Food models

Any kind of model is a simplification that "attempts to formulate the behaviour of systems from knowledge of the properties of their component parts." (van Boekel, 2009). These simplifications should help to analyze specific isolated mechanisms or components, without unwanted interactions in the complex food medium. This can help us to understand food reactions, but it will only approximate reality. In food science many different models are used as tools: *in vivo* (animal or human study), *in vitro* (e.g. digestion model, cell culture model), *ex vivo* (tissue models), *in silico* (mathematical model) or model foods (simplified food systems).

1.4.1 *In silico* models

The understanding of what food quality is changed over time, but is always determined by the expectation of the consumer (van Boekel, 2008). Nowadays food safety, high nutritional quality, handling properties, shelf life, texture, colour, appearance, flavour and taste are important quality attributes of food, which food scientist try to influence (van Boekel, 2009). The quality of a food itself changes over time too, by chemical reactions (e.g. oxidation), microbial reactions (growth of undesired microorganisms), biochemical reactions (e.g. enzymatic browning) and physical reactions (e.g. sedimentation) (van Boekel, 2009). These changes can be described by kinetics and can be mathematically modelled. Such models can be used to optimize food changes in the chain (a.o., industrial processes) in terms of nutritional value or to improve the accuracy of epidemiological studies. If the

degradation mechanism of a food component is known, the content of all kind of components in food can be estimated at the moment of consumption.

Mathematical models can be used to simulate quality determining reactions in foods by describing their kinetics as equations with different parameters. These equations can be based upon underlying chemical and physical mechanisms (mechanistic or white box approach) or can be obtained by fitting suitable mathematical equations to experimental data without knowledge about the underlying mechanisms (empirical or black box approach) (Perrot, Trelea, Baudrit, Trystram, & Bourgine, 2011). After estimation of parameters by fitting experimental data to these mechanistic models, the obtained parameters depend on the food characteristics and, as they are based on mechanistic knowledge, they can be used outside the studied conditions. In addition, mechanistic models can be used to obtain more knowledge on the underlying mechanisms themselves, because mechanistic kinetic models give insight in this; this, however, was not the aim for the research described in this thesis. In opposite to empirical models, the parameters of mechanistic models have a physical meaning, e.g., a diffusion constant, a reaction rate constant or a permeability constant (van Boekel, 2009). A reaction rate constant as, for instance, for thermal degradation, describes how quick a component will be degraded. Reaction rate constants are temperature dependent. An Arrhenius equation can be used to describe the temperature dependency of several reaction rate constants. The activation energy, introduced by the Arrhenius equation, describes the energy necessary, to start the reaction.

It has to be noted that mechanistic models are generally developed for simple, dilute, homogeneous systems. In contrast, foods are often containing various components, in low or high concentrations and different compartments at the same time (van Boekel, 2009). Mechanistic models can be still used for foods, but then the estimated parameters might not be the 'true' parameters as in an ideal environment and should be handled carefully and cautiously (van Boekel, 2009).

In contrast, empirical models do not require mechanistic knowledge, they are obtained by fitting , for instance, polynomial functions or artificial neural networks to the data set (Perrot et al., 2011). The obtained parameters are only describing the studied data set and it is not well possible to use such empirical models for predictions for other conditions.

1.4.2 *In vitro* digestion models

In vitro models for digestion mimic the conditions in and the behaviour of food or food components, in the digestive tract of the human body. Although *in vitro* models can never be as accurate as *in vivo* studies, they are a faster, labour-saving and hence less expensive and non-invasive alternative. *In vitro* studies can be divided into static models, which do not copy exactly the physiological and physical processes taking place *in vivo*, and dynamic models, where the experimental set-up is as close as possible to human physiology (Kong & Singh, 2008). Advanced dynamic models, which mimic not only the complex movements during digestion such as grinding and peristaltic movements in the stomach, but also enzyme concentrations based on the digested food, are developed, for instance, by the Dutch research institute TNO (Minekus, Marteau, Havenaar, & Huis in 't Veld, 1995), by the Institute of Food Research (IFR) (Wickham, Faulks, Mann, & Mandalari, 2012) and the French INRA (Ménard et al., 2014). Whether a static or dynamic model is selected, depends on the research question for which the *in vitro* model is used.

1.4.3 *In vivo* chewing study

Human digestion is a highly complicated process, which was studied extensively in either fed or fasted state or in health and disease. *In vivo* studies in humans are the "gold standard" for studying human digestion and absorption of food components, but taking gastric or intestinal samples is invasive and has to be ethically approved. Furthermore only liquid meals can be collected and the collecting devices are difficult to place in the right location (Wickham et al., 2012). *In vivo* studies can be divided into human studies and animal studies. Animal studies with rats, mice, hamsters, guinea pigs, primates or pigs have been used especially to investigate toxic components, due to the similar digestion and absorption processes to humans (Lefebvre et al., 2014). The translation of the results from animal studies to human health is complex and also these studies need to be approved by ethical committees.

The first step in the digestion, starts with chewing of food. During mastication the food structure is disrupted and lubricated with saliva as well as water contained in the food. In addition, aromas and flavours of the food are further released from the food structure (Chen, 2009). The chewing time and the number of chewing cycles can vary between individuals (Mishellany, Woda, Labas, & Peyron, 2006) and is influenced by the physical properties of foods (Chen, 2009).The formed food ball ready to swallow

is called bolus. By studying the mechanisms by which the food properties are affected by chewing it can be included into *in vitro* digestion models or *in silico* models.

1.4.4 DREAM project

The research described in this thesis was embedded in an EU-Framework Program 7 called DREAM, which stands for "Development of REAlistic food Models with well characterized micro- and macro-structure and composition". Models were developed, by integrating experimental and mathematical approaches. These models are intended to be used as predictive tools for the assessment of food associated risks and nutritional quality, e.g. to optimize processes in the industry and to design epidemiological studies in the future. In epidemiological studies, instead of using the GS content of unprocessed foods or using a mean value for processed foods, by using the model the GS content could be corrected for the effect of processing individually. In this way the sensitivity of the study could be enhanced by decreasing the variability of the GS intake between subjects.

Four categories of products were covered: Fruits and vegetables (filled cellular model), dairy products (combined gelled/dispersed/aerated systems model), meat (proteinous cellular network model) and bakery products (open solid foam model).

DREAM was a trans-disciplinary project with partners in nine countries. The nine assembled work packages are shown in figure 1.1. The research described in the present thesis was part of work package 2, in which we worked together with partners from INRA (Nantes and Avignon) and IFR (Norwich).

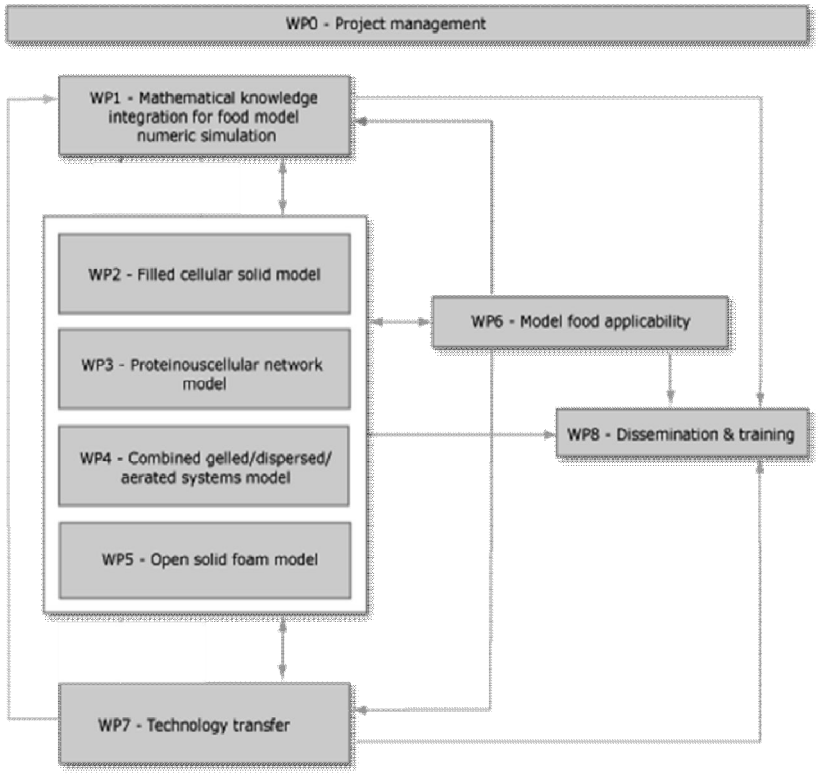


Figure 1.1: Structure of interactions in the EU 7th framework program DREAM (adapted from the DREAM proposal).

1.5 Objective and outline of the thesis

For food scientists it is an important goal to improve food quality. Food quality attributes can be differentiated in extrinsic factors, which are associated with the food, but are not part of it, like the price or brand name, and intrinsic factors, which can be found in the product itself, like the nutritional value or shelf life (van Boekel, 2009). Intrinsic factors can often be measured scientifically and can sometimes be enhanced, to achieve an improved food quality. One intrinsic factor which has gained more and more importance is the nutritional quality of foods (Verkerk et al., 2009). As GS containing foods are assumed to be cancer-preventive, an appropriate content of GSs in the prepared food is associated with an increased food quality. In this research the

fate of GSs in some selected *Brassicaceae* crops was studied, from processing to digestion. To better understand and quantify the mechanisms related to the GS content and bioaccessibility, different models were used as tools. These models aim to describe the fate of GSs in the food chain and could therefore be used to monitor and enhance food quality.

During processing the content of GSs can be substantially diminished by thermal degradation and leaching of GSs into a water phase. Much is known about the fate of GSs during processing and some studies investigated the bioavailability of GSs in humans, but a study with a chain-approach from processing to digestion was still missing in literature.

Different processes influence the fate of GSs during processing and digestion. During industrial and/or domestic processing, thermal treatment degrades GSs and inactivates the endogenous enzyme myrosinase and the presence of water combined with temperature leads to leaching of GSs into the cooking water. If myrosinase is still active, it will hydrolyze released GSs during chewing. During the whole digestion, the food components might have an influence on the bioaccessibility of GSs and their breakdown products. To investigate the mechanisms during processing is a typical approach of food technology. On the other hand the mechanisms during digestion would fall in the area of nutrition science, which is why an interdisciplinary approach was necessary.

With this global study the key points of interest for the fate of GSs could be identified and further surveyed. For this aim, different models were used as tools: an *in silico* mathematical model, an *in vitro* digestion study and an *in vivo* chewing study.

A mathematical model describing the thermal degradation of GSs and leaching of GSs into the cooking water during thermal processing of *Brassica* vegetable is described in **chapter 2**. In **chapter 3**, this model was fitted to data sets of individual GS contents in vegetable and cooking water of red cabbage, white cabbage, Brussels sprouts and broccoli. The reaction rate constants and activation energies were estimated and compared between GSs and between vegetable.

The stability and bioaccessibility of GR, SF and SFN during digestion of differently processed broccoli with and without additional meal components was studied with a static *in vitro* model (**chapter 4**). The oral phase was investigated in detail in **chapter 5**: an *in vivo* study was used to investigate the hydrolysis of GR from raw and steamed

broccoli to SF and SFN during chewing. With these studies the fate of GSs was investigated during digestion.

In **chapter 6**, the main findings of this research and their implications are summarized and discussed and recommendations for future research are given.

Chapter 2

Modelling the fate of glucosinolates during thermal processing of *Brassica* vegetables

Sarvan, I., Verkerk, R., Dekker, M. (2012) LWT-Food Science and Technology 49(2), 178-183

Abstract

Glucosinolates are secondary metabolites of *Brassica* vegetables that have been associated with health benefits. The concentrations of these compounds are strongly affected by processing of the vegetables. Various mechanisms are responsible for these changes: Lysis of plant cells and compartments, diffusion and leaching of glucosinolates and myrosinase (a plant enzyme able to hydrolyse glucosinolates) into the cooking water, enzymatic hydrolysis of glucosinolates, inactivation of myrosinase and thermal degradation of glucosinolates. This publication presents a dynamic mathematical model that includes these mechanisms with their estimated parameters and the effect of temperature on them.

Simulations made by the model for several process conditions show losses of glucosinolates as a consequence of domestic boiling of 69%, microwaving of 8%, industrial blanching of 37% and by industrial sterilisation of 82%.

The model can assist in adapting the processing conditions like the time-temperature profile and the vegetable-water ratio to optimise industrial and domestic processing of *Brassica* vegetables in terms of health benefits. In addition, this model can help to add the effect of vegetable preparation practices on the quantitative glucosinolate intake in epidemiological studies.

2.1 Introduction

Mathematical modelling can be used as a tool to describe the kinetics of quality related changes during food processing as a function of the specific processing conditions. Different type of modelling strategies can be applied, such as empirical approaches just using experimental data or mechanistic approaches in which the underlying physical, chemical and biochemical phenomena are translated into mathematical equations and experimental data is used to estimate the parameters in the model. Mechanistic models have the advantage of giving insight in the various sub-processes and allow for more flexibility and accuracy in use outside the studied experimental settings. In this paper an example of modelling of the effect of process conditions of a quality attribute is given by a case study on the concentration of beneficial components in *Brassica* vegetables (glucosinolates (GSs)).

The health effect of GSs was described by epidemiological studies, which indicate that a diet rich in *Brassica* vegetables can reduce the risk of several cancers (Steinbrecher, Nimptsch, Husing, Rohrmann, & Linseisen, 2009; Steinmetz & Potter, 1991; Verhoeven, Goldbohm, van Poppel, Verhagen, & van den Brandt, 1996). However up to now, epidemiology can only correlate protection against certain cancers or other diseases with specific vegetables, subgroups or individual components weakly (Steinbrecher, et al., 2009).

GSs are thioglucosides present in *Brassica* vegetables such as broccoli, cauliflower, radish, Brussels sprouts, mustard, turnip and cabbage. GSs co-exist with, but are locally separated from, the endogenous enzyme myrosinase (thioglucoside glucohydrolase; EC 3.2.1.147) in the intact *Brassica* plant. Upon mechanical injury of the tissue, due to e.g. chewing or cutting, the enzyme comes in contact with its substrate and hydrolyses it (Mithen, Dekker, Verkerk, Rabot, & Johnson, 2000; Verkerk et al., 2009). The products of GS hydrolysis, particularly the isothiocyanates, have been shown in animal and cell studies to act as anticarcinogens by inhibition of phase I enzymes responsible for bioactivation of carcinogens and by induction of phase II detoxification enzymes that affect xenobiotic transformations. Research is ongoing to establish the biological activities of dietary glucosinolates and breakdown products, their bioavailability and metabolism (Mithen, et al., 2000; Verkerk, et al., 2009).

Even if GSs are hydrolysed by myrosinase to its bioactive break down products, GSs in food may also be hydrolysed to isothiocyanates by the gut flora (Conaway et al., 2000; Getahun & Chung, 1999; Rouzaud, Young, & Duncan, 2004; Shapiro, Fahey, Wade, Stephenson, & Talalay, 1998). It is important not only to know about the GSs and active myrosinase content in food, but also what influences the different steps of processing have on enzyme, substrate and their bioactive breakdown products. A large number of *Brassica* vegetables are cooked before consumption, and the amounts of glucosinolates and active myrosinase are usually reduced considerably in heat treated vegetables.

The effects of different ways of industrial as well as domestic processing was estimated in previous research and an alteration of the concentration of the component of interest in foods to approx. 5-10 fold each was found, but if cultivar/cultivation differences were included in the total variability in the same vegetable at the moment of consumption the concentration can alter up to a factor of 100 (Dekker, Verkerk, & Jongen, 2000).

Different studies have shown strong effects of cooking *Brassica* vegetables on the levels of GSs (Jiao, Yu, Hankin, Low, & Chung, 1998; Mullin & Sahasrabudhe, 1978; Rosa & Heaney, 1993; Volden, Wicklund, Verkerk, & Dekker, 2008). These studies emphasize the importance of assessing the intake of GSs after processing prior to consumption. Therefore, assessment of accurate dietary intake of GSs can play a crucial role in understanding their connection with reduced cancer risks.

The knowledge about effects of the complete production chain on the GSs content in a food can be used industrially to optimise the production chain with respect to nutritional quality. Furthermore epidemiological studies can possibly be improved by estimating GS intake from product intake data by correcting for the effect on the concentration of GSs by different steps in the food production chain. The aim of this publication is to describe a realistic dynamic mathematical food model of the fate of GSs as well as myrosinase in vegetable tissue and cooking water during thermal processing at various temperature-time profiles.

2.2 Materials and Methods

Experimental data to estimate the parameters for thermal degradation was taken from Oerlemans et al (2006) and for leaching as well as for myrosinase kinetics from Verkerk (2002). In 2.2.1 till 2.2.5 the methods are described in short.

2.2.1 Thermal degradation

Samples of red cabbage were microwaved as described by Verkerk (2004) to inactivate the myrosinase, frozen with liquid nitrogen and ground into powder. A small amount of fresh material was frozen immediately with liquid nitrogen and ground to define the GS pattern in the initial material.

5 g of frozen material was filled in sealed 15 ml tubes and placed in a heating block for different times (0-360 min) at 80, 90, 100, 110, 118, 120 and 123 °C. The temperature of the samples was measured with thermocouples placed in the tubes. After the heat treatment the samples were extracted as described in 2.2.5. (Oerlemans, et al., 2006)

2.2.2 Leaching

Red cabbage was cut into pieces of approximately 1 cm^2. 150 g material was placed in 800 ml beakers and 400 ml of hot tap water was added. The beakers were placed in a water bath at 40, 60, 80 and 100 °C for 10, 20, 40, and 60 min. After incubation the samples were cooled on ice and the vegetables were separated from the cooking water. Leaching of cell contents was determined by conductivity measurement at 23 °C (Verkerk, 2002).

2.2.3 Myrosinase activity

Red cabbage juice was prepared with a juice centrifuge. The endogenous GSs were removed by incubation for 1 h at 40 °C. Part of the juice was incubated at 100 °C for 15min to inactivate the containing endogenous myrosinase and to use this juice for dilution purposes.

The GSs free cabbage juice was equilibrated at 20, 40, 60 and 80 °C. Hydrolysis was started by adding 1 ml of 6 mol/L sinigrin standard and stopped by addition hot

methanol. The remaining sinigrin was measured by HPLC as described in 2.1.5 and the rate of hydrolysis calculated (Verkerk, 2002).

2.2.4 Inactivation of myrosinase by temperature

5 g GS-free red cabbage juice was incubated at 20, 40, 60 and 80 °C for 0, 5, 10 and 20 min. To determine the inactivation of myrosinase the hydrolysis was started by adding 1 ml sinigrin standard (6 mol/L, dissolved in water). This method was adapted from Oerlemans et al (2006). The reaction was stopped by adding hot methanol. The juice was centrifuged (5000 x g for 10 min at room temperature) and remaining sinigrin was isolated from the collected supernatant and analyzed by HPLC as described in 2.2.5. The rate of hydrolysis was calculated from the remaining intact sinigrin (Verkerk, 2002).

2.2.5 Glucosinolate analysis

Glucosinolates present in the fresh cabbage were analyzed using high performance liquid chromatography (HPLC) following on-column desulphation as described by Verkerk et al (2001). Glucotropholin (3 mol/L, in water) was added as internal standard since it is not present in the explored vegetables.

To determine the thermal degradation of GSs in the water phase, the cooking water from the experiment described in 2.2.2 was used. To 30 ml of cooking water 1 ml of glucotropaeolin standard (3 mol/L, in water) was added. 2 ml of this solution was placed on a DEAE sephadex column as described by Verkerk (2001) without further extraction.

2.2.6 Determination of parameters and simulations

Parameter estimations were taken from literature (Oerlemans, et al., 2006; Verkerk, 2002) and are summarised in table 3. In short modelling reaction kinetics and estimating parameters was done by integral fitting of the data sets using the determinant criterion (Stewart, Caracotsios, & Sorensen, 1992). Different temperature-time datasets were modelled simultaneously.

The software package Athena Visual Workbench (www.athenavisual.com) was used for numerical integration of differential equations as well as parameter estimation of

the rate constants in the differential equations following minimization of the determinant in order to obtain the parameters.

Simulations of various processes with specific temperature-time profiles were done by the same software package by using this profile and the estimated parameters as inputs.

2.3 Model description

Different mechanisms take place during cooking, which are schematically shown in figure 2.1.

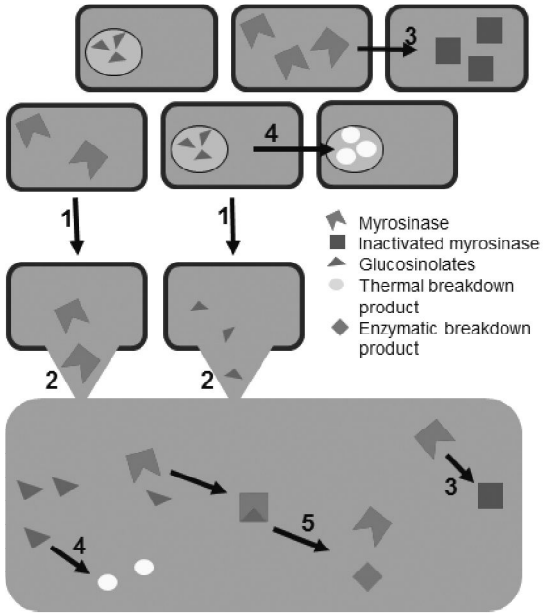


Figure 2.1: Schematic illustration of the most important processes determining the fate of glucosinolates during cooking (1. cell lysis, 2. leaching, 3. enzyme inactivation, 4. GS thermal degradation, 5. enzyme hydrolysis). After: (Dekker, et al., 2000)

The heat will damage the cell walls and membranes of vegetable cells and compartments (lysis) leading to leaching of the cell contents. The cellular content of both myrosin cells and cells containing GSs in their vacuoles will be distributed between the volume of lysed cells within the plant tissue and the cooking water forming one continuous water pool. If the myrosinase is still active, enzymatic

hydrolysis can take place in this water pool. Myrosinase will hydrolyse GSs into breakdown products. At higher temperatures the myrosinase will inactivate, either in the cooking water or in the still intact cells. In addition the GSs can be degraded thermally either in cooking water or in the intact cells.

2.3.1 Translation into mathematical equations

To build a model of the fate of GSs during cooking, the mechanisms, as shown in figure 2.1, have to be modelled separately and finally linked together into one processing model. The mechanisms were divided into thermal degradation of GSs and inactivation of myrosinase in the intact cells as well as in the water pool, leaching of GSs and myrosinase into cooking water and enzymatic breakdown of GSs. The variables are described in table 2.1.

An overview of the different sub-processes, the required experiments and the necessary parameters are presented in table 2.2.

Table 2.1: Overview of the variables used in equation 1-14

F_i	Fraction of intact cells (-)
F_l	Fraction of lysed cells (-)
k_l	Rate constant of cell lysis (min^{-1})
M_w	Mass of free water (g) consisting of the cooking water plus the lysed cell content
$M_{v,0}$	Initial mass of vegetable (g)
$C_{g,w}$	Concentration of GSs in the free water (μmol/g)
$C_{g,v}$	Concentration of GSs in the intact part of the vegetable (μmol/g)
$C_{e,w}$	Concentrations of myrosinase in the free water phase (mg/g)
$C_{e,v}$	Concentrations of myrosinase in the vegetable tissue (mg/g)
$k_{d,w}$	Breakdown rate constant of GSs in the free water pool (min^{-1})
$k_{d,v}$	Breakdown rate constant of GSs in vegetable (min^{-1})
$k_{d,e,w}$	Rate constant of myrosinase inactivation in released water (min^{-1})
$k_{d,e,v}$	Rate constant of myrosinase inactivation in intact cells (min^{-1})
k_a	Rate constant of maximum enzyme activity (min^{-1})
K_m	Michaelis-Menten constant (µmol/g) of the myrosinase
k_x	Rate constant of reaction x (min^{-1})
$k_{x,ref}$	Rate constant at T_{ref} (min^{-1})
$E_{a,x}$	Apparent activation energy of reaction x (kJ/mol)
R	Gas constant (kJ/mol.K)
T_{ref}	Reference temperature is set at 373 K for the cell lysis and GSs degradation rates and at 313 K for the enzyme activity and inactivation rate
T	Absolute temperature (K)
C_{GS}	Concentration of GSs in the whole vegetable (in the lysed part as well as in the intact tissue part)
$\|L$	Change of GSs or myrosinase due to leaching
$\|b$	Change of GSs or myrosinase due to thermal breakdown
$\|a$	Change of GSs due to enzymatic hydrolysis
$\|d$	Changes of myrosinase activity due to thermal inactivation

Table 2.2: Schematic representation of the experimental set-up for the parameter estimation

Experiment	Range of time and temperatures	Analysis	Parameters	Equations
Cell lysis/ leaching[a]	$T_{313\text{-}373K}$ $t_{0\text{-}60\ min}$	Conductivity in cooking water	k_l $E_{a,l}$	(1, 2, 3, 4, 5)
Enzyme activity[b]	$T_{293\text{-}353K}$ $t_{0\text{-}20\ min}$	Hydrolysis of GS	k_a K_m $E_{a,a}$	(8)
Enzyme inactivation[b]	$T_{298\text{-}343K}$ $t_{0\text{-}60\ min}$		$k_{d,e}$ $E_{a,d}$	(9, 10)
Thermal degradation[a]	$T_{353\text{-}396K}$ $t_{0\text{-}360\ min}$	Thermal degradation of GS	$k_{d,p}$ $k_{d,w}$ $E_{a,b}$	(6, 7)

[a] In red cabbage material; T = Temperature (K); t = time (min)

[b] In juiced red cabbage

2.3.2 Lysis and leaching

Based on literature (Verkerk, 2002) and former experiments in our group (not shown) cell lysis can be described by a first order equation (Eq. 2.1). It is assumed that cells containing GSs or myrosinase have the same leaching behaviour and rate, which is the reason why only one rate constant k_l is used. The fraction of lysed cells is calculated from the balance of the fractions of intact and lysed cells (Eq. 2.2).

$$\frac{dF_i}{dt} = -k_l \cdot F_i \qquad (2.1)$$

$$F_l = 1 - F_i \qquad (2.2)$$

It is assumed that the contents of lysed cells will form one continuous phase with the cooking water. This water phase is called "free water phase" and is composed of the cooking water together with the content of the lysed cells in the vegetable tissue (the volume of the latter phase will continue to increase as more cells are lysing). The change in the amount of this combined free water phase is described by equation 2.3.

$$\frac{\mathrm{d}M_w}{\mathrm{d}t} = -\frac{\mathrm{d}F_i}{\mathrm{d}t} M_{v,0} = k_l \cdot F_i \cdot M_{v,0} \tag{2.3}$$

Diffusion processes are assumed to be much quicker than the lysis process as long as small *Brassica* parts are processed (e.g. cut cabbage leaves). Therefore leaching of GSs and myrosinase is the direct consequence of this cell lysis. For larger pieces of vegetables (e.g. broccoli and Brussels sprouts) this assumption might not be valid. For these vegetables the model can still be used, but the estimated lysis rate constant will then be an apparent rate constant in which the diffusional effects of leaching are lumped. The GSs and myrosinase content of the lysed cells is added to the total free water phase. No differences in leaching behavior of individual GSs are expected. Based on these assumptions, leaching of GSs and myrosinase from the lysed cells is described by equations 2.4 and 2.5, respectively, considering both the leaching and the dilution due to the increasing volume of the free water phase.

$$\frac{\mathrm{d}C_{g,w}}{\mathrm{d}t}|L = \left(\frac{k_l \cdot F_i \cdot M_{v,0} \cdot C_{g,v}}{M_w}\right) - \left(\frac{k_l \cdot F_i \cdot M_{v,0} \cdot C_{g,w}}{M_w}\right) = \frac{k_l \cdot F_i \cdot M_{v,0}\left(C_{g,v} - C_{g,w}\right)}{M_w} \tag{2.4}$$

$$\frac{\mathrm{d}C_{e,w}}{\mathrm{d}t}|L = \left(\frac{k_l.F_i \cdot M_{v,0} \cdot C_{e,v}}{M_w}\right) - \left(\frac{k_l \cdot F_i \cdot M_{v,0} \cdot C_{e,w}}{M_w}\right) = \frac{k_l \cdot F_i \cdot M_{v,0}\left(C_{e,v} - C_{e,w}\right)}{M_w} \tag{2.5}$$

2.3.3 Thermal degradation

Thermal degradation of GSs and myrosinase during cooking can occur in the intact vegetable tissue or after leaching in the free water phase. Thermal degradation of GSs was found to be described well by a 1st order equation (Oerlemans, et al., 2006). Breakdown rates could be different in vegetable and cooking water that is why different rate constants are used (Eq. 2.6 and 2.7):

$$\frac{dC_{g,v}}{dt}\Big|b = -k_{d,v} \cdot C_{g,v} \tag{2.6}$$

$$\frac{dC_{g,w}}{dt}\Big|b = -k_{d,w} \cdot C_{g,w} \tag{2.7}$$

2.3.4 Enzymatic degradation of GSs

The hydrolysis of GSs by myrosinase is described by the Michaelis-Menten equation (Eq. 2.8).

$$\frac{dC_{GS}}{dt}\Big|a = -\frac{k_a \cdot C_{e,w}}{\frac{K_m}{C_{GS,w}}+1} \tag{2.8}$$

2.3.5 Myrosinase inactivation

Inactivation of myrosinase due to heat treatment can be described with several different models, such as 1st order equation, consecutive step model and the distinct isozyme model (Ludikhuyze, Ooms, Weemaes, & Hendrickx, 1999; van Boekel, 2009; van Eylen, Oey, Hendrickx, & Van Loey, 2007). In literature, inactivation of myrosinase from broccoli showed the best fit with the consecutive step model (Ludikhuyze, et al., 1999). It specifies two irreversible consecutive reactions. The used data did not support the estimation of two reaction rate constants, therefore the 1st order equation was used to describe the inactivation of myrosinase. Using the 1st order

equation reduces the amount of parameters in the model and simplicity is one of the aims of modelling (principle of parsimony) (van Boekel, 2009) .

The 1st order reaction for the inactivation of myrosinase in the intact cells and the free water phase is described in equation 2.9 and 2.10 respectively.

$$\frac{dC_{e,v}}{dt}\Big|d = -k_{d,e,v} \cdot C_{e,v} \tag{2.9}$$

$$\frac{dC_{e,w}}{dt}\Big|d = -k_{d,e,w} \cdot C_{e,w} \tag{2.10}$$

2.3.6 Temperature effects and integration of mechanisms

Lysis, leaching, thermal degradation of GSs and myrosinase as well as enzymatic hydrolysis of GSs by myrosinase are temperature dependent reactions. The rearranged Arrhenius-like equation is used to describe the temperature dependency of all the reaction rate constants (Eq. 2.11). By introducing T_{ref} it is possible to use data points of different temperatures for estimating a rate constant of the reaction x, assuming E_a not to be temperature dependent (van Boekel, 2009). The Arrhenius equation can only be used on simple elementary reactions. If it is used for complex reactions such as in this case, the activation energy is a collective of several activation energies and should therefore be considered as an apparent activation energy.

$$k_x = k_{x,ref} \cdot e^{\frac{E_{a,x}}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)} \tag{2.11}$$

For the degradation of GSs during cooking in intact vegetable tissue only thermal degradation occurs as described in equation (Eq. 2.6). To describe the changes of GSs in the free water phase the equations of leaching (Eq. 2.4), thermal degradation (Eq. 2.7) and enzymatic degradation (Eq. 2.8) were combined into one equation (Eq. 2.12)

$$\frac{dC_{g,w}}{dt} = \frac{k_l F_i M_{v,0}(C_{g,v} - C_{g,w})}{M_w} - k_{d,w} C_{g,w} - \frac{k_a C_{e,w}}{1 + \frac{Km}{C_w}} \quad (2.12)$$

The change in active myrosinase concentration in the vegetable tissue is only changing by the inactivation reaction (Eq. 2.9). In the free water phase the changes are due to both leaching (Eq. 2.5) and inactivation (Eq. 2.10) and are combined in one equation (Eq. 2.13).

$$\frac{dC_{e,w}}{dt} = \frac{k_l C_{c,i} M_{v,0}(C_{e,v} - C_{e,w})}{M_w} - k_{d,e} C_{e,w} \quad (2.13)$$

The overall concentration of GSs in the total vegetable tissue consists of two parts: the intact vegetable cells and the lysed cells containing the concentrations of the free water pool. To calculate this overall concentration in the vegetable tissue equation 2.14 is used.

$$C_{GS} = C_{g,w} \cdot C_l + C_{g,v} \cdot (1 - C_l) \quad (2.14)$$

2.4 Simulations and discussion

2.4.1 Model predictions after different processes of heat treatment

The proposed model of the fate of GSs during thermal processing was applied to estimates of red cabbage, taken from literature (Oerlemans, et al., 2006; Verkerk, 2002). Exemplary the parameters of sinigrin were chosen for use in the simulation, after recalculation to a reference temperature of 100 ºC, and are summarized in table 2.3. In this simulation the rate constants of the inactivation of sinigrin in the vegetable and in the cooking water $k_{d,v}$ and $k_{d,w}$ were assumed to be equal.

Table 2.3: Overview of the parameter estimation of the different cooking mechanisms from literature ([a]Oerlemans, Barrett, Suades, Verkerk & Dekker, 2006; [b]Verkerk, 2002)

Mechanism	Parameters	
Cell lysis[2,b]	k_l	0.11 min^{-1}
	$E_{a,l}$	53 kJ/mol K
Myrosinase activity[1,b]	k_a	0.55 min^{-1}
	$E_{a,a}$	32 kJ/mol·K
Myrosinase inactivation[1,b]	$k_{d,e,v}$ [3]	6.78 * 10^{-3} min^{-1}
	$E_{a,a}$	155 kJ/mol·K
Thermal degradation[2,a]	$k_{d,v}$ [3]	2.34 * 10^{-3} min^{-1}
	$E_{a,b}$	190 kJ/mol K

[1] at 313 K

[2] at 373 K

[3] $k_{d,w}$ and $k_{d,e,w}$ are assumed to be equal to respectively $k_{d,v}$ and $k_{d,e,v}$.

Figure 2.2A-D shows simulations of sinigrin and myrosinase concentrations in red cabbage and processing water during different thermal processes of food preparation such as cooking, in-can sterilization, blanching and microwaving. Heating up and cooling down periods were taken into account.

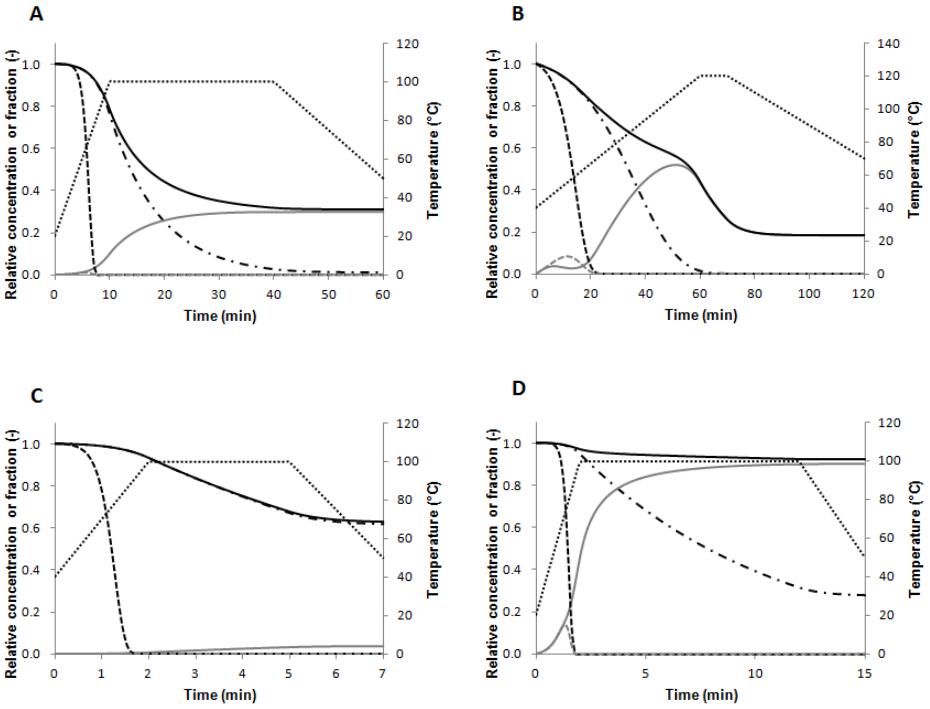


Figure 2.2: Simulation of the changes of sinigrin in vegetable (black continuous line) and water (grey continuous line), enzyme activity in the vegetable (black dashed line) and cooking water (grey dashed line), and cell lysis (dashed and dotted line) during A: domestic cooking (vegetable-water ratio of 1:2), B: in-can sterilization (vegetable-water ratio of 2:1), C: blanching (vegetable-water ratio of 1:9) and D: microwaving (vegetable-water ratio of 20:1) of red cabbage. The temperature profile is shown in dotted lines.

2.4.1.1 Domestic cooking

For this model the time of domestic cooking was estimated to be 30 min at 100 ºC with a heating up time of 10 min and a cool down phase of 20 min. The vegetable-water ratio was defined to be 1:2. As can be seen in figure 2.2A the sinigrin content reduces to 31% of its initial amount after processing. After the heating up time still approximately 80% sinigrin is present in the vegetable, but will leach into the cooking water continuously. The myrosinase is already inactivated after 10 min, before the desired temperature was reached.

2.4.1.2 In-can sterilization

For the simulation of in-can sterilization a total time of 120 minutes was estimated. The heating up time of the can from 40 ºC to 120 ºC was set to 60 min, for 10 min the temperature would be kept constant, followed by a cooling down phase of 50 min (Fig. 2.2B). Till the temperature peak is reached, approximately 60% of sinigrin in the vegetable and processing water are broken down. After the completed sterilization only approximately 18% of sinigrin is present in the vegetable. The myrosinase is inactivated after 20 min in processing water and red cabbage.

2.4.1.3 Blanching

Blanching was described by a vegetable-water ratio of 1:9 (Fig. 2.2C). After a short warming up time of 2 min and constant temperature of 100 ºC for 3 min and a 2 min cooling down phase, the sinigrin is predicted to be reduced to 63% of its initial concentration. With increasing water volume higher losses due to leaching are expected. The myrosinase is inactivated before 2 min of processing.

2.4.1.4 Microwaving

During microwaving (Fig. 2.2D) for 10 min with a warming up time of 2 min and a cooling down phase of 3 min and a vegetable-water ratio of 20:1 the concentration of sinigrin in the vegetable and processing water is diminished by approximately 8% of its initial amount. Compared to the other modeled processing methods, these are the lowest losses. After 2 minutes of processing all myrosinase is inactivated.

2.4.2 Myrosinase inactivation

The processes simulated in figure 2.2 are at high temperatures, which is why myrosinase will be inactivated rather fast. Till the time point when myrosinase is inactivated, most of the cells are still intact, which stops the enzyme from getting in contact with its substrate. Therefore it is assumed that enzymatic hydrolysis plays only a minor role during these processes.

This fits with the experiment of Yen and Wei (1993), who measured the inactivation of myrosinase from red cabbage and found that it is more stable than myrosinase from white cabbage but both were reduced by 90% after heating at 70 °C for 30 min.

2.5 Conclusion

This publication proposes a model of the fate of GSs during heat treatment. Because of the fast inactivation of myrosinase while most of the cells are still intact, only a very small amount of enzymatic breakdown products are expected. Even so, intact GSs can also be hydrolyzed to a smaller extent by the human gut flora (Conaway, et al., 2000; Getahun & Chung, 1999; Rouzaud, et al., 2004; Shapiro, et al., 1998). Therefore an optimized process would not only contain as much active myrosinase but also as much intact GSs as possible. The simulations of different processing methods show higher sinigrin losses in the vegetable tissue after in-can sterilization than after domestic cooking, microwaving or blanching. For a more accurate prediction of the fate of GSs a complete data set of one batch of a *Brassica* vegetable cultivar at different times and temperatures for all mechanisms taking place during cooking including standard deviations would be preferable. Possible differences in the losses of distinct GSs could be investigated as well. Furthermore a validation of the proposed model with a different data set should follow. It could be also interesting to compare different *Brassica* vegetables.

This mathematical model can be used as a tool for the industry to optimize processes in terms of GSs or to improve the design of epidemiological studies.

Acknowledgement

We thank Prof. Dr. Tiny van Boekel for critical reading the manuscript. The research leading to these results has received funding from the European Community's Seventh Framework Program (FP7/2007-2013) under the grant agreement nºFP7-222 654-DREAM.

Chapter 3

Comparison of the degradation and leaching kinetics of glucosinolates during processing of four *Brassicaceae* (broccoli, red cabbage, white cabbage, Brussels sprouts)

Sarvan, I., Verkerk, R., van Boekel, M. A. J. S., Dekker, M. (2014) Innovative Food Science and Emerging Technologies 25, 58–66

Abstract

Glucosinolates (GSs) are secondary metabolites of *Brassica* vegetables that are associated with health benefits. The concentrations of these compounds are strongly affected by processing of the vegetables.

During thermal treatment of *Brassicaceae*, such as domestic cooking, different mechanisms affecting the content of GSs can take place and were modelled in the present study: Lysis of plant cells and compartments, leaching of GSs into the cooking water and thermal degradation of GSs in both the intact vegetable tissue and in the cooking water. These mechanisms were described mathematically and the model parameters for broccoli, Brussels sprouts, red cabbage and white cabbage were estimated based on experimental results. Differences between the thermostability of the same GSs originating from different *Brassicaceae* could be detected, as well as differences between the thermostability of the same GSs in the vegetable matrix compared to that in the cooking water.

This mathematical model and the estimated parameters can be used to simulate the different glucosinolate contents in prepared foods considering the processing method. This should be a useful tool in food research and industry to make predictions about the nutritional quality of foods and to optimize their health related quality attributes.

3.1 Introduction

A diet rich in *Brassicaceae* has been shown to reduce the risk of cancer in epidemiological studies (Steinbrecher et al., 2009; Verhoeven et al., 1996). The beneficial health effect is supposed to be induced by enzymatic breakdown products of glucosinolates (GSs), which are secondary plant metabolites especially rich in *Brassicaceae*. GSs are β-thioglucoside-N-hydroxy sulphates, which are sulfur-linked to a β-D-glucopyranose moiety, and with an indolic, aliphatic or aromatic side chain characterizing the molecule.

Enzymatic degradation can take place either by rupture of cells, e.g., during chewing or cutting, so that the locally separated endogenous enzyme myrosinase (β-thioglucosidase, EC 3.2.1.147) can get in contact with the GSs, or by hydrolysis in the human intestinal flora (Rouzaud, 2004; Shapiro et al., 1998; Shapiro, Fahey, Wade, Stephenson, & Talalay, 2001). If GSs are hydrolysed, an instable intermediate product occurs that is transformed to nitriles, thiocyanates, epithionitriles, oxazolidines or isothiocyanates, depending on its environmental conditions of the reaction. Isothiocyanates are assumed to cause the beneficial health effects after regular *Brassicaceae* consumption.

During processing of *Brassicaceae* for food preparation the most commonly used technique is cooking. During cooking, three mechanisms affecting the GSs can take place at the same time: lysis of cells and leaching of the cellular contents, enzymatic hydrolysis of GSs and thermal degradation. Intracellular components such as GSs and myrosinase can leach in the cooking water. The ratio of vegetable to cooking water and the size of the vegetable pieces will affect the rate and final amount of leaching. If active myrosinase and GSs are present in the cooking water, myrosinase may hydrolyse its substrate resulting in the formation of nitriles, thiocyanates, epithionitriles, oxazolidines or isothiocyanates. At higher temperature, myrosinase can be inactivated by heat (Van Eylen, Oey, Hendrickx, & Loey, 2008) and GSs can be thermally degraded (Oerlemans, Barrett, Suades, Verkerk, & Dekker, 2006; Sarvan, Verkerk, & Dekker, 2012; Verkerk, Knol, & Dekker, 2010).

The degradation of GSs during processing is widely studied, but the linkage between the different degradation mechanisms in a mathematical model was only proposed recently (Sarvan et al., 2012). In that study the parameter estimations were taken from different experiments described in literature. But a study of the differences between

the parameter estimates during processing of the same cultivars is lacking. As has been shown by Dekker et al. (2009) and Hennig et al. (2012), differences in degradation rate constant and activation energy can be found between *Brassicaceae* cultivars and cultivation years. In these studies, only the thermal degradation in the vegetable was investigated. No information on the lysis and leaching kinetics and the thermal degradation in the cooking water during processing for different *Brassica* cultivars has been reported in literature until now.

In this paper a new dataset was used with one batch of material for thermal degradation and leaching experiments. Thermal degradation was studied separately to define these parameter more accurately, and were then used in the overall process model to determine the parameter of leaching. Such a model could help to understand the differences in thermal degradation as well as losses of GSs due to leaching between different *Brassica* vegetables. Furthermore, simulations of different processes could be used by the industry to forecast the GSs content in the ready to eat product and help to optimize processing steps in terms of GSs.

3.2 Materials and methods

3.2.1 Materials

Broccoli (*Brassica oleracea var. italic var. sirtaki*) was purchased from a local grower in Raamsdonk, Netherlands, while Brussels sprouts (*Brassica oleracea var. gemmifera var. Franklin*), red cabbage (*Brassica oleracea var. capitata f. rubra var. Pesara*) and white cabbage (*Brassica oleracea L. var. capitata L. f. alba DC var. Mandy*) were kindly provided by Bejo Zaden B.V., Warmenhuizen, Netherlands. Brussels sprouts, red cabbage and white cabbage were harvested in autumn 2010 from the same field, while broccoli was harvested in spring 2011.

Methanol and acetonitrile were purchased from Biosolve (Valkenswaard, The Netherlands). Sodiumacetate anhydrous, tetramethylammoniumchloride and acetic acid were used from Merck (Darmstadt, Germany). DEAE Sephadex A-25, sulfatase from helix pomatia were delivered by Sigma-Aldrich (Zwijndrecht, The Netherlands). Glucotropaeoline was purchased from the Laboratory of Biochemistry, Plant Breeding and Acclimatization Institute (Radzikow, Blonie, Poland).

3.2.2 Methods

3.2.2.1 Sample preparation

Fresh broccoli heads were cut into florets with a 2 cm stem and mixed. From red cabbage, white cabbage and Brussels sprouts the outer leaves were removed. The heads of red cabbage and white cabbage were cut in quarters before cutting them as well as half of the Brussels sprouts into 5 mm thin slices. The material was prepared for each experimental day freshly.

3.2.2.2 Thermal degradation study

Samples of broccoli, Brussels sprouts, red cabbage and white cabbage were microwaved (microwave Daewoo, model KOC-870T) in 300 g batches for 4 min 50 s at 900 W as described by Verkerk et al. (2004) to inactivate the myrosinase, frozen with liquid nitrogen and ground into frozen powder. A small amount of fresh material was frozen immediately to define the GSs pattern in the initial material.

Frozen material (5 g) was filled in 15 mL stainless steel tubes, sealed airtight and placed in a preheated heating block for different times (0 - 150 min) at 90 °C, 100 °C, 110 °C and 120 °C. The heating time started when the desired temperature was reached. The temperature of the samples was measured with thermocouples placed in the tubes. All experiments were performed in triplicate. After the heat treatment 2 g of the samples were extracted as described in 3.2.2.4.

3.2.2.3 Leaching study

The cut material as well as whole Brussels sprouts heads were separated in 100 g batches and placed in 800 mL wide neck bottles containing 400 mL of preheated distilled water. The bottles were placed in a water bath at 50 °C, 65 °C, 85 °C and 100 °C for 0 – 180 min, respectively. After incubation, the vegetables were separated from the cooking water and weighed separately to determine the evaporation losses. All experiments were performed in triplicate using an incomplete block design. The vegetables were frozen with liquid nitrogen and ground to a frozen powder for later GSs determination as described in section 3.2.2.4. Differences in initial material due to storage between the cooking days were modelled with the software SAS (SAS Institute Inc.) and corrected for.

3.2.2.4 GLS extraction and determination by HPLC

GSs were extracted with hot methanol (70%) and analyzed using high performance liquid chromatography (HPLC) following on-column desulphation as described by Verkerk et al (2001) with the following small adjustments. The column used was a GRACE Smart RP18 with a flow rate of 1 mL/min. One run took 31 min starting with 100% eluent A (milliQ water with 0.05% tetramethylammoniumchlorid) for the first minute, then changing till 21 min to 100% eluent B (60% milliQ water 40% acetonitrile, 0.05% tetramethylammoniumchloride) and till 26 min changed again to 100% eluent A. Till the end of the run, the mobile phase consisted of eluent A. The UV spectrum was measured over the whole run time at 229 nm.

The aliphatic glucosinolate glucotropaeolin (3 mol/L, in water) was added as internal standard since it is not present in the investigated vegetables. GSs were quantified against the internal standard glucotropaeolin using relative response factors as given in ISO 9167 1:1992.

3.2.2.5 Mathematical model

The model describing the lysis/leaching and thermal degradation in the vegetable tissue and in the cooking water is described by Sarvan et al. (2012). In that model also enzymatic hydrolysis and enzyme inactivation is described. In the present study the enzymatic processes were neglected, since the temperature increase during the leaching and thermal degradation studies was so fast that no enzymatic degradation is expected. For the cooking water of 50˚C and 65˚C this could not be assumed, these measured concentrations in the cooking water were therefore neglected in the parameter estimations (table 3.1).

Table 3.1: Overview of the variables used in equation 1-8

F_i	Fraction of intact cells (-)
F_l	Fraction of lysed cells (-)
k_l	Rate constant of cell lysis (min^{-1})
M_w	Mass of free water (g) consisting of the cooking water plus the lysed cell content
$M_{v,0}$	Initial mass of vegetable (g)
$C_{g,w}$	Concentration of GSs in the free water (μmol/g)
$C_{g,v}$	Concentration of GSs in the intact part of the vegetable (μmol/g)
$k_{d,w}$	Breakdown rate constant of GSs in the free water pool (min^{-1})
$k_{d,v}$	Breakdown rate constant of GSs in vegetable (min^{-1})
k_x	Rate constant of reaction x (min^{-1})
$k_{x,ref}$	Rate constant at T_{ref} (min^{-1})
$E_{a,x}$	Apparent activation energy of reaction x (kJ/mol)
R	Gas constant (kJ/mol.K)
T_{ref}	Reference temperature is set at 373 K for the cell lysis and GSs degradation rates and at 313 K for the enzyme activity and inactivation rate
T	Absolute temperature (K)
C_{GS}	Concentration of GSs in the whole vegetable (in the lysed part as well as in the intact tissue part)
$\|L$	Change of GSs due to leaching
$\|b$	Change of GSs due to thermal breakdown

In this model cell lysis was described by a first order equation (Eq. 3.1). The fraction of lysed cells is calculated by subtracting the fraction of still intact cells from the total cells (Eq. 3.2).

$$\frac{\mathrm{d}F_i}{\mathrm{d}t} = -k_l \cdot F_i \tag{3.1}$$

$$F_l = 1 - F_i \tag{3.2}$$

The content of lysed cells is assumed to be in equilibrium with the cooking water, the sum of these volumes of water was called "free water phase" (Eq. 3.3). Therefore leaching of GSs is the direct consequence of cell lysis and the GSs content of the lysed cells is added to the total free water phase (Eq. 3.4). (Sarvan et al., 2012)

$$\frac{\mathrm{d}M_w}{\mathrm{d}t} = -\frac{\mathrm{d}F_i}{\mathrm{d}t} M_{v,0} = k_l \cdot F_i \cdot M_{v,0} \tag{3.3}$$

$$\frac{\mathrm{d}C_{g,w}}{\mathrm{d}t}|L = \left(\frac{k_l \cdot F_i \cdot M_{v,0} \cdot C_{g,v}}{M_w}\right) - \left(\frac{k_l \cdot F_i \cdot M_{v,0} \cdot C_{g,w}}{M_w}\right) = \frac{k_l \cdot F_i \cdot M_{v,0}\left(C_{g,v} - C_{g,w}\right)}{M_w} \tag{3.4}$$

As described in literature (Oerlemans et al., 2006; Volden, Wicklund, Verkerk, & Dekker, 2008), thermal degradation of GSs was expressed as a 1st order equation. Because the medium in which the reaction takes place could have an influence on the reaction, equation 3.5 described thermal degradation of GSs in the intact vegetable cells and equation 3.6 in the free water phase.

$$\frac{\mathrm{d}C_{g,v}}{\mathrm{d}t}\Big|b = -k_{d,v} \cdot C_{g,v} \tag{3.5}$$

$$\frac{dC_{g,w}}{dt}\Big|b = -k_{d,w} \cdot C_{g,w} \quad (3.6)$$

The measured concentration in the vegetable will consist of two parts: the concentration in the intact cells and the concentration in the lysed cells. This is calculated by equation 3.7.

$$C_{GS} = C_{g,w} \cdot C_l + C_{g,v} \cdot (1 - C_l) \quad (3.7)$$

To describe the temperature dependency of lysis, leaching and thermal degradation reactions, a rearranged Arrhenius equation is used, although it is acknowledged that these reactions are not simple chemcial reactions for which the Arrhenius equation is derived (van Boekel, 2009)(Eq. 3.8). By combining the temperature dependency equation with the rate equations, it is possible to use all the data points of different temperatures for estimating both the reference rate constants and the activation energies. E_a is the collective of several activation energies and should therefore be considered as an apparent activation energy. Both, k_{ref} and E_a describe the velocity of a degradation curve according to the Arrhenius equation. The k_{ref} defines the rate at a reference temperature, the E_a defines the change in this rate when the temperature changes. Because the k_{ref}'s are compared at a reference temperature (100° C), a smaller k_{ref} means a higher thermostability of a GSs.

$$k_x = k_{x,ref} \cdot e^{\frac{E_{a,x}}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)} \quad (3.8)$$

The detailed rationale behind these equations is described elsewhere (Sarvan et al., 2012).

The parameters were estimated from the experimental data of each individual GS in each vegetable type. In short, estimating parameters was done by integral fitting of the data sets of all time-temperature combination for each specific GS in each vegetable type. The data of the GS concentration in the vegetable and in the water was used

simultaneously in a multi response model. To correct for the effect of correlations between the responses, minimization of the determinant criterion was used to optimise the parameter estimates (Stewart, Caracotsios & Sorensen, 1992). By minimization of the cross-products considering all responses of the determinant matrix, maximum likelihood estimates of the parameters are archived (van Boekel, 1996). The software package Athena Visual Workbench (www.athenavisual.com) was used for numerical integration of differential equations as well as parameter estimation of the rate constants in the differential equations following minimization of the determinant in order to obtain the parameters as described by Sarvan et al. (2012).

For the estimation of the parameters of the degradation in the vegetable, the raw data of all four temperatures were modelled simultaneously with a first order equation (Sarvan et al., 2012) and the rate constant of glucosinolate degradation in vegetable (k_{dv}) as well as the activation energy (E_{a_kdv}) at the reference temperature of 100 °C were estimated.

The data set of the leaching study contained the GSs concentrations of broccoli, Brussels sprouts, red cabbage and white cabbage after different incubation times at four temperatures (50 °C, 65 °C, 85 °C and 100 °C) in the vegetable and at two temperatures (85 °C and 100 °C) in the cooking water. The complete data set was modelled simultaneously, in which the parameter estimates of the thermal degradation (k_{dv} and E_{a_kdv}) were used from the thermal degradation study and set as fixed constants. Parameters estimated by the leaching study were the rate constants of GSs degradation in the cooking water (k_{dw}) and of the leaching kinetics (k_l) as well as their associated activation energies (E_{a_kdw} and E_{a_kl}, respectively). Initial estimates of parameters used for the estimation procedure were based on previous results (Oerlemans et al., 2006; Sarvan et al., 2012; Verkerk, Dekker, & Jongen, 2001).

3.2.2.6 Statistical analysis

The parameter estimates obtained with Athena Visual Workbench as described in Section 3.2.2.5 consist of the estimated values and their confidence intervals. To determine whether or not the estimated parameters are significantly different between the different *Brassicaceae* studied, the method based on Julious (2004) is used.

First, the standard errors of the parameter estimates are calculated from the confidence intervals. The test statistic for the difference between two means is then

based on the square root of the sum of squares of these standard errors using an α of 5%.

3.3 Results and discussion

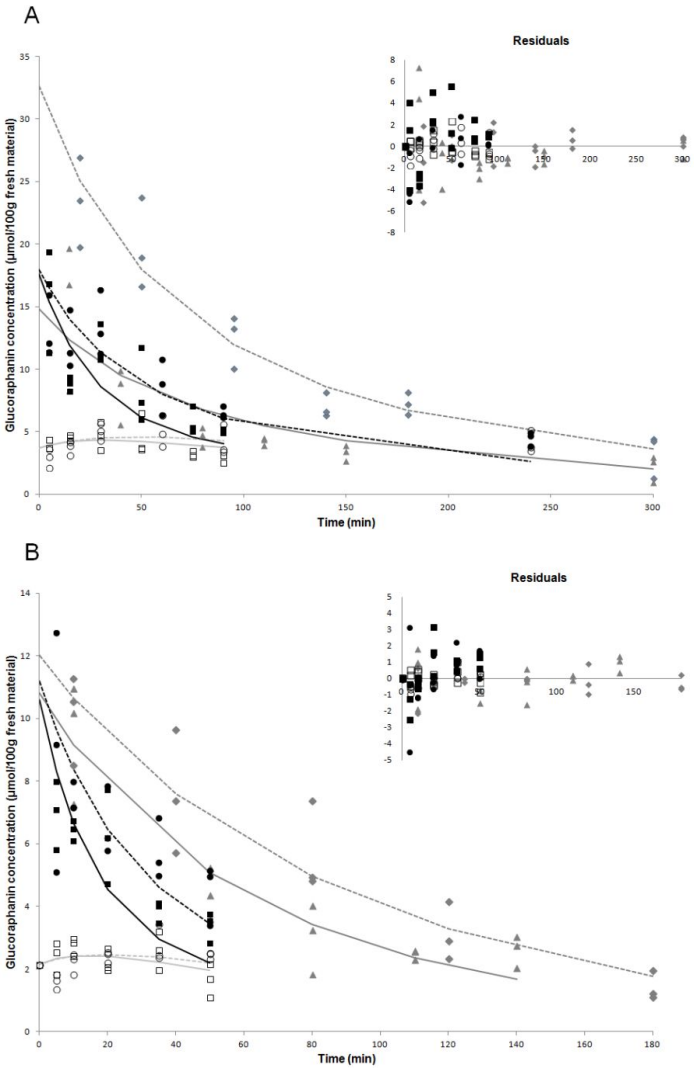
All parameter were estimated from experimental data of different *Brassicaceae* under constant heat treatment (Tab. 3.2). As an example, the models of glucoraphanin and its residuals are shown in figure 3.1. For the model of each cultivar one batch of vegetable was used, which was grown in the same field and sawn and harvested at the same time. In a different year, for a field with different environmental conditions or for a different cultivar it cannot be assumed that the estimates would be the same (Hennig et al., 2012). Nevertheless it was shown that the model can be used to describe the reactions of GSs during thermal treatment and the differences between the parameter of various cultivars could be compared.

Table 3.2: Parameter estimates of glucosinolates from broccoli, Brussels sprouts, red cabbage and white cabbage at the reference temperature of 100°C: rate constant vegetable (k_{dv}), rate constant cooking water (k_{dw}), rate constant for leaching (k_l), activation energy vegetable (E_{a_kdv}), activation energy cooking water (E_{a_kdw}), activation energy for leaching (E_{a_kl}), estimated initial concentration in vegetable incubated at 50°C (C_{GS_50}), 65°C (C_{GS_65}), 85°C (C_{GS_85}) and 100°C (C_{GS_100}) and estimated initial concentration of glucosinolates in the cooking water ($C_{CW,initial}$).

	$k_{d,v}$	$k_{d,w}$	k_l	$Ea_{kd,v}$	$Ea_{kd,w}$	Ea_{kl}	C_{GS_50}	C_{GS_65}	C_{GS_85}	C_{GS_100}	$C_{CW,initial}$
						Broccoli					
Raphanin	0.0128	0.0041	0.0194	106.63	0.00	3.79	32.67	14.85	17.95	17.59	3.07
st. dev.	0.0010	0.0005	0.0095	7.88	0.00	10.96	2.63	2.77	2.15	2.34	0.48
Glucobrassicin	0.0182	0.0024	0.2410	95.80	0.20	65.84	5.33	5.98	5.70	4.76	0.54
st. dev.	0.0008	0.0011	0.1555	4.64	13.97	15.70	0.48	1.10	1.57	1.62	0.40
4-methoxy-Glucobrassicin	0.0137	0.0072	0.1780	95.70	14.43	44.63	3.37	3.47	1.86	1.97	0.19
st. dev.	0.0013	0.0037	0.1911	10.46	12.30	25.53	0.60	0.99	0.98	1.05	0.25
NeoGB	0.0152	0.0032	0.8366	73.30	12.99	86.85	12.37	13.97	17.51	14.88	0.00
st.dev.	0.0008	0.0014	0.3064	5.13	14.26	8.84	1.25	2.57	1.21	1.21	0.00

	$k_{d,v}$	$k_{d,w}$	k_l	$Ea_{kd,v}$	$Ea_{kd,w}$	Ea_{kl}	C_{GS_50}	C_{GS_65}	C_{GS_85}	C_{GS_100}	$C_{CW,\ initial}$
Brussels sprouts											
Progoitrin	0.0198	0.0105	0.0396	59.70	0.00	21.58	95.89	90.61	88.44	83.08	10.96
st. dev.	0.0040	0.0021	0.0106	22.69	0.00	6.39	3.99	7.57	6.71	7.55	1.47
Sinigrin	0.0152	0.0082	0.0309	18.70	0.00	31.30	108.86	102.35	101.92	83.67	11.54
st. dev.	0.0019	0.0024	0.0097	14.69	0.00	8.23	4.40	8.35	7.25	8.05	1.53
Raphanin	0.0244	0.0096	0.0327	67.30	0.00	17.01	12.01	10.81	11.19	10.59	2.16
st. dev.	0.0083	0.0027	0.0172	38.58	0.00	12.57	0.83	1.53	1.35	1.51	0.29
Napin	0.0240	0.0114	0.0317	62.50	0.00	19.64	56.01	53.15	55.79	49.24	5.71
st. dev.	0.0043	0.0034	0.0116	20.20	0.00	8.67	2.84	5.57	5.04	5.70	1.05
4-OH-Glucobrassicin	0.0385	0.0282	0.0286	97.20	20.91	0.00	4.25	3.11	6.38	4.66	0.68
st. dev.	0.0047	0.0108	0.0077	14.05	10.34	0.00	0.65	1.03	0.73	0.91	0.16
Glucobrassicin	0.0249	0.0118	0.0621	48.70	0.00	35.85	72.43	74.79	89.58	67.02	12.77
st. dev.	0.0041	0.0026	0.0263	18.98	0.00	11.04	4.44	9.67	10.06	10.94	2.22
4-methoxy-Glucobrassicin	0.0420	0.0099	0.0478	79.80	0.00	24.74	96.08	93.86	95.90	101.88	9.95
st. dev.	0.0100	0.0021	0.0146	27.30	0.00	7.27	4.37	8.47	8.29	9.75	1.88
Red cabbage											
Progoitrin	0.0123	0.0153	0.0437	74.40	0.00	12.59	57.19	42.11	43.79	54.11	2.89
st. dev.	0.0010	0.0030	0.0098	7.24	0.00	5.59	3.40	5.32	4.14	4.81	0.96
	$k_{d,v}$	$k_{d,w}$	k_l	$Ea_{kd,v}$	$Ea_{kd,w}$	Ea_{kl}	C_{GS_50}	C_{GS_65}	C_{GS_85}	C_{GS_100}	$C_{CW,}$
Sinigrin	0.0145	0.0156	0.0348	77.40	0.00	10.79	43.21	30.23	32.30	36.16	2.21
st. dev.	0.0012	0.0038	0.0095	7.81	0.00	6.58	2.48	3.97	3.15	3.64	0.71
Raphanin	0.0162	0.0128	0.0309	65.20	0.00	5.68	64.93	46.04	44.86	52.26	4.08
st. dev.	0.0014	0.0024	0.0085	8.21	0.00	6.76	3.91	5.65	4.19	4.77	0.94

Napin	0.0162	0.0153	0.0335	66.60	0.00	9.03	38.49	27.16	30.53	34.50	1.97
st. dev.	0.0011	0.0039	0.0097	6.56	0.00	7.12	2.49	3.88	3.06	3.53	0.68
4-OH-Glucobrassicin	0.0385	0.0227	0.0088	97.20	4.41	6.93	1.11	0.86	0.90	1.09	0.01
st. dev.	0.0000	0.0937	0.0227	0.00	0.00	55.52	0.17	0.38	0.33	0.44	0.07
Glucobrassicin	0.0237	0.0150	0.0422	61.80	0.00	13.52	7.03	5.45	5.87	6.48	0.49
st. dev.	0.0019	0.0029	0.0121	8.03	0.00	6.92	0.40	0.66	0.56	0.65	0.13
4-methoxy-Glucobrassicin	0.0417	0.0107	0.0240	66.80	0.00	7.53	2.64	2.59	2.40	2.58	0.23
st. dev.	0.0039	0.0029	0.0111	10.10	0.00	10.90	0.17	0.28	0.23	0.28	0.05
White cabbage											
Iberin	0.0046	0.0174	0.0330	95.10	0.00	9.41	92.13	65.33	64.86	67.11	6.54
st. dev.	0.0034	0.0027	0.0000	0.00	0.00	475.61	8.26	5.11	5.47	1.26	1.55
Sinigrin	0.0060	0.0183	0.0402	84.38	0.00	13.20	64.64	41.92	43.43	47.41	3.64
st. dev.	0.0007	0.0067	0.0155	9.98	0.00	9.06	5.37	8.97	7.12	8.24	1.78
Raphanin	0.0059	0.0180	0.0341	93.42	0.00	10.52	26.11	16.50	15.60	15.30	1.75
st. dev.	0.0012	0.0043	0.0093	15.82	0.00	6.36	1.28	2.08	1.62	1.80	0.38
Napin	0.0065	0.0196	0.0399	77.64	0.00	11.79	8.94	5.63	6.12	6.69	0.43
st. dev.	0.0007	0.0064	0.0134	9.90	0.00	7.94	0.68	1.11	0.88	1.01	0.22
Glucobrassicin	0.0105	0.0115	0.0447	84.70	0.00	18.50	2.56	2.10	3.40	3.19	0.49
st. dev.	0.0029	0.0049	0.0249	24.94	0.00	14.77	0.39	0.70	0.63	0.70	0.15


A
Residuals
Glucoraphanin concentration (µmol/100g fresh material)
Time (min)
B
Residuals
Glucoraphanin concentration (µmol/100g fresh material)
Time (min)

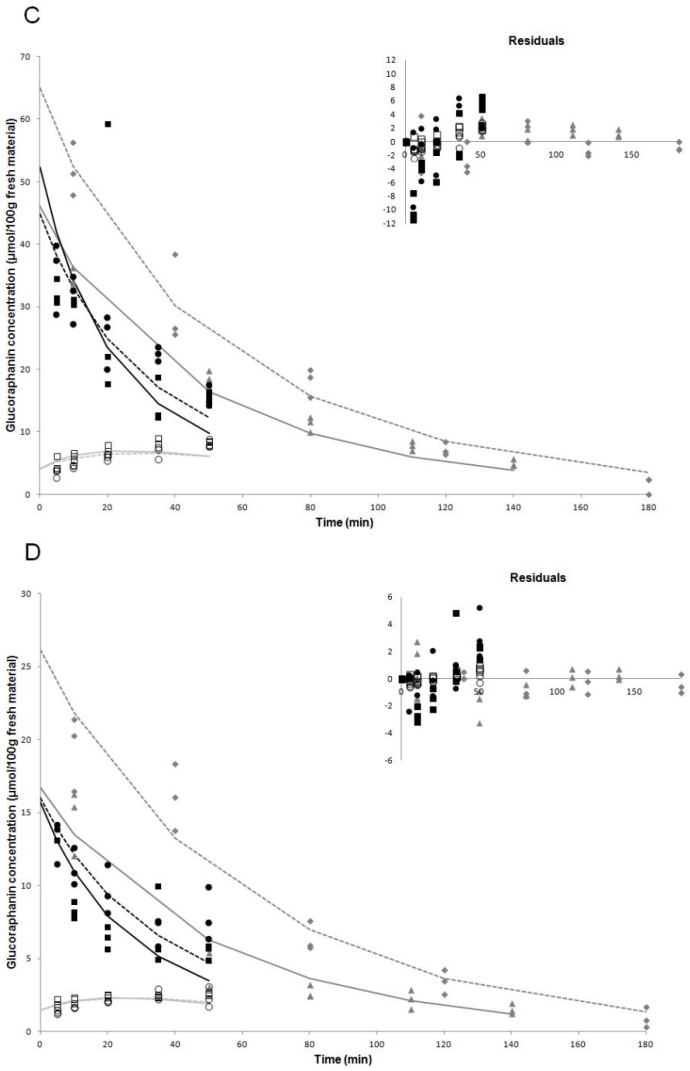
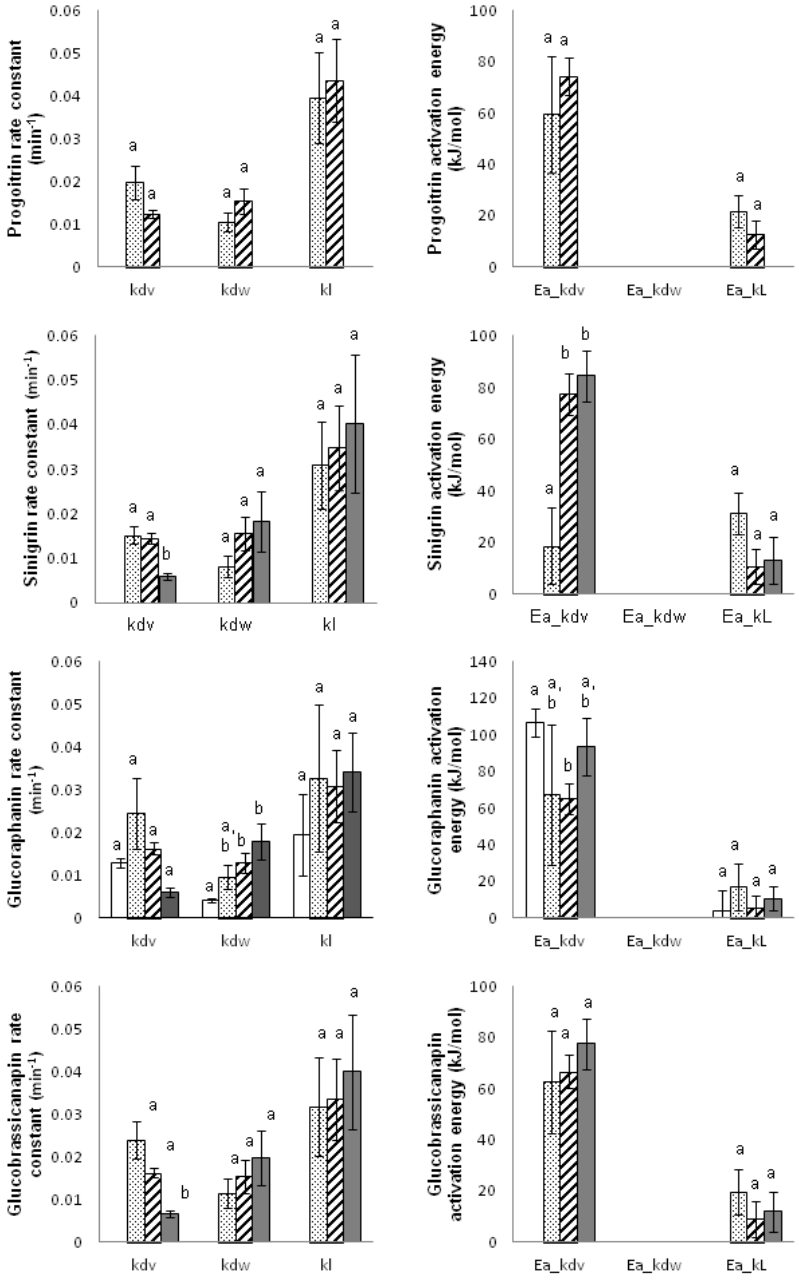


Figure 3.1: Degradation of glucoraphanin concentration (µmol/100 g fresh material) from broccoli (A), Brussels sprouts (B), red cabbage (C) and white cabbage (D) after incubation for different times in vegetable at 50 ˚C (grey diamond, model: grey-dashed line), 65 ˚C (grey triangle, model: grey line), 85 ˚C (black circle, model: black-dashed line) and 100 ˚C (black square, model: black line) and cooking water at 85 ˚C (black-rimmed circle, model: light grey-dashed line) and 100 ˚C (black-rimmed square, model: light grey line) and the residuals of the model.

3.3.1 Thermal degradation of GSs in the vegetables

The k_{dv} of Brussels sprouts and red cabbage were similar to each other for progoitrin, sinigrin, glucoraphanin, glucobrassicin and 4-methoxy-glucobrassicin, a significant difference could be detected only between the rate constants of glucobrassicin from Brussels sprouts and red cabbage (Fig 3.2). In contrast, the k_{dv}'s of GSs from white cabbage were found to be always significantly lower, meaning that the thermal degradation of the same GSs in white cabbage was slower than in red cabbage or Brussels sprouts. Furthermore the $E_a\_k_{dv}$ of GSs from white cabbage were similar or higher than from Brussels sprouts or red cabbage. In a study by Fuller et al. (2007) GSs in white cabbage were also found to degrade slowly during thermal processing. After microwaving roughly chopped white cabbage for either 2 min or 5.5 min, no difference could be found in the total GSs content. Opposing results were found by Volden et al. (2008). Shredded white cabbage was cooked with tap water (45 kg vegetable and 50 L water) up to 150 min and leaching as well as thermal degradation was modelled. The degradation of GSs was far quicker (50% reduction of aliphatic GSs and 59% reduction of indole GSs compared to fresh broccoli after 2 min cooking) than in the recent study. The rate constants of all GSs in the vegetables were approximately 10 times higher. Due to the big volume of water used in this study, the heating up time might have been longer than in the present study and therefore the myrosinase would be later inactivated and would hydrolyse more GSs. In a study in which the different thermal degradation kinetics in Chinese kale and broccoli were estimated in two different seasons, the rate constants were in the same range as in the recent study (Hennig et al., 2012).


Progoitrin rate constant (min-1)
Progoitrin activation energy (kJ/mol)
Sinigrin rate constant (min-1)
Sinigrin activation energy (kJ/mol)
Glucoraphanin rate constant (min-1)
Glucoraphanin activation energy (kJ/mol)
Glucobrassicanapin rate constant (min-1)
Glucobrassicanapin activation energy (kJ/mol)
kdv
kdw
kl
Ea_kdv
Ea_kdw
Ea_kL

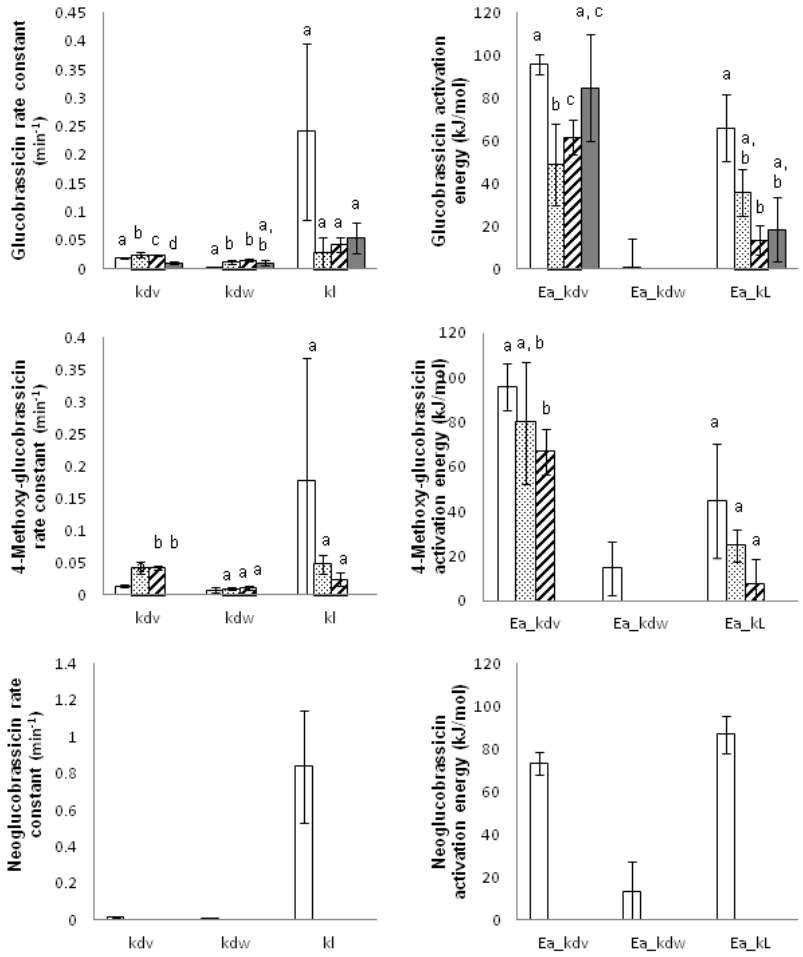


Figure 3.2: Rate constants (min^{-1}) and activation energies (kJ/mol) at the reference temperature of 100˚C of glucosinolates from broccoli (white bar), Brussels sprouts (dotted bar), red cabbage (striped bar) and white cabbage (grey bar). Bars marked with different letters are significantly different ($p<0.05$).

Reasons of this higher thermostability of GSs in white cabbage than in red cabbage or Brussels sprouts in the present study might be due to different environments in the cells or components in the cell structure that protect the degradation of GSs. Hanschen et al (2012) found, for instance, different stabilities of the same GSs after heat treatment in media with different pH.

The k_{dv} and $E_a\_k_{dv}$ of broccoli were for glucoraphanin from broccoli similar to glucoraphanin from Brussels sprouts, in glucobrassicin and 4-methoxy-glucobrassicin the k_{dv}'s were lower but the E_a's similar. This finding suggests a slightly higher thermostability of glucobrassicin and 4-methoxy-glucobrassicin in broccoli than in Brussels sprouts.

3.3.2 Thermal degradation of GSs in the cooking waters

The rate constants of thermal degradation obtained from cooking water were similar to each other, only the k_{dw} of glucoraphanin and glucobrassicin from broccoli were lower than from Brussels sprouts, red cabbage or white cabbage. The activation energy of thermal degradation in cooking water could mostly not be estimated, as the data set was limited to two temperatures for GSs concentrations in cooking water due to the possibility of myrosinase activity at the two lowest temperatures. If the differences in the degradation of GSs are only due to the environment in the cells and the cell structure, then the similar results for the rate constants in the cooking waters of the same GS from different vegetables are to be expected. However, there can still be a difference due to the leaching of other cellular compounds into the cooking water that might affect the degradation rate of GSs.

Between the thermostability of GSs in vegetable and cooking water a distinction between the kinetics of different *Brassica* vegetables could be found. While all GSs from Brussels sprouts are less stable in the vegetable than in the cooking water, the GSs from white cabbage are more stable in the vegetable compared to the cooking water. The k_d's of GSs from red cabbage in the vegetable or cooking water were similar to each other and a big difference in their thermostability is therefore not expected.

3.3.3 Leaching of GSs from the vegetables to the cooking waters

Cell lysis was described in this model by a first order equation. This assumption was based on literature (Verkerk, 2002) and former experiments in our group (not shown). The mechanism of leaching of GSs into the cooking water due to cell lysis contains of multiple reactions. Heat treatment leads to complex chemical changes in the cell wall. The chemical changes are influenced by its environment such as pH and present salt ions, but also by the presence of polysaccharides, which can increase an uptake of water in the cells and therewith reduce the cohesiveness of the cell wall matrix (Rao &

Lund, 1986). As lysis of cells is a complex mechanism and not a simple chemical reaction, using a first order equation to model the leaching of GSs into the cooking water can only be a simplification. The reaction rate constants for leaching are therefore not real reaction rate constants, but a collective of reaction rates (van Boekel, 2009).

The parameters of leaching were similar between all *Brassica* vegetables with the exception of the E_{a_kl} of glucobrassicin from broccoli, which was significantly different from the E_{a_kl} obtained from Brussels sprouts, red cabbage or white cabbage. Brussels sprouts, red cabbage and white cabbage was cut into thin strips before usage, a difference in the leaf structure seems to have no influence on the leaching kinetics. Broccoli on the other hand was incubated as florets. One would expect the leaching rate to be slower from the larger sized florets, this was however not the case. The structure of the broccoli matrix seems to be so soft that no substantial difference in the leaching rate between broccoli florets and cut cabbage or Brussels sprouts material could be detected. A study about the softness of broccoli was done previously (Lin, 2005), but not in comparison to other *Brassicaceae*. It is suggested that a study about the differences in texture and organization of the cell matrix between the various cultivars has to be carried out.

3.4 Conclusion

The proposed mathematical model (Sarvan et al., 2012) was successfully used for four different *Brassicacea*e and its parameters could be estimated, with an exception of the $E_a\_k_{dw}$'s.

It was found that the thermostability of GSs varied not only in different media such as the intact vegetable tissues or the cooking water, but also changed with the vegetable in which the GS is present. The environment in the cells or cell components of the different cultivars is likely to have an influence on the thermostability of GSs. To specify these components further research is necessary. A review about mathematical modelling of foods can be found elsewhere (Perrot et al., 2011).

Acknowledgement

The research leading to these results has received funding from the European Community's Seventh Framework Program (FP7/ 2007e2013) under the grant agreement n° FP7-222 654-DREAM.

Chapter 4

Sulforaphane formation and bioaccessibility is more affected by steaming time than meal composition during *in vitro* digestion of broccoli

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Abstract

Broccoli is a rich source of the secondary plant metabolite glucoraphanin (GR). After hydrolysis of the glucosinolate GR by the endogenous enzyme myrosinase, depending on the environmental conditions, sulforaphane (SF) or sulforaphane nitrile (SFN) are produced. SF has been reported to be involved in the reduction of the risk of several cancers in humans. To study the effect of steaming and meal composition on the conversion of GR and the bioaccessibility of released breakdown products, an *in vitro* digestion model was used. GR, SF and SFN were analyzed in broccoli (raw or steamed for 1, 2 or 3 min) broccoli after *in vitro* digestion with and without addition of protein (bovine serum albumin (BSA)) and/or lipid (olive oil). The main formation of SF and SFN from GR occurred during the *in vitro* oral phase. The content of GR, SF and SFN did not degrade after digestion. SF concentrations were up to 10-times higher in raw and 1-min steamed broccoli samples after the digestion compared to broccoli that was steamed 2 or 3 min. The addition of BSA and olive oil had no influence on the formation and bioaccessibility of SF or SFN. In the present study most SF was produced during *in vitro* mastication even when only 14% of the initial myrosinase activity was still present in the broccoli. Its concentration did not degrade throughout the subsequent digestion stages. In the gastric phase the endogenous myrosinase was irreversible inactivated, which lead to no further hydrolysis of GR. Additions of BSA and olive oil had no effect on the SF bioaccessibility in the *in vitro* digestive system. Meal preparation seems to have a much more pronounced effect on SF formation and bioaccessibility compared to meal composition.

4.1 Introduction

The isothiocyanate sulforaphane (SF) was shown *in vitro* cell and in animal studies to affect several mechanisms that could reduce the risk of developing cancer. In epidemiological studies the intake of cruciferous vegetables that contain glucosinolates (GS), precursors of isothiocyanates, are associated with a decreased risk of several cancers (Steinbrecher et al., 2009; Traka & Mithen, 2008; Verhoeven et al., 1996; Verkerk et al., 2009; Voorrips et al., 2000). SF can be produced by enzymatic hydrolysis of glucoraphanin, which is a glucosinolate naturally occurring in e.g. broccoli (*Brassica oleracea var. italic*). Enzymatic hydrolysis can take place by the plant enzyme myrosinase (β thioglycosidase, E.C. 3.2.1.147), contained in myrosin cells (Kissen et al., 2008), or to a lesser content by microbiota with myrosinase-like activity in the human gut. If the cell structure of the plant is disrupted e.g. during cutting or chewing, myrosinase can get in contact with glucoraphanin and hydrolyze it to SF or sulforaphane nitrile (SFN), depending on environmental conditions (Verkerk et al., 2009). At neutral pH mainly SF will be formed, at pH 4 and/or in the presence of iron-ions and active endogenous epithiospecifer protein, SFN formation is favoured (Matusheski, Juvik, & Jeffery, 2004). SF is believed to alter carcinogen metabolism, induce cell cycle arrest and apoptosis, inhibit angiogenesis and metastasis, change the histone acetylation status, and induce antioxidant, anti-inflammatory, and immunomodulatory activities (Dinkova-Kostova & Kostov, 2012).

Much is known about the mechanisms of action of SF when it has entered the gut wall, but limited research has been performed on the effect of the harsh conditions (i.e. pH dynamics and enzymatic activity) within the human digestive tract on the formation and fate of SF (i.e. if SF is available for uptake: bioaccessibility) before it is absorbed into the human body. The aim of the recent study was to investigate the effect of different steaming times and the presence of other meal components during *in vitro* digestion on the formation, stability and bioaccessibility of SF.

To mimic the digestion process, a static, three stage *in vitro* digestion system was employed, that was used before to assess the bioaccessibility of chemicals from toys (Brandon et al., 2006) and release of vitamins (Brandon et al., 2014). The digestion started with the oral phase (pH 6.8), followed by a gastric phase (pH 2) and the intestinal phase (pH 6.5). The *in vitro* design allows to take samples at different time points, and is thus a good non-invasive, standardized method to study the processes taking place during human digestion.

4.2 Material and methods

Broccoli (*Brassica oleracea var. italic*) was used from one batch of 13 heads grown in Spain in winter 2012/13.

4.2.1 Sample preparation

Fresh broccoli heads were cut into florets with a 2 cm stem and mixed thoroughly. The florets were divided into portions of 100 g, some raw portions set aside and the other steamed in an electrical steam oven (Miele, Steam oven DG 1050) for 1 min, 2 min and 3 min. The oven was preheated to 100°C before the material was added. A thermocouple was introduced in the core of the stem of one of the florets to measure the temperature profile during steaming. After steaming, the broccoli florets were placed in aluminium dishes on ice to cool till room temperature.

Two batches of each steaming time were taken to determine the myrosinase activity after steaming and cooling. The other samples were frozen in liquid nitrogen, blended (Waring Commercial, Torrington, USA) and stored at -20 ˚C until analysis for GR, SF and SFN.

4.2.2. Gastro intestinal-tract set up in vitro

Broccoli (4.5 g), frozen and blended after different steaming times, was added to the *in vitro* digestion system. To samples of each steaming time either 1 g bovine serum albumin (BSA) or 0.72 ml olive oil was added or they were placed in the digestion model without further additions. Each treatment was repeated twice in the digestion system. The individual amounts of BSA and olive oil were chosen to mimic the amounts found in a meal of lean minced meat and broccoli, (same amounts of broccoli and minced meat, calculated with (Aign, Muskat, Elmadfa, & Fritzsche, 2010)).The *in vitro* digestion was performed in three subsequent steps i.e. an oral phase, a gastric phase and an intestinal phase, after Versantvoort et al. (2005). For the oral phase, 6 ml artificial saliva solution at 37 ˚C was added to the frozen broccoli sample and shaken gently with a head-over-heel rotation for 5 min at 37 ˚C (pH 6.8). After incubation 12 ml gastric juice was added and adjusted to pH 2 with HCL (37%). After incubation for 2 hours at 37 ˚C in the head-over-heel, the gastric phase was completed. For the intestinal phase 12 ml duodenal juice, 6 ml bile solution and 2 ml sodium bicarbonate solution (all at 37 ˚C) were added, the pH adjusted to 6.5 with

NaOH (1 M) and incubated in the head-over-heel rotation at 37 ˚C for 2 hours. Samples were taken after the mouth phase, after adjustment to pH 2 but before gastric incubation, after the gastric phase and after the intestinal phase. Samples were frozen at -20 ˚C till further analysis of GR, SF and SFN.

4.2.3 Myrosinase activity

The myrosinase activity was determined by the method described by Oliviero et al. (2014). To extract the myrosinase, 0.5 g of ground broccoli sample was added to 140 ml of potassium phosphate buffer (pH 7, 50 mM) and incubated over night at 15 °C. After incubation the samples were centrifuged at 2670g for 10 min and filtered through filter paper. Three ml of this solution was added to a centrifugal filter tube (Millipore, Ultracel 30kD) and centrifuged at 4000g for 10 min to remove dissolved components smaller than myrosinase. The myrosinase in the filter was dissolved in 470 µl phosphate buffer (pH 7, 50 mM) and stored on ice.

A D-glucose enzyme kit (Biocontrol Europe, Enzyplus EZS 781+) was used to determine the glucose released during hydrolysis of sinigrin by myrosinase at wavelength 340 nm for 7 min in the spectrophotometer. The activity was determined based on the slope of the linear part of the curve of absorbance versus reaction time. To quantify the myrosinase activity, an external calibration determined by following the same procedure for the samples analysis. Activity was expressed as U/mg dry weight, where one unit produces 1 µmol glucose/min from sinigrin at pH 6 and 25 ˚C.

4.2.4 Glucoraphanin extraction and determination

Glucoraphanin was extracted with hot methanol (70%) and analyzed using high performance liquid chromatography (HPLC) following on-column desulphation as described by Verkerk et al (2001). Glucotropaeolin (3 mol/L, in water) was added as internal standard since it is not present in the explored vegetables. The column used was a Merk (Darmstadt, Germany) LiChroCART (RP-18 125x4mm) with an attached LiChroCART guard column (RP-18, 4x4mm). One run took 25 min with 100% milliQ water as solvent for the first 2 min, increasing to 8% acetonitrile till 7.5 min, further increasing to 25% acetonitrile till 14 min, the gradient held till 18 min and decreased to 100% water again at 20 min.

4.2.5 Breakdown-product determination

The SF and SFN extraction and determination was done as described in chapter 5, with modifications. In short, trichloracetic acid (5% in sample) and benzyl-isothiocyanate (0.3 mol/L), as internal standard, were added to the digestion juice containing broccoli and incubated at room temperature for 1 h. After the incubation, dichloromethane was added, the solutions shaken and centrifuged. The dichloromethane layer was removed and extraction repeated two more times with the water layer. The combined dichloromethane layers were dried with sodium sulfate, filtered and evaporated to 0.5 ml. Half of this solution was used for the detection of SFN and half for the determination of SF by gas chromatography-mass spectrometry (GC-MS), both on a RxiR-5HT (30m, 0.25nm ID, 0.25 μm df) column.

Due to the lack of an appropriate standard, SFN could be only measured semi-quantitatively. The SF and SFN concentration of fully hydrolyzed GR from broccoli was reported to be in the same order in literature (Wang et al., 2012), hence the areas of SFN were considered to represent a similar range of concentrations compared to the SF concentrations.

Statistical analysis of the experiments was performed by a two-way analysis of variance and Fisher's protected LSD with $\alpha<0.05$ using the statistical software package SPSS 17.0 (IBM, New York, USA).

4.3 Results

Broccoli florets were either digested raw or after using different steaming times (1 min, 2 min or 3 min). The maximum temperatures reached in the core of the broccoli florets were 95 °C after 3 min steaming (Fig 4.1). The surface temperature of the broccoli will increase faster and be higher, with a maximum of 100°C, compared to this centre temperature.

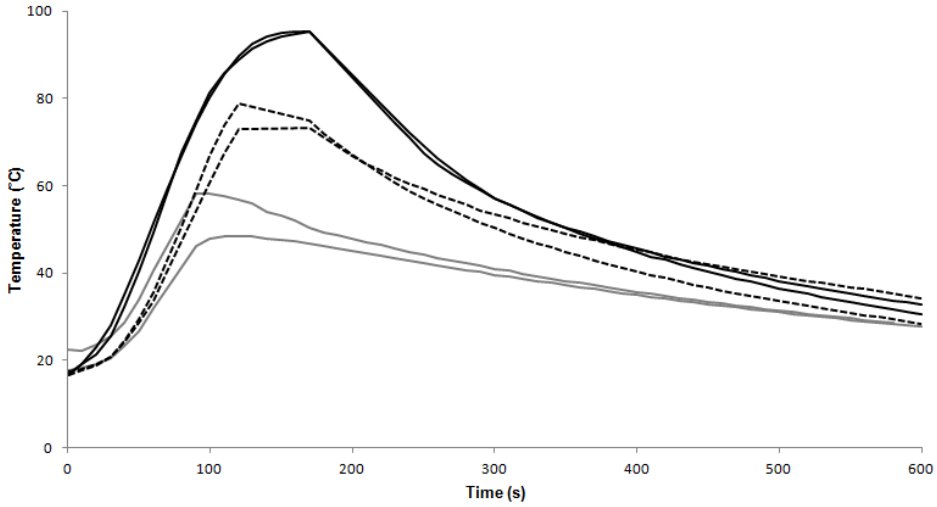

Figure 4.1: Temperature development in the core of the stem of the broccoli florets during different steaming times (60 sec: grey lines, 120 sec: dashed lines, 180 sec: black lines) and cooling on ice.

The raw broccoli had a myrosinase activity of 12.5 U/g fresh weight (FW). It decreased to 14% after 1 min steaming and approx. 1% after 2 min steaming compared to the raw sample (Fig. 4.2). After 3 min steaming of broccoli no myrosinase activity was detectable.

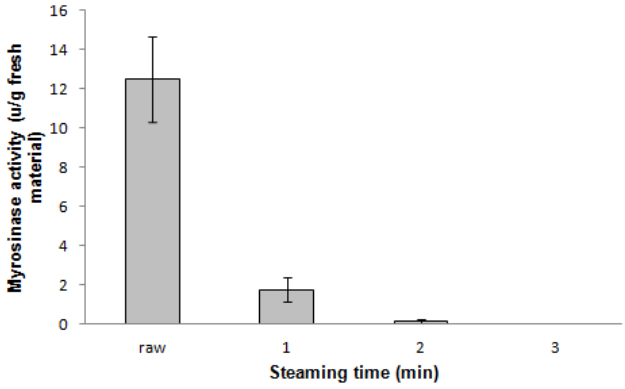

Figure 4.2: Myrosinase activity (U/g fresh material) in raw and steamed broccoli (1 min, 2 min and 3 min).

In raw and steamed broccoli before digestion (no significant differences between raw and steamed broccoli), the GR content was 181.0± 14.6 µmol/100g FW, the content of SF was 2.77 ± 0.93 µmol/100g FW and the area of SFN was 2.03 ± 0.02

Steaming time had a significant effect on the GR, SF and SFN content measured after the *in vitro* mouth phase. As shown in figure 4.3 and table 4.1, raw and 1-min steamed broccoli samples had the lowest GR concentrations, while in 2-min steamed samples higher concentrations could be found. The highest GR concentration could be detected in 3 min-steamed samples. The highest SF concentrations were found in raw and 1-min steamed broccoli. 2-min and 3-min steamed broccoli samples were not significantly different from each other and showed the lowest SF concentrations. The detected areas of SFN were up to 10-times higher for raw and 1-min steamed broccoli than 2 and 3-min steamed broccoli. Remarkably, both SF concentration and SFN areas, seem to increase for all steaming times in the final digestion phase.

The content of GR, SF and SFN were not degraded after completed intestinal digestion compared to the content after the oral phase.

No significant differences in the measured GR, SF and SFN content between the addition of BSA or olive oil or without addition during the *in vitro* digestion were observed, except for the SF content in the samples after the gastric phase (Tab. 4.1). The results shown in figure 4.3 are therefore obtained by pooling the samples (n=6), regardless of their additions, for each digestion stage and steaming time.

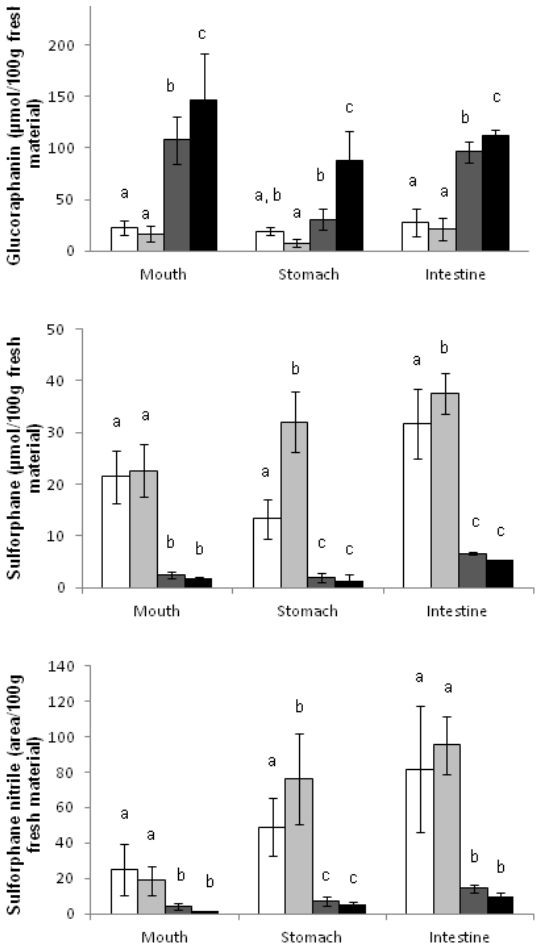

Figure 4.3: Glucoraphanin (µmol/100g fresh broccoli) and sulforaphane (µmol/100g fresh broccoli) concentration as well as sulforaphane nitrile area of raw (white bar) and steamed broccoli samples (1 min: light grey bar, 2 min: dark grey bar and 3 min: black bar) after different stages of *in vitro* digestion (mouth, stomach and intestine). The results were pooled regardless of their meal addition (n=6). Different letters indicate significant differences between the values after that digestion stage ($p<0.05$).

Table 4.1: Contents of GR (μmol/100g FW), SF (μmol/100g FW) and SFN (area/area internal standard), with standard deviations, after the digestion stages of raw and steamed (1 min, 2 min and 3 min) broccoli with and without the addition of olive oil or BSA

Oral phase

	Raw			1 min steaming			2 min steaming			3 min steaming		
	Olive oil	BSA	No addition	Olive oil	BSA	No addition	Olive oil	BSA	No addition	Olive oil	BSA	No addition
GR	27.5 ± 11.1	19.4 ± 2.2	20.6 ± 8.6	18.2 ± 3.1	11.9 ± 9.4	20.1 ± 10.6	102.8 ± 20.7	89.7 ± 13.3	130.8 ± 14.9	129.9 ± 42.6	122.4 ± 0.8	186.9 ± 59.6
SF	24.7 ± 3.4	21.4 ± 8.2	18.1 ± 3.6	23.6 ± 0.7	17.2 ± 3.8	27.2 ± 3.0	2.2 ± 0.1	3.4 ± 0.3	2.0 ± 0.01	1.8 ± 0.1	2.1 ± 0.2	1.5 ± 0.1
SFN	24.0 ± 14.4	33.8 ± 21.1	16.6 ± 8.9	13.3 ± 0.4	17.9 ± 7.2	25.0 ± 11.3	5.4 ± 1.6	2.6 ± 0.3	3.9 ± 2.3	1.6 ± 0.4	1.2 ± 0.5	1.5 ± 0.8

Gastric phase

	Raw			1 min steaming			2 min steaming			3 min steaming		
	Olive oil	BSA	No addition	Olive oil	BSA	No addition	Olive oil	BSA	No addition	Olive oil	BSA	No addition
GR	20.0 ± 0.3	15.3 ± 2.7	22.3 ± 0.2	11.7 ± 1.2	6.5 ± 2.4	4.6 ± 1.3	34.9 ± 16.1	30.3 ± 14.2	27.4 ± 0.7	73.4 ± 21.2	115.4 ± 30.5	74.4 ± 22.6
SF	10.4 ± 0.3	18.1 ± 0.1	11.5 ± 2.3	36.2 ± 1.8	28.9 ± 6.0	31.1 ± 8.5	2.8 ± 1.4	1.6 ± 0.005	1.7 ± 0.05	1.3 ± 0.03	1.3 ± 0.1	1.3 ± 0.1
SFN	49.9 ± 16.7	52.9 ± 24.3	44.1 ± 19.0	76.3 ± 22.7	62.3 ± 19.2	89.8 ± 40.3	8.4 ± 5.3	5.8 ± 0.9	6.8 ± 0.4	4.5 ± 0.04	4.9 ± 1.2	7.1 ± 0.7

Intestinal phase

	Raw			1 min steaming			2 min steaming			3 min steaming		
	Olive oil	BSA	No addition	Olive oil	BSA	No addition	Olive oil	BSA	No addition	Olive oil	BSA	No addition
GR	26.3 ± 3.8	16.4 ± 7.1	40.8 ± 13.2	27.5 ± 2.6	7.7 ± 1.1	29.3 ± 2.0	95.3 ± 16.9	102.3 ± 8.8	92.4 ± 7.6	112.6 ± 4.9	115.4 ± 5.5	107.7 ± 6.7
SF	28.6 ± 6.6	38.3 ± 6.6	28.2 ± 2.3	33.6 ± 2.5	41.7 ± 1.7	37.5 ± 0.8	6.8 ± 0.3	6.4 ± 0.1	6.7 ± 0.1	5.4 ± 0.1	5.2 ± 0.01	5.2 ± 0.3
SFN	113.3 ± 56.8	60.3 ± 0.3	71.5 ± 2.5	88.3 ± 12.3	84.3 ± 0.4	113.6 ± 12.6	13.4 ± 0.1	13.8 ± 1.4	15.4 ± 4.0	10.9 ± 0.7	11.5 ± 0.1	7.0 ± 1.0

4.4 Discussion

An *in vitro* digestion model was used to study the differences in bioaccessibility of GR, SF and SFN for different steaming times of broccoli and with and without the addition of olive oil and bovine serum albumin.

The *in vitro* digestion model used consisted of a three stage system, with a static pH during each stage. In literature it was used before to assess the bioaccessibility of chemicals from toys (Brandon et al., 2006) and the release of vitamins (Brandon et al., 2014).To mimic chewing in the present research, ground frozen broccoli samples were placed in a plastic tube and warm artificial saliva was added, the tubes closed and placed in a turning machine inside an incubator for 5 min. This time was chosen, to secure hydrolysis of GR in amounts similar to *in vivo* conditions (Sarvan et al., 2014). Mincing of broccoli as advised by Minekus (2014) resulted in total hydrolysis of GR (results not shown) and was therefore considered inappropriate to mimic *in vitro* chewing. In further digestion stages, samples were shaken with constant frequency in a head-over-heel machine. This simplifies the movements during digestion in humans, as these are very complex (i.e. chewing in the mouth and peristaltic movements in stomach and intestine).

The found myrosinase activity in raw broccoli samples was 12.5 U/g FW. In literature different broccoli cultivars had very variable myrosinase activities. Charron et al. (2005) measured myrosinase activities between 51 – 151 U/g FW, while Oliviero et al. (2014) found 59 U/g DW, which accounts for approximately 6 U/g FW. As described in chapter 5 of this thesis, 3.5 U/g FW were found in the present research. The present study focused on the differences between the myrosinase activities after different steaming times. The 1-min and 2-min steamed samples contained 14% and 1%, respectively, of the initial myrosinase activity. This is comparable with my own experiments (described in chapter 5 of this thesis) where 13% remaining activity was found after 1 min steaming and 2.5% after 2 min steaming in broccoli. In a study by Rungapamestry et al. (2007a) the myrosinase activity of raw broccoli was not reported, but 2-min steamed broccoli contained approximately 0.4 U/g fresh broccoli and 5.5-min steamed broccoli 0.1 U/g fresh broccoli active myrosinase. Steaming conditions were performed in a microwave with 150g broccoli and 16 ml water, which is different than in the present study.

The average GR concentration in steamed broccoli before chewing was comparable to concentrations found in literature. Jones et al. (2010) found concentrations in two varieties of approximately 131 - 280 µmol/100g FW. The concentration of GSs can vary in *Brassicaceae* depending on the variety, but also the growing conditions and the storage (Verkerk et al., 2009).

The individual GR concentrations found after the different *in vitro* digestion stages are shown in figure 4.3. The differences between raw and 1-min steamed broccoli and longer steamed broccoli can be explained by the different myrosinase activities. It is remarkable, that the 1-min steamed samples showed the same amount of GR degradation than raw broccoli, although after 1 min steaming only 14% myrosinase activity remained in the sample. It seems that myrosinase is in excess present in broccoli, and with 14% activity compared to the raw samples, a similar percentage of hydrolysis of GR can be obtained after *in vitro* chewing.

After the oral phase the GR concentrations did not degrade after completed intestinal digestion compared to the content after the oral phase. The low pH in the gastric phase inactivates myrosinase. This inactivation is irreversible since the activity cannot be restored by increasing the pH again. In a pilot study, buffers with different pH (2-7) were added to crushed broccoli, incubated at room temperature over night and the myrosinase activity determined. At pH 5-7 myrosinase is active, while after the pH decreases to pH 2, an increase in pH to 5 did not reactivate the enzyme again (results not shown). This explains, why no further hydrolysis of GR occurred after the pH rose in the intestinal phase. No other factors besides myrosinase seem to influence the GSs concentration. A stable GR concentration throughout *in vitro* digestion of rape seed was found by Maskell and Smithardt (1994). Contrary, Iori et al. (2004) found decreasing aliphatic glucosinolate concentrations during *in vitro* gastric digestion of 27% and a further decrease after intestinal digestion of total GSs content. The group of Rodriguez-Hernandez (2013) digested different parts of raw freeze-dried broccoli *in vitro* (starting with gastric digestion) and analyzed the GR, SF and SF-metabolite concentration in dialyzed and non-dialyzed fractions. In one cultivar ("Naxos") the GR and SF (SF and SF-metabolites) concentration decreased during digestion of broccoli florets, in another cultivar ("Viola") GR decreased by 7% and SF and its metabolites increased by 23%. It has to be noted that in this *in vitro* digestion an oral step was excluded and GR concentrations were very low already in the undigested samples. Especially because the used material in the study was freeze-dried raw

broccoli powder, it might be possible that the short time to adjust the pH to gastric conditions (pH 2) was sufficient to hydrolyze some GR present in the sample.

The average SF concentration in raw and steamed unchewed material was similar to results found in literature. In chapter 5 of the present research, concentrations of 2.7 – 3.2 μmol/100g FW were found, while Jones et al (2010) found SF concentrations in two broccoli variates of approximately 1.1 and 2.9 μmol/100g FW.

The highest SF concentration and SFN areas were detected in raw and 1-min steamed broccoli after the oral phase. In raw and short steamed broccoli, myrosinase was still active and could hydrolyze GR into its breakdown products. In literature, highest SF concentrations were detected in shortly heated broccoli samples (Ghawi, Methven, & Niranjan, 2013; Matusheski et al., 2004). Significant differences were observed in GR concentrations between the 2-min and 3-min steamed samples, therefore also differences for the breakdown products were expected, but these were not found.

All samples showed a further increase of SF concentration and SFN areas after the intestinal phase by 1.5 - 3 fold and 3 - 7 fold respectively. This further increase in hydrolysis products is remarkable. Formation of SF and SFN in this stage is not expected since no further GR hydrolysis occurs, it might be explained by an increased release from the matrix due to the longer digestion time.

The present *in vitro* digestion model does not contain the colonic stage. It has been reported in literature that GR can be hydrolyzed by enzymes from the colonic bacterial flora leading to SF formation and absorption in the colon (Getahun & Chung, 1999; Shapiro et al., 1998).

No effect of the addition of olive oil or BSA could be found on the bioaccessibility of GR, SF or SFN. In an *in vivo* study by Rungapamestry (Rungapamestry et al., 2007a) no differences in bioavailability of glucosinolates could be found with different meal compositions. Volunteers ingested either 2 or 5.5-min microwaved broccoli with or without the addition of lean beef. In the 24-h-urine SF-mercapturic acid as the urinary metabolite of SF, was measured. No differences were found between the addition to meals for the different heat treatments. They hypothesized that the lipophilic isothiocyanates might interact with meal composition, if they are ingested preformed and not when they are formed in the intestine by the human microflora as in their setup. This hypothesis could not be confirmed by the present study. SF was mainly produced in the chewing phase in the present *in vitro* model, as can be seen in figure

4.3 and therefore our hypothesis is, that meal composition does not change the bioaccessibility of SF, no matter at what phase in the digestion it is formed.

4.5 Conclusion

The present study showed that the main factor for SF formation and bioaccessibility from broccoli is the presence of active myrosinase. In raw and 1-min steamed broccoli with active myrosinase, up to 10-times higher SF concentrations could be found after the mouth phase than in longer steamed broccoli containing very low or no detectable myrosinase activity. The addition of BSA or olive oil did not alter the extractable amounts and therefore the bioaccessibility.

Acknowledgements

The research leading to these results has received funding from the European Community's Seventh Framework Program (FP7/ 2007e2013) under the grant agreement n° FP7-222 654-DREAM and from the Dutch Ministry of Economic affairs (for RIKILT-Wageningen UR authors). We thank Peter Hollman for critical discussions on the research.

Chapter 5

The effect of chewing on oral glucoraphanin hydrolysis in raw and steamed broccoli

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Abstract

Raw broccoli contains myrosinase, an enzyme which converts the glucosinolate glucoraphanin (GR) into the biological active sulforaphane (SF), a potent health-promoting compound. Upon chewing of broccoli, the cells get disrupted and myrosinase can effectively hydrolyze glucosinolates. The amount of glucosinolates and active myrosinase can be substantially altered by processing and preparation of broccoli. The aim of this research was to study how chewing time and steaming time of broccoli affected GR hydrolysis and SF as well as sulforaphane nitrile (SFN) formation in the mouth. To study the effect of chewing time on differently steamed broccoli, 10 g of broccoli was chewed for 11 s, 22 s, 30 s and 40 s. To determine the effect of steaming time, raw broccoli as well as broccoli that had been steamed for 0.5-min, 1-min 2-min and 3-min respectively, was chewed for 22 s. GR, SF and SFN were analyzed in all samples. Chewing time influenced the amount of hydrolysis of GR in raw and short steamed broccoli that contains active myrosinase (raw, 0.5-min and 1-min steamed), but not in broccoli that had been steamed longer. Steaming time showed to influence the oral hydrolysis of GR. Both chewing time and steaming time influence the enzymatic breakdown of GR in the mouth. Longer chewing times of raw and short steamed broccoli (0.5-min and 1-min), which contains active myrosinase, lead to more hydrolysis. It was shown that 0.5-min steamed broccoli had the highest hydrolysis rate of GR (38%).

5.1 Introduction

To determine the effect of health beneficial components from vegetables on the human body it is not only important to know the amount in the food, but also the release from the food matrix into the digestive system. The release from the food matrix, called bioaccessibility, can be affected before consumption by storage, methods of food preparation (raw, cooked, or processed) (Dekker et al., 2000; Dekker & Verkerk, 2003), source of the nutrient (synthetic, fortified or naturally occurring), food structure (Parada & Aguilera, 2007) and composition of the meal.

One group of beneficial components in vegetables are glucosinolates. These are secondary plant metabolites and are especially prevalent in plants of the family *Brassicaceae* to which broccoli belongs. Epidemiological and case-control studies with a diet rich in *Brassica* vegetables have suggested a reduced risk of several cancers (Bosetti et al., 2012; Miller & Snyder, 2012). This health promoting activity is not caused by the biologically inactive glucosinolates themselves, but by their hydrolysis products isothiocyanates (ITCs). ITCs are formed from glucosinolates trough hydrolysis by a class of endogenous enzymes called myrosinase (EC 3.2.1.147). Myrosinase is physically separated from glucosinolates in intact plant cells (Kissen et al., 2009). If *Brassica* vegetables are chopped or processed, myrosinase can interact with glucosinolates and hydrolyse them (Verkerk et al., 2009). The mechanism during chewing is illustrated in figure 5.1. Depending on the environmental conditions in which the reaction takes place, ITCs, nitriles, epithionitriles or thiocyanates will form (Hayes et al., 2008).

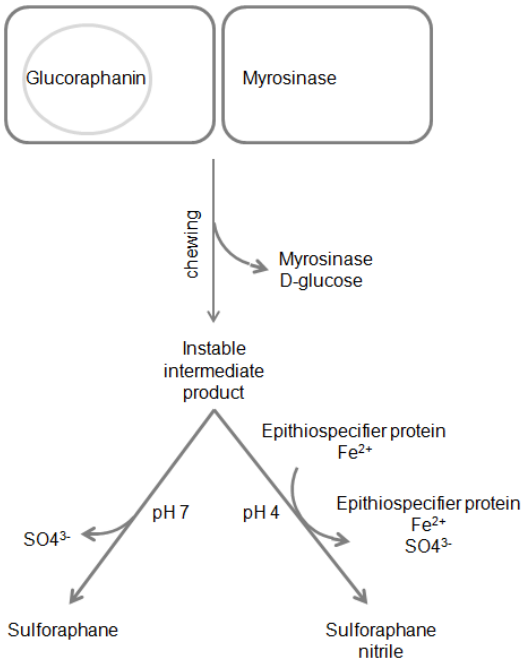

Figure 5.1: Hydrolysis of glucoraphanin by myrosinase to sulforaphane and sulforaphane nitrile (adapted from Matusheski et al., 2006)

One of the most studied ITCs is sulforaphane (SF), the breakdown product of the glucosinolate glucoraphanin (GR). SF can affect cancer development at different steps: in the initiation phase, the cell proliferation and the apoptosis. (reviewed by: Fimognari & Hrelia, 2007; Hayes et al., 2008)

Brassica vegetables are often eaten after a heat treatment. Depending on the processing time and temperature, this treatment degrades the amount of glucosinolates and inactivates the endogenous enzyme myrosinase (Oerlemans et al., 2006; Van Eylen, Oey, Hendrickx, & Van Loey, 2007).

The glucosinolate-myrosinase system is further influenced by digestion of the vegetables in the human body. Digestion starts with chewing of the food. During mastication the food structure is disrupted and lubricated with saliva as well as water contained in the food. When broccoli is chewed, cells get disrupted and glucosinolates can get in contact with the endogenous myrosinase and lead to hydrolysis (Mithen, Dekker, Verkerk, Rabot, & Johnson, 2000; Verkerk et al., 2009).The hydrolysis rate is

expected to be influenced not only by the content of active myrosinase and glucosinolates in the product, but also by the chewing intensity and the time the food remains in the mouth as well as the conditions such as pH, presence of other meal components or saliva in the mouth. More chewing cycles lead to increased cell damage, and furthermore, a longer retention time in the mouth enhances the possibilities for myrosinase to hydrolyze glucosinolates.

The aim of this study is to investigate the breakdown of glucosinolates from raw and thermally processed broccoli during chewing, and to find out which factors are affecting this breakdown as well as the occurrence of SF and SFN. The glucosinolate-myrosinase system has been studied in different processing methods such as steaming, blanching or boiling (Oerlemans et al., 2006; Rungapamestry, Duncan, Fuller, & Ratcliffe, 2007b; Van Eylen et al., 2008; Verkerk & Dekker, 2004; Volden, Borge, et al., 2008). However, little is known about the breakdown of glucosinolates during digestion and needs further investigation. The present study focuses on the interaction of thermal treatments of the product and the first step in digestion: oral processing.

5.2 Materials and Methods

5.2.1 Sample preparation

Two batches of broccoli (*Brassica oleracea var. italic*) were purchased from a local retailer (Wageningen, the Netherlands) and stored until treatment at 4°C. The first batch was used for the characterization of the material (section 5.2.2), the second batch was used to determine the effect of chewing time on GR hydrolysis (section 5.2.3) as well as the effect of chewing on GR breakdown with five volunteers (section 5.2.4).

Fresh broccoli heads were cut into florets with a 2 cm stem and mixed thoroughly. The florets were divided into portions of 100 g and steamed in an electrical steam oven (Miele, Steam oven DG 1050) for 0 min, 0.5 min, 1 min, 2 min and 3 min. The oven was preheated to 100°C, before the material was added. After steaming the broccoli florets were placed in aluminum dishes on ice to cool.

5.2.2 Characterization of initial material

To characterize the material, the temperature during steaming was recorded. One floret of each steaming cycle was pierced with a thermocouple 1 cm into the stem and the core temperature during steaming and resting on ice was determined for 10 min. Furthermore GR, SF and SFN were measured in raw and steamed broccoli and the myrosinase activity ascertained. All measurements were done in duplicate.

5.2.3 Determining the effect of chewing time on GR hydrolysis and breakdown product formation

The aim of this pilot study was to determine the effect of chewing time on the glucosinolate content in processed broccoli. Portions of 10 g raw or steamed broccoli (0.5 min, 1 min, 2 min and 3 min) were placed in a 8 cm long latex bag and chewed by one volunteer (24 years) for different times (11 s, 22 s, 30 s, 40 s) with one bite each second. Samples were collected in duplicates and placed in liquid nitrogen directly after chewing. The frozen samples were ground into a fine powder with a blender (Waring Commercial, Torrington, USA) and stored at -20°C until further analysis.

5.2.4 Determining the effect of chewing on GR and breakdown product concentration with five volunteers

Portions of 10 g raw or steamed broccoli florets were placed in approximately 8 cm long latex lab-glove fingers and closed. No artificial saliva was added. Five volunteers (22-25 years) chewed on these filled latex bags for 22 s with a frequency of one bite per second. This chewing time and frequency was determined in a pilot study with ten volunteers as average values for chewing broccoli (results not shown). All samples were collected in triplicates.

After chewing, half of the sample material was placed in liquid nitrogen to stop further enzyme activity. After the samples were removed from the plastic sacks, they were ground to a fine frozen powder with a blender (Waring Comercial, Torrington, USA) and stored at -20 ˚C until further determination.

The other half of the chewing material was used to determine cell lysis by measuring the conductivity at 23 ˚C. To 10g of chewed broccoli 100 ml milliQ water was added and after 1 hour the conductivity was determined by a conductivity meter (inoLab Cond 730) with an attached conductivity cell (Terta Con 325) (Verkerk et al., 2010).

5.2.5 Myrosinase activity

The myrosinase activity was determined after the method described by Oliviero et al. (2014). To extract the myrosinase, 0.5 g of ground broccoli sample was added to 140 ml of potassium phosphate buffer (pH 7, 50 mM). The solutions were incubated over night at 15 °C and stirred constantly. After incubation, the samples were centrifuged at 2670g for 10 min (Thermo scientific, Heraeus multifuge X3R) and filtered through filter paper (Whatman, Grade 595 1/2 folded filters, Ø125 mm) to remove plant material.

Three ml of this solution was added to a centrifugal filter tube (Millipore, Ultracel 30kD) and centrifuged at 4000g for 10 min to remove dissolved components smaller than myrosinase. The myrosinase retained by the filter was dissolved in 470 µl phosphate buffer (pH 7, 50 mM) and stored on ice.

A D-glucose enzyme kit (Biocontrol Europe, Enzyplus EZS 781+) was used to determine the glucose released during hydrolysis of sinigrin by myrosinase. 0.1 ml myrosinase extract was added to Millipore water, NaCl–ascorbic acid (Merk, Darmstadt, Germany) solution, imidazole buffer, solution of NAD^+ and ATP, hexokinase (300 U/ml) and glucose-6-phosphate dehydrogenase (600 U/ml). After the solutions were mixed, 0.05 ml sinigrin solution (0.079 mol/L) was added and the mixture placed in a cuvette in a spectrophotometer. The emerging glucose of the hydrolysis of sinigrin by myrosinase is phosphorylated by the hexokinase. The newly emerged glucose-6-phosphate will be transformed in the presence of $NADP^+$ by glucose-6-phosphate dehydrogenase to gluconate-6-phosphate and the reduced $NADPH+H^+$. The latter can be measured by the absorbance at wavelength 340 nm for 7 min.

The activity was determined based on the slope of the linear part of the curve of absorbance versus reaction time. To quantify the myrosinase activity, an external calibration was used determined by following the same procedure as for the samples analysis. In the reaction mixture 50 µL a standard myrosinase solution (from Sinapsis alba seeds Sigma-Aldrich, calibration solutions ranged from 0.02 to 1.2 U/mL) instead of the myrosinase sample extract was added. Activity was expressed as U/mg fresh weight of broccoli, where one unit produces 1.0 µmol glucose per min from sinigrin at pH 6.0 at 25 °C.

5.2.6 GR extraction and determination by HPLC

GR were extracted with hot methanol (70%) and analyzed using high performance liquid chromatography (HPLC) following on-column desulphation as described by Verkerk et al (2001). Glucotropaeolin (3 mol/L, in water, Laboratory of Biochemistry, Plant Breeding and Acclimatization Institute, Radzikow, Blonie, Poland) was added as internal standard since it is not present in broccoli. The column used was a Merck (Darmstadt, Germany) LiChroCART (RP-18 125x4mm) with an attached LiChroCART guard column (RP-18, 4x4mm). One run took 25 min with 100% milliQ water as solvent for the first 2 min, increasing to 8% acetonitrile till 7.5 min, further increasing to 25% acetonitrile till 14 min, the gradient held till 18 min and decreased to 100% water again at 20 min.

5.2.7 Breakdown product determination

For the extraction of ITCs, 4 ml dichloromethane and 15 µl benzyl-ITC (0.3 mol/L) as internal standard was added to 2 g of chewed broccoli sample in a 15 ml plastic tube and shaken for 10 min at room temperature. After the incubation, the samples were centrifuged (Heraeus multifuge X3R, Thermo Scientific, USA) at 1363g for 5 min and the dichloromethane layer taken out and stored in a separate tube. The extraction was repeated three times. The combined dichlormethane layers were dried with sodium sulfate and filtered through a 0.2 µm filter before evaporation. Approximately 8 ml of the obtained dichloromethane was evaporated under nitrogen to 0.5 ml. Half of this solution was used for the detection of SFN and half for the determination of SF by gas chromatography-mass spectrometry (GC-MS). A Trace GC 2000 (Thermo Scientific, USA) connected to a TriPlus autosampler (Thermo Scientific, USA) and DSQ-ll detector (Thermo Scientific, USA) was used and operated with Automated Mass Spectral Deconvolution and Identification System software (AMDIS, version 2.64, National Institute of Standards and Technology, USA) for qualification and the software Excalibur (Thermo Scientific, USA) for quantification.

For detection of SF, 2 µl were injected in the programmed temperature vaporizing (PTV) liner with a temperature program starting at 30 °C and rising to 230 °C in 7.5 °C/s. The oven was kept at 35 °C for 3 min and then increased to 230°C in 7.5 °C/min with a 15 min hold time. The flow was 3 ml/min in the first minute and thereafter 1 ml/min. The column was a RxiR-5HT (30m, 0.25nm ID, 0.25 µm df) and the measurement was carried out with a splitflow (30 ml/min for 1 min) in selective

iron monitoring (SIM) mode (8 min till 15 min SIM 42, 85, 113; 15 min till 22 min SIM 65, 91, 149; 22 min till 48 min SIM 55, 72, 160). The retention time of SF was 25 min, of 18.6 min and of butenyl-ITC 10.8 min. Butenyl-ITC, the thermal breakdown product of SF, was measured to investigate the possible breakdown of SF during injection or in the column. The found SF areas were corrected for the internal standard benzyl-ITC and quantified with a five-point calibration curve of SF/benzyl-ITC solutions (0.05 – 1.2 μmol/μmol).

For the SFN measurement 1 μl of dichlormethane extract was injected in the PTV liner. The same temperature program, flow program and column was used as for the SF measurements. SFN was measured in splitless mode as full scan (50 - 250 mass, 1 scan/min).The retention time of SFN was 21.5 min and of benzyl-ITC 18.8 min. SFN could not be quantified, instead the area was determined and divided by the internal standard benzyl-ITC.

All data sets were tested for and cleaned from outliers (Hoaglin, Iglewicz, & Tukey, 1986). Statistical analysis of the experiments was performed by a two-way analysis of varience and Fisher's protected LSD with $\alpha<0.05$ using the statistical software package SPSS 17.0 (IBM, New York, USA).

5.3 Results

5.3.1 Characterization of steamed initial material

Broccoli florets were steamed before chewing for different times (0.5 min, 1 min, 2 min and 3 min). The core temperature of one floret in each batch is shown in figure 5.2. The highest temperatures were reached in the 3-min steamed samples with approximately 95 °C, while the 0.5-min steamed samples reached maximum temperatures of 35 - 40 °C.

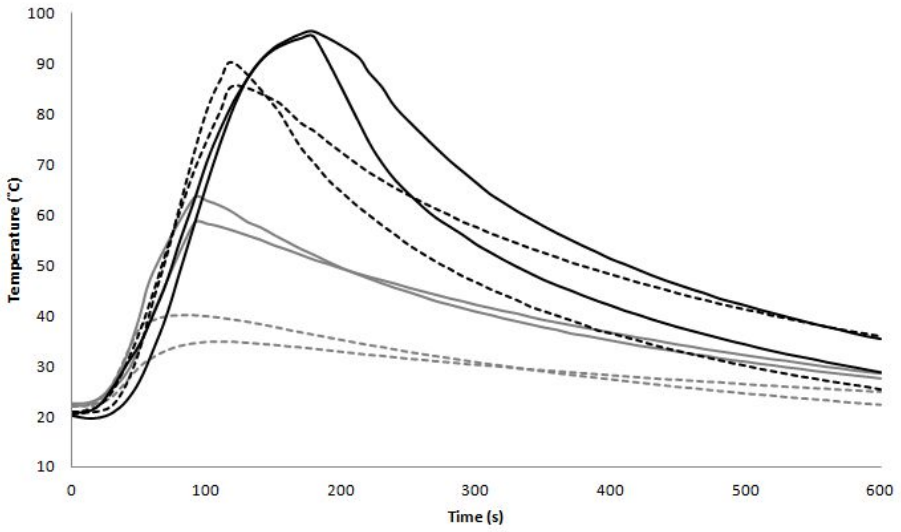

Figure 5.2: Core temperature profile in a broccoli floret during steaming for 0.5 min (grey dashed line), 1 min (grey line), 2 min (black dashed line) and 3 min (black line), all followed by cooling on ice (n=2 for each steaming time).

The range of GR concentrations in the steamed samples was between 46 – 74 μmol/100g fresh maerial. An increase in GR concentration is visible in the steamed broccoli samples compared to the raw material (Tab 5.1), however not statistical significant (p=0.274).

Table 5.1: Glucoraphanin and sulforaphane concentration (umol/100g fresh material) as well as amount of sulforaphane nitrile (area) in broccoli after different steaming times (n=2)

Steaming time (min)	Glucoraphanin (µmol/100g FW)	Sulforaphane (µmol/100g FW)	Sulforaphane nitrile (area)
0	46.1 ± 4.63	2.937 ± 0.029	1.568 ± 0.331
0.5	58.0 ± 5.48	3.061 ± 0.125	1.259 ± 1.001
1	62.1 ± 5.19	3.215 ± 0.077	0.804 ± 0.200
2	59.3 ± 2.51	2.802 ± 0.040	0.571 ± 0.014
3	73.9 ±21.8	2.742 ± 0.027	0.629 ± 0.246

The SF concentration in raw broccoli was between 2.7 – 3.2 µmol/100g fresh weight (Tab 5.1). The detected SFN areas were between 0.57 – 1.6 and did not significantly differ ($p=0.321$) at varying steaming times (tab 5.1).

The myrosinase activity of raw and steamed broccoli samples is illustrated in figure 5.3. Raw broccoli has a myrosinase activity of 3.4 U/g fresh weight. The myrosinase activity after 0.5 min of steaming decreased to 67% compared to fresh broccoli while, after a steaming time of 1 min the enzyme activity decreased to approximately 13%. Very low myrosinase activity (2.5%) was measured in broccoli steamed for 2 min and no activity for 3 min.

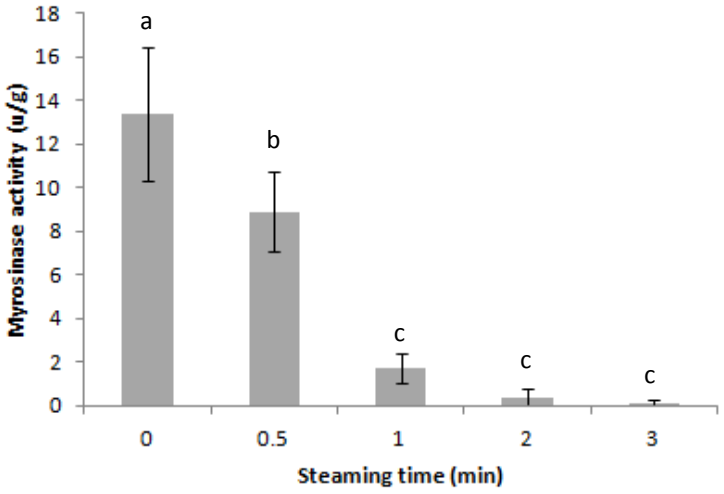

Figure 5.3: Inactivation of myrosinase in broccoli after steaming for different times (raw, 0.5 min, 1 min, 2 min, 3 min). Bars marked with different letters are significant different ($p<0.05$) from each other.

5.3.2 Pilot study: Effect of chewing time on GR and breakdown product concentration

A chewing study with different chewing times (11 s, 22 s, 30 s, 40 s) was accomplished with one volunteer. A subject chewed in a small enclosed latex bag one broccoli floret that was raw or steamed for 0.5 min, 1 min, 2 min or 3 min in duplicate.

The concentration of GR decreased in raw, 0.5-min steamed and 1-min steamed samples after 40 s chewing to 44%, 30% and 47% respectively (Fig 5.4). In samples steamed for 2 and 3 min, no significant change could be detected in the GR concentration at different chewing times, but compared to the raw unchewed broccoli a decrease between 10 and 25% could be detected.

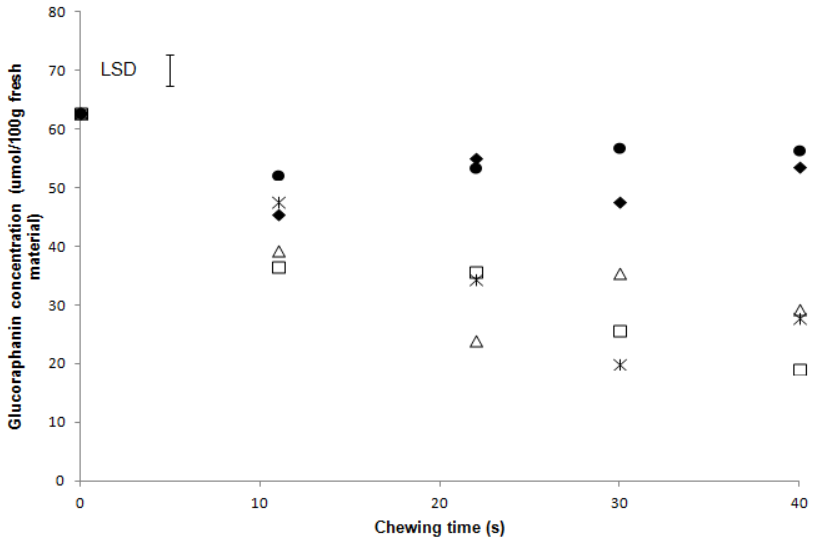

Figure 5.4: Glucoraphanin concentration (umol/100g fresh material) of unchewed broccoli (x-axis=0) and raw (asterisk), 0.5-min steamed (square), 1-min steamed (triangle), 2-min steamed (filled diamond) and 3-min steamed (filled circle) samples after different chewing.

The concentration of SF, the isothiocyanate released from GR, was determined in each chewing sample. Between different chewing durations of the same material no significant difference could be found. In contrast, steaming time was a significant factor ($p<0.05$) between the samples. Broccoli steamed for 1 min and 3 min were significantly lower than raw, 0.5-min steamed and 2-min steamed broccoli.

In the same samples the areas of SFN were measured. No significant differences could be found between chewing times for samples of the same material ($p=0.962$). In contrast steaming time was highly significant between samples ($p<0.05$). The measured areas of SFN were highest for raw and 0.5-min steamed chewed broccoli samples. 1-min steamed samples were lower in concentration than raw broccoli ($p<0.05$), but not than 0.5-min steamed broccoli ($p=0.087$). Samples chewed after steaming times of 2 and 3 min show very low SFN areas and were significantly lower than the shorter steamed samples (all $p<0.05$).

5.3.3 Determining the effect of chewing on GR and breakdown product concentration with five volunteers

To determine the effect of chewing on the GR concentration and its breakdown products, five volunteers chewed raw and steamed broccoli for 22 s with one bite/s. Between the volunteers no significant difference between the GR concentration of the same steaming time could be found ($p=0.543$), hence the results of each steaming time were pooled ($n=5*3=15$). In contrast, the influence of steaming time before chewing was highly significant ($p<0.05$). The lowest GR concentrations were found in 0.5-min steamed samples (36.0 µmol/100 g), followed by raw broccoli (46.9 µmol/100 g) and 1-min steamed samples (49.6 µmol/100 g) (Fig. 5.5). The 2-min steamed samples (79.73 µmol/100 g) and 3-min steamed samples (80.59 µmol/100 g) showed higher amounts of GR after chewing.

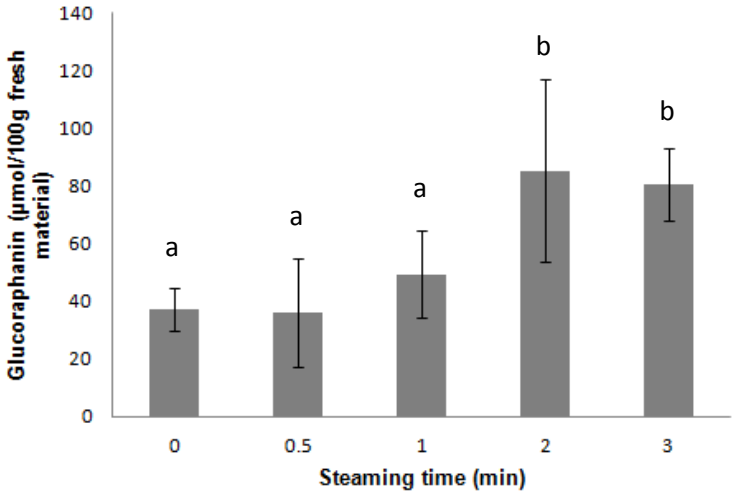

Figure 5.5: Effect of steaming time (0 min, 0.5 min, 1 min, 2 min and 3 min) on glucoraphanin concentration in broccoli chewed by five subjects for 22 s in triplica (for each steaming time n=3*5=15).

Figure 5.6 shows the SF concentration in broccoli samples steamed for different times and chewed by five volunteers. Compared to raw chewed broccoli the average SF concentration of 0.5-min and 1-min steamed chewed broccoli increased marginally, but not significantly, by 41% and 62% respectively. SF concentration of chewed 2-min steamed samples increased significantly by 188%, compared to raw chewed samples

and were different from all other samples (all $p<0.05$). After 3 min of steaming, only 91% of the SF concentration compared to raw broccoli could be determined. All steaming times had high standard deviations.

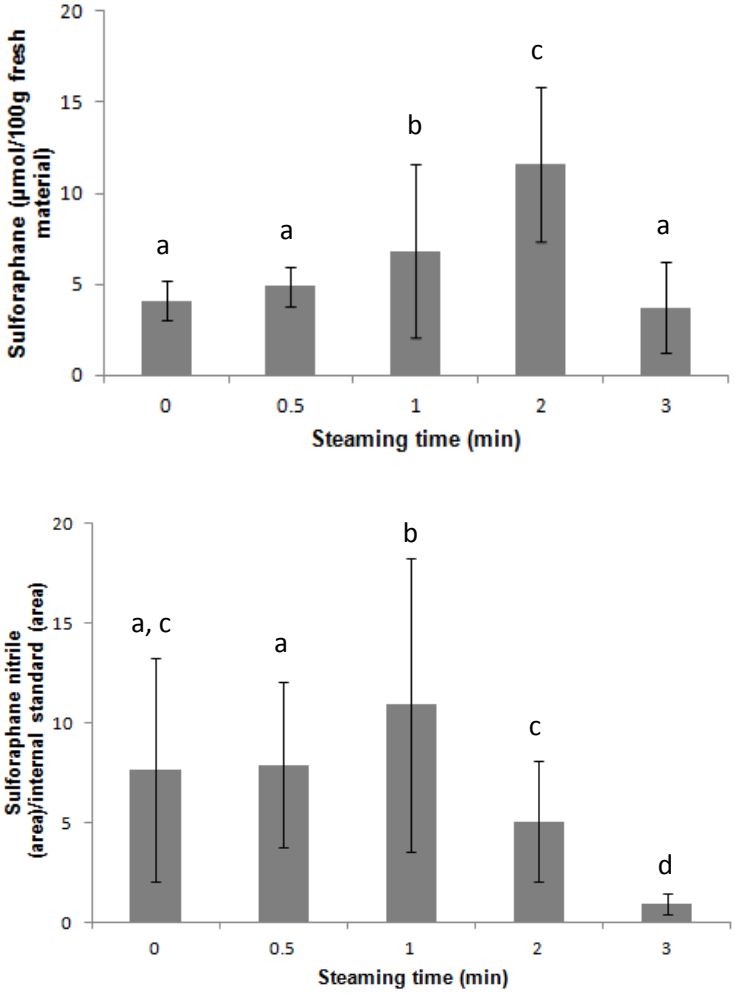

Figure 5.6: Sulforaphane concentration (µmol/100 g fresh material) and sulforaphane nitrile area in raw and steamed (0.5 min, 1 min, 2 min and 3 min) broccoli florets after chewing by 5 volunteers in triplicate (for each steaming time n=15).

The same chewed samples were used to measure the SFN content (Fig 5.6). SFN was only determined qualitatively by the area found, corrected for the internal standard. Significant differences could be found between the steaming times ($p<0.001$). The average area of SFN increased for samples steamed 0.5 min and 1 min compared to the raw chewed broccoli by 46% and 43% respectively. After steaming for 2 min the measured areas of SFN were reduced by 33%. In samples chewed after 3 min of steam treatment only 12% of the areas found in raw broccoli samples were detected.

5.4 Discussion

5.4.1 Experimental design of chewing studies

In the present study volunteers were asked to chew broccoli in a latex bag without presence of saliva. Chewing with food inside a bag could hinder the repositioning and mingling of the food by the tongue and hence might interfere with the natural chewing behavior of the subjects. Nevertheless chewing in an enclosed lab-glove finger was preferred over natural chewing, because it was important to collect all cell components. Especially the leached cell contents were of interest, because here most of the enzyme activity is expected to take place. Literature reports after natural chewing and rinsing of the mouth weight losses of the material of 60% (Peyron, Mishellany, & Woda, 2004). By chewing on a product inside a bag variations caused by saliva are eliminated as well, as saliva can be very diverse in composition and volume between and within subjects (Lentner, 1984).

In literature volunteers are often asked to chew the food till they would swallow it, but instead spitting it out (Lemmens, Van Buggenhout, Van Loey, & Hendrickx, 2010; Mishellany et al., 2006; Peyron et al., 2004). This resulted in various chewing times and cycles between volunteers. Instead in the present study subjects were asked to chew for a fixed time period (11 s, 22 s, 30 s, 40 s) with a fixed frequency (1 chew/s). In the chewing study with five volunteers the time period was set to 22s for each chewing cycle, which was the average time of 20 people chewing raw broccoli before they would swallow it (data not shown). Similar time spans of chewing were found by Hoebler (1998) for bread and pasta with chewing times of 27.0 and 20.3 s respectively. Standardization of the chewing time was done to ensure a comparability between

samples with higher and lower myrosinase activity, as the time is an important factor for the metabolic rate of the enzyme.

It has to be noted that SFN was not quantified. Although the areas were merely divided by the internal standard, the measured areas of SF and SFN were in the same order.

5.4.2 Pilot study: Effect of chewing time on GR and breakdown product concentration

Significant differences between GR concentration in samples of different chewing times could be found for raw samples and steaming times of 0.5 and 1 min (Fig 5.4). A trend to lower GR concentrations with longer chewing times was visible. More cell breakdown is expected to occur with longer chewing times, resulting in more GR and myrosinase being released from the cells enabling hydrolysis of GR as long as the myrosinase is active.

For all chewing times, higher hydrolysis was found for non-steamed broccoli or short steaming times of 0.5 and 1 min, while lower GR hydrolysis was found for broccoli steamed for longer times of 2 or 3 min. This finding can be explained by the residual myrosinase activity in fresh and short steamed broccoli. After cell rupture during chewing, myrosinase hydrolyses glucosinolates. It is remarkable that, although the enzyme activity in the 1-min steamed samples was only 13% of the initial activity, the hydrolysis of GR is comparable to that in raw broccoli. It might be possible that a softer texture due to steaming leads to a quicker breakdown of cells during chewing, which increases cell lysis and leaching of cell contents. The leached GR and myrosinase can get in contact with each other and the GR will be hydrolyzed. In the 2-min steamed samples only a little and in the 3-min steamed samples no myrosinase activity could be found. This explains why the GR content did not decrease during cell rupture by chewing after 3 min steaming and only little degradation, even if not significantly different from the 3-min steamed samples, could be found in the 2 min steamed broccoli.

Between different chewing times no significant difference could be found in the SF concentration or the area measured of SFN. Because the chewing time had no influence on the breakdown product formation, in the chewing study with five volunteers an average chewing time of 22 s was chosen.

5.4.3 Determining the effect of chewing on GR and breakdown product concentration with five volunteers

Each steaming time had high standard deviations for GR, SF and SFN due to the heterogeneity in the way of chewing of different individuals and, to a lesser content, to the processing conditions. Steaming led to a standard deviation of approximately 10% in the characterization of the initial material, but cannot account for the high found standard deviations after processing and chewing.

No significant difference between the chewing samples of the five subjects could be found, hence pooling of the results of one steaming time was possible. In literature conflictive studies are described. Peyron et al (2004) showed that the particle size of food boluses is dependent on the foods (nuts compared to vegetable) and varies little between or among subjects. In contrast another group (Lemmens et al., 2010) found an inter-subject variability in size distribution in boluses during chewing raw and cooked carrot cubes and separated the volunteers in "good chewers" and "bad chewers". Both experimental set ups varied from the present set up by letting the subjects chew the food till they would swallow, instead of a fixed frequency. Mishellany et al (2006) let 10 subjects produce a ready-to-swallow bolus of six foods and documented the duration and sequence of the mastication. They found a high variability between subjects in these mastication parameters.

For GR significant differences between steaming times were found. Broccoli steamed for shorter times (raw, 0.5 min, 1 min) with active myrosinase, contained lower GR concentrations after chewing than broccoli steamed for longer times (2 min and 3 min). These findings confirm the results found in the pilot study with different chewing times (section 5.3.1). A decrease in the GR concentration compared to raw chewed broccoli can be detected for samples after 0.5-min steaming, although myrosinase activity is already reduced to 67% in these samples. This GR decrease might be explained by a texture change of the samples compared to raw broccoli, which may lead to more leaching of cell content. Increased leaching of chewed material after longer steaming times compared to shorter steaming times was detected by an additional conductivity measurement (results not shown). After 1 min steaming more cell content leached out of the cells, but only 13% of the initial myrosinase activity was left to hydrolyze GR. Remarkably this led to nearly the same amount of hydrolysis than in raw chewed broccoli. For 2-min and 3-min steamed broccoli the GR content was 38% and 40% higher respectively, compared to the raw unchewed

broccoli. This suggests a higher release of GR for heat treated samples. Similar results were found in the characterization of the initial material, where the GR concentration increased after 2 min steaming by 29% and after 3 min steaming by 60%. This phenomenon is described in literature (Ciska & Kozłowska, 2001; Verkerk et al., 2010) and explained by an increase in chemical extractability.

The effect of steaming time on the occurrence of SF and SFN found in the chewing study with different chewing durations could be confirmed in the chewing study with 5 volunteers (Fig 5.6). The raw chewed material showed a low content of SF and SFN compared to 0.5-min and 1-min steamed and chewed samples. If broccoli samples are shortly heat treated but myrosinase is still active, as in the 0.5-min and 1-min steamed samples, this leads to more hydrolysis products compared to raw broccoli samples. This can be explained by a lower degree of cell lysis after chewing the raw compared to the steamed broccoli. Approximately 10% of the GR is converted into SF by chewing these samples. After 2 min of steaming the SF content was significantly higher than in the raw broccoli samples, even if the residual myrosinase activity was only 2.5%. This low myrosinase activity led still to 15% of GR degradation. High degradation rates of myrosinase in spite of low active myrosinase could be found as well in literature. In a study dried broccoli with different myrosinase activities were rehydrated. Even with low myrosinase activity the hydrolysis products of GR and glucoiberin, sulforaphane and iberin respectively, are formed (Oliviero, Verkerk, Vermeulen, & Dekker, 2014). This formation of sulforaphane in shortly heat treated samples might be explained by the inactivation of the endogenous thermolabile epithiospecifier protein, in which absence the production of SF is favored over the SFN production (Matusheski et al., 2004; Wang et al., 2012). After 3 min of steaming, very little amounts of SF or SFN were found. This matches the findings of no active myrosinase and therewith no enzymatic degradation in the 3-min steamed samples. The amounts in the 3-min steamed samples reflect the found amounts of SF and SFN in the initial material (Tab 5.1).

5.5 Conclusion

The present study clearly shows that the steaming time has a significant influence on the hydrolysis of GR and the occurrence of the health beneficial component SF upon chewing. The highest SF production after chewing was found in 2-min steamed

broccoli samples, even though the myrosinase activity was very little in these samples compared to raw broccoli. This could lead to a recommendation to steam broccoli for a short time before consumption.

Acknowledgement

The research leading to these results has received funding from the European Community's Seventh Framework Program (FP7/ 2007e2013) under the grant agreement n° FP7-222 654-DREAM. No authors declare a conflict of interest.

Chapter 6

General discussion

6.1 Introduction

GSs are secondary metabolites especially rich in the plants of *Brassicaceae*. Some of their breakdown products, isothiocyanates, which can be formed after rupture of the intact plant cells due to hydrolysis by the endogenous enzyme complex myrosinase or by enzymes from the gut flora, are believed to reduce the risk of several cancers. The most well-known isothiocyanate is SF, one of the breakdown products of GR. The concentration of GSs, isothiocyanates and the myrosinase activity can be influenced by breeding and cultivation, but also by industrial and/or domestic processing (Verkerk et al., 2009). After ingestion, the bioaccessibility of isothiocyanates could be affected by the action of digestion on the vegetable matrix and may be influenced by additional meal components.

One aim of this thesis was to describe the fate of GSs, as one food quality attribute, in the food chain. This research shows that it is possible to combine a chain-approach with interdisciplinary research questions.

Another aim was, to use food technology and nutrition science approaches with different models (*in silico*, *in vitro, in vivo*) as tools. This research shows that these models can be used to monitor and enhance food quality. First, *in silico*, a mathematical model of thermal degradation and leaching was built and fitted to individual GSs concentrations during thermal processing in four different *Brassica* vegetables. Although this model consists of a semi-mechanistic or semi-empirical approach for cell lysis, it could be used to describe the fate of GSs during processing. Furthermore, the differences between the individual reaction rate constants and activation energies of different GSs in various cultivars could be compared. Second, the bioaccessibility of GR and SF was investigated during digestion with an *in vitro* digestion model. It was revealed that the oral phase is the most important phase of GSs hydrolysis by the endogenous myrosinase. Third, the fate of GR was explored *in vivo* during chewing of broccoli as a representative for *Brassica* vegetables, in a study with five subjects.

This discussion begins with the main findings of this research, followed by methodological considerations and discussion and interpretation of the results. Finally, future prospects and main conclusions of the thesis are described.

6.2 Main findings

In **chapter 2** a mathematical model describing the fate of GSs during thermal processing was proposed and the individual rate constants and activation energies of this model were estimated from experimental results obtained with GSs from red cabbage, white cabbage, broccoli and Brussels sprouts in **chapter 3**. Different GSs from the same *Brassica* vegetable after thermal processing showed different degradation rates, e.g., glucobrassicin was degraded quicker than glucoraphanin in broccoli. Furthermore, the thermostability varied for the same GSs, not only in different media such as the intact vegetable tissue or the cooking water, but also in different *Brassica* vegetables, for instance, glucobrassicanapin in Brussels sprouts was less stable compared to the same GS in white cabbage.

The results of GR hydrolysis and occurrence of SF and SFN are shown in **chapter 4** during *in vitro* digestion of raw and steamed broccoli (1 min, 2 min and 3 min). Enzymatic hydrolysis by the endogenous myrosinase took place only during the oral phase. The low pH of the gastric phase inactivated myrosinase irreversibly. The SF concentration, produced in the oral phase, and the remaining GR concentration were constant throughout the digestive model. The addition of olive oil or bovine serum albumin to the digestive system did not influence the hydrolysis GR or the formation or bioaccessibility of SF.

During the *in vivo* chewing study presented in **chapter 5**, the GR hydrolysis by the endogenous enzyme myrosinase and the formation of SF and SFN was studied during chewing of raw and steamed (0.5 min, 1 min, 2 min and 3 min) broccoli. Even low amounts of active myrosinase, e.g., 2.5% of the initial activity in the 2-min steamed samples caused substantial GR hydrolysis. No significant differences between subjects could be found, but steaming time showed a clear influence on GR hydrolysis and SF formation. The highest SF concentration could be found in 2-min steamed broccoli.

6.3 Methodological considerations

6.3.1 Mathematical model *in silico*

Mathematical modelling can be used in food science to describe reactions important for quality attributes, such as colour, or quality performance indicators, such as

lactulose concentration as indicator for the intensity of heat treatment in milk (van Boekel, 2009). In addition, changes in quality attributes and quality performance indicators can be controlled and predicted with mathematical models as tools (van Boekel, 2009). They can be implemented in quality management systems, such as hazard analysis critical control points (HACCP) to predict critical control points, ISO systems and good manufacturing practice (GMP) (van Boekel, 2009). Even if mathematical models can be helpful tools to predict and control food quality, foods are very complex systems in a physical and chemical sense (van Boekel, 2009). To involve all possible factors in a model would result in an overparameterisation and lead to models with limited validity. To prevent too extensive models and focus on the key factors influencing quality attributes, it would be useful at times, to employ systems mimicking foods (van Boekel, 2009).

In the mathematical model of the fate of GSs during thermal processing, a total of eleven parameters (rate constants and activation energies of thermal degradation of GSs in vegetable and cooking water as well as for leaching, the initial concentration of GSs in the cooking water and in the vegetable) were estimated. If all parameters would be estimated with the same data set, the parameters would be less accurate. To increase the accuracy of the parameters, two different experimental set-ups were chosen to study the mechanisms of thermal degradation and leaching independently where possible.

Cell lysis was described in the mathematical model of the fate of GSs in *Brassicaceae* during thermal processing (**chapter 2**) as a first order differential equation, as described in previous research (Verkerk, 2002) and confirmed by former experiments in our group (unpublished results). Leaching is described in the model by a steady state between the cell content of the lysed cells and the water surrounding the tissue. In reality, leaching of GSs into the cooking water is an accumulation of different processes due to chemical/physical changes in the cell wall and cell and organelle membranes. The chemical changes are influenced by environmental conditions such as pH and the presence of ions, but also by the presence of polysaccharides, which can increase an uptake of water in the cell walls and therewith reduce the cohesiveness of the cell wall matrix (Rao & Lund, 1986). The estimated rate constants for lysis are therefore not real lysis rate constants, but lumped parameters (van Boekel, 2009) including diffusion rates. For larger pieces of vegetables, e.g. florets of broccoli, diffusion limitation might limit leaching rather than the rates of membrane and cell

wall degradation. Because of these limitations in the estimation of the parameters, the model is not a purely mechanistic model, but a semi-empirical or semi-mechanistic model.

For the GSs concentration in cooking water, the data set was limited to two temperatures (85 °C and 100 °C) due to the possibility of myrosinase activity in the two lower temperatures (50 °C and 65 °C). Because of this reduced data set, the activation energy of thermal degradation in cooking water could mostly not be estimated or had high standard deviations (glucobrassicin, 4-methoxyglucobrassicin and neoglucobrassicin in white cabbage). Literature data on the activation energy of GSs in cooking water is not present, but if the differences in the thermal degradation of GSs are determined by the cell content and cell matrix, then similar activation energies are expected for GSs in cooking water.

The rate constants of leaching of the four *Brassica* vegetable were not significantly different from each other. This was to be expected, as red cabbage, white cabbage and Brussels sprouts were comparable in sample size (cut into stripes). As soon as the membrane breaks and the cells walls get permeable, all GSs were expected to have a similar leaching behaviour. In contrast, broccoli was prepared as florets, and because of their bigger size, was expected to leach slower into the cooking water. However, this was not the case. The similar leaching rates between broccoli and the cut *Brassica* vegetable stripes might have been caused by the softened cell matrix of broccoli due to cooking. The leaching rate constants would result from the kinetics of cell matrix softening, which is highly temperature dependent, and diffusion, which is not very temperature sensitive.

It cannot be assumed that the estimated parameters in **chapter 3** would be the same in a different cultivar, in a different year (Hennig et al., 2012) or grown under other environmental conditions (Verkerk et al., 2009). Various *Brassica* cultivars could be grown under different conditions (e.g. lighting hours, fertilisation of the ground, exposed to insects), the GSs profiles determined in raw and processed vegetables and the data used to predict the parameters, to test the influence of these factors on the parameters. Nevertheless, the present model could be used to describe the reactions of GSs during thermal treatment and the differences between the parameter of various cultivars could be compared.

A validation of this model for real industrial processes is recommended, after which the production processes can then be optimized in terms of GSs. For consumers it is recommended to steam *Brassica* vegetables shortly, before eating. It would be further interesting to include texture changes during heat treatment and its correlation to GSs and/or myrosinase release, from the cell into the cooking water. Consumers will use other criteria for vegetable quality (like texture, taste and colour) when preparing them, besides health aspects. Therefore it is important to design optimal preparation methods by integrating these quality aspects as well, for example as done in the study of Bongoni et al (2014) on broccoli.

6.3.2. *In vitro* digestion model

The used static *in vitro* model (**chapter 4**) is a simplification of the digestion in the human body. The digestion was especially simplified in terms of movement, and hence mechanical stress on the vegetable matrix, and pH.

The complex movements of the human digestion, from chewing in the mouth to grinding and peristaltic movements in the stomach and intestine, were not mimicked in the present *in vitro* research. Especially the cutting, grinding and movement of broccoli by the tongue in the mouth would have been interesting to mimic more precisely, as all SF formation took place there. Mincing of broccoli, as advised by Minekus et al., (2014) was considered, but resulted in total hydrolysis of GR due to the large residence time in the machine, and was therefore omitted. Instead, frozen blended broccoli was immersed in warm saliva and shaken at 37 °C for 5 min. This resulted in a similar GR and SF profile after the mouth phase *in vitro* as in the *in vivo* chewing study (**chapter 5**).

The pH of the digestion model was static in each digestion stage. In the mouth phase the pH was 6.8 after addition of artificial saliva to ground broccoli, in the gastric phase the pH was adjusted to 2 and in the intestinal phase to pH 6.5. *In vivo,* especially the pH of the stomach varies during digestion. In the empty human stomach the pH is approximately 2, but when food enters the stomach, the pH rises quickly to pH 5 and decreases over approximately 1 hour to pH 2 again, depending on the food (Malagelada, Fongstreth, Summerskill, & Go, 1976). For the hydrolysis of GSs the pH of the stomach plays an important role, because up until pH 5 myrosinase is active and can hydrolyse GSs. In contrast, at a constant pH = 2 as in the present *in vitro* digestion model, myrosinase is quickly and irreversibly inactivated. Recently, a

standard *in vitro* digestion model was proposed (Minekus et al., 2014), in which a static pH of 3 was proposed for the stomach.

In contrast to the *in vitro* results, it might therefore be possible that *in vivo* glucosinolate hydrolysis can also occur after the oral stage of the digestion, if active myrosinase is still present in the ingested broccoli. Further research with a dynamically varying pH especially in the gastric phase *in vitro* could give further insight into gastric GSs hydrolysis. This could be done e.g. by titration of acid into a vessel with gastric conditions. Furthermore, no colonic microflora was included in the digestion model, although it is known that *in vivo* colonic microflora leads to further hydrolysis of intact GSs. To see the whole picture of GS hydrolysis during digestion, this step should be included in the *in vitro* digestion as well, for instance by incorporating a digestion step with intestinal bacteria.

Other food components could be tested whether or not they have an influence on the GS and isothiocyanate bioaccessibility. BSA and olive oil were chosen as standard protein and lipid. Other proteins or phytochemicals, or lipids in emulsion could be tested as well.

6.3.3. *In vivo* study

A study was performed to get a better insight in the extent of cell rupture and the resulting GR hydrolysis during chewing (**chapter 5**). Raw and steamed (1 min, 2 min and 3 min) broccoli was chewed by 5 volunteers, standardized in terms of chewing frequency (1 chew/s) and duration (10, 22, 30 or 40 s). This standardization of time is an intervention of the natural chewing behaviour. It was chosen to get comparable results concerning the various myrosinase activities, as the residence time is an important factor for the metabolic conversion by the enzyme.

Another interference with the natural chewing behaviour was the choice to let the subjects chew on broccoli enclosed in latex glove fingers. This way, the natural mixing of the food by the tongue cannot be assured, but it was necessary to get all cell compounds of the chewed broccoli. Particularly the leached cell contents, in which most of GR hydrolysis was expected, would have been lost to a large extent otherwise. Saliva could have an effect on the fate of GSs in the oral phase, for instance by amylase binding to GSs or its breakdown products. But because the contents of

human saliva can vary drastically inter- and intra-individually (Lentner, 1984), saliva was left out of the experimental set-up to have a standardized procedure.

6.4 Discussion and interpretation of results

The aim of this research was to study the fate of glucosinolates from raw *Brassica* vegetable till the gut wall, using different modelling approaches. To achieve this goal, an interdisciplinary approach was necessary (another aim of this study) and different models were used as tools.

Representing the processing of food with a mathematical model of the fate of GSs during thermal treatment as in **chapter 2 and 3**, is a typical research approach in food technology to simulate and optimize food processing. Otherwise the *in vitro* and *in vivo* study described in **chapter 4 and 5**, respectively, are nutritional or food science approaches to get insight into the degradation of GR and the production of SF in the mouth and their bioaccessibility. The integration of both fields can increase the knowledge of the fate of GSs during industrial or domestic thermal treatment, during chewing and during the digestion till they reach the gut wall. This chain approach helped to identify the oral phase as a key stage of GSs hydrolysis during digestion, if active myrosinase is still active (for raw and shortly steamed vegetable). The integration of two fields can be sometimes challenging, as priorities can be different between food technologists and nutritionists, but for this research, and for my personal development as a scientist, it was very beneficial.

In this research, different models were used as tools. Any kind of models, if *in vitro*, *ex vivo* or *in silico*, can be used to represent a simplified reality. The better the model is adjusted to the underlying mechanisms and to the research question, the nearer is the output of the model to the reality. On the other hand a model should be as simple as possible. Mathematical models should be simple, to have no over parameterization, which could lead to an indiscriminate model and large variances of the parameters (principle of parsimony) (van Boekel, 2009). In addition, it would be very challenging and laborious to include all possible mechanisms and this would lead to a very complex mathematical model. In the present research, the used mathematical model can be seen as a semi-mechanistic or semi-empirical model. If *in vitro* models are kept simple, it is easier to analyse the samples and interpret the results, furthermore less labour is needed and therefore expenses are lower.

The present mathematical model could be used by the food industry to improve their foods by processing them in a way that leads to more beneficial GSs profiles. In addition, epidemiological studies could be improved with these kinds of models (Dekker et al., 2000). Epidemiological studies, for instance, on the prevention of fruit and the association between vegetable consumption and cancer, show often inconsistent results (Benetou et al., 2008; Boffetta et al., 2010; Löf et al., 2011; Steinbrecher et al., 2009). Besides other reasons, one of the explanations of this inconsistency could be the fact that fruit or vegetable intake is taken as a variable, while we know that the phytochemical content of fruit and vegetable products can vary with the way they are processed. Therefore epidemiological studies do not consider the real intake of potential cancer-preventive components in processed or prepared foods (Dekker & Verkerk, 2005). Often either the food intake is just associated with cancer incidence, or, if the content of a assumed cancer-preventive component is measured, then concentrations are measured in unprocessed foods or average values of processed foods are used. For GSs, the content in the food will change dramatically with processing (temperature, time, cutting size and water used) and would cause a high variability between the real GSs intake of subjects. If instead the modelled contents per subject are used, this would lead to an enhanced sensitivity of the epidemiological study (Dekker & Verkerk, 2005). For using such a model it would be necessary to include information on processing and preparation in the data gathering step in epidemiological studies. Variability due to cultivar and cultivation would still be present in the data. Nevertheless, such a model would still only mimic the real GSs content in the food, but it would reproduce the variability between subjects.

In **chapter 4**, the influence of food components on the bioaccessibility of GR, SF and SFN was tested during *in vitro* digestion. Our starting hypothesis of food components reacting with GR, SF and SFN could not be shown in our study, instead steaming time affected the bioaccessibility significantly.

6.4.1 Relating thermal processing to health aspects

To compare samples with different myrosinase activities, all experiments were performed with raw and heat treated *Brassica* vegetables, in which myrosinase was partly or fully inactivated. Heat treatment changes not only the myrosinase activity in *Brassica* vegetables, but has also various effects on the processed vegetable and the

production of isothiocyanates. These effects are summarized in figure 6.1 and are explained in the following.

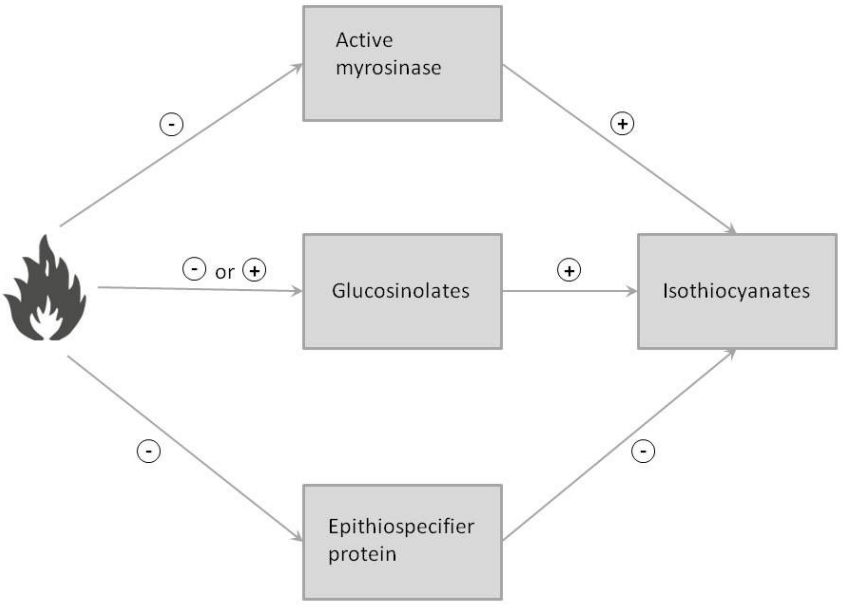

Figure 6.1: Positive (+) and negative (-) influence of thermal processing on the formation of isothiocyanates in *Brassica* vegetable.

Heat treatment has a direct influence on the GSs concentration of *Brassica* vegetables. On the one hand, cell walls soften after heat treatment. This softening could be caused by an uptake of water by polysaccharides, which can reduce the cohesiveness of the cell wall matrix and decrease the intercellular adhesion (Rao & Lund, 1986) and chemical breakdown of cell wall components. It was hypothesised that this softening of the cell walls explains the higher leaching rates of longer steamed and chewed broccoli, compared to shorter steamed and chewed broccoli samples, which was detected in the *in vivo* chewing study (**chapter 5**).

On the other hand, heat treatment appears to have a direct effect on the GSs content found in vegetables. In a study by Verkerk et al. (2004) increasing total GSs concentrations, and especially sinigrin concentrations, after different microwave treatments in red cabbage were found. This increase was explained by a higher

chemical extractability due to heat treatment. Experiments with steamed broccoli by the same authors showed also an increase in GSs concentration compared to the raw material, with a maximum at 10 min steaming (Verkerk et al., 2010). Ciska & Kozłowska, (2001) detected an increase in GS content in cooking water and cabbage after 15 min up to 30 min of cooking. They hypothesized that GSs are bound to cell walls and are released after thermal treatment. This increase in chemical extractability could explain the results of the longer steamed broccoli samples in the *in vitro* chewing study (**chapter 5**). GR concentrations for raw, 0.5 min steamed and 1 min steamed broccoli after chewing were similar, but the SF and SFN contents for 1 min steamed and chewed broccoli were significantly higher than of raw and 0.5 min steamed and chewed broccoli. Furthermore the GR concentration of 2 min and 3 min steamed broccoli were, compared to raw broccoli, approximately 40% higher. This suggests a higher release of GR in heat treated samples.

Heat treatment for 10 min at 50 °C was shown to significantly decrease the endogenous ESP (Matusheski et al., 2004). In literature, active ESP and iron ions directed the myrosinase catalyzed hydrolysis reaction of purified GR towards SFN (Matusheski et al., 2006). The presence of ESP could explain the results described in the *in vivo* chewing study (**chapter 5**). Increasing SF concentrations could be detected in the 1-min and 2-min steamed and chewed broccoli samples. Broccoli steamed for 1 min and 2 min reached maximum core temperatures of approx. 60 °C and approx. 90 °C, respectively. This would suggest a partly inactivation of ESP after 1 min and further inactivation after 2-min steaming, while myrosinase is still active, causing a preferred production of SF over SFN. The data of the SFN content was not as clear. Similar reasoning is described in literature. In a study with four heat treated broccoli cultivars, the SF and SFN concentration were analyzed after chopping and autolysis in water (Wang et al., 2012). All but one cultivar showed an increase in SF concentration after 1 min steaming, but after 5 min steaming all SF concentrations were lower or the same as in raw broccoli. SFN concentrations decreased with steaming time. Differences in the time window for heat treatments to enhance SF concentrations in broccoli were found (0-3 min steaming) between the cultivars. It was concluded that these differences among cultivars are caused not only by different initial GR concentrations, but also by differences in myrosinase and ESP.

Effects as the increased chemical extractability of GSs after heat treatment and the inactivation of epithiospecifier protein, could be observed in the experiments, but were not studied individually.

6.4.2 Are there negative health effects of GSs?

Besides the beneficial health effects of GSs, they need also to be discussed for their negative health effect. Goitre as well as malfunction of kidneys and liver were found after feeding high levels of GSs to animals (Verkerk et al., 2009). In a few *in vitro* animal studies, hydrolysis products of neoglucobrassicin from broccoli extracts were found to be mutagenic (Latté, Appel, & Lampen, 2011). Feeding of 600 g raw broccoli to pigs, caused an increase in DNA strand breaks by 50% (Lynn, Collins, Fuller, Hillman, & Ratcliffe, 2007). In a study in which mice and rats were fed raw or steamed broccoli (on average 6 g/day and 45 g/day, respectively; steamed broccoli with residual myrosinase activity), one definite and a further possible spot for DNA adduct could be determined in broccoli preparations by ^{32}P-postlabelling and multidirectional thin-layer chromatography (Baasanjav-Gerber et al., 2010). Because the mice and rats had free access to raw and steamed broccoli supplementary to the standard laboratory feed, it is not known how much each animal ingested. In a different study, broccoli derived neoglucobrassicin and its breakdown product neoglucobrassicin alcohol were orally administered to mice and resulted in the formation of DNA adducts (Schumacher et al., 2014). The lowest given dose was 20 μmol/kg body mass, which would relate in humans (50 kg) to a dose of 1 mmol. 100 g broccoli contains between 4–170 μmol indole GSs, which are in broccoli mainly neoglucobrassicin and glucobrassicin (Verkerk et al., 2009).

Mutagenicity of neoglucobrassicin breakdown products was also shown in *in vitro* studies in *Salmonella typhimurium* strains TA 100 with pak choi sprout juice (Wiesner, Schreiner, & Glatt, 2014), and with fresh broccoli juice or vegetable homogenates (Baasanjav-Gerber, Hollnagel, Brauchmann, Iori, & Glatt, 2011; Glatt et al., 2011). However, the correlation between mutagenicity in these cell studies and in the human body has to be established.

In low amounts, an increase of GSs in the diet will have a beneficial health effect. Further intake does not increase this positive health effect and increasing the daily intake will lead to negative health effects (Holst & Williamson, 2008). The range of the beneficial GSs intake is not known, but the daily doses over a longer time span

necessary for humans to develop a goitre was calculated by Watzl (2001) to be very high: either 400 g cabbage, 2 kg china cabbage or 2.8 kg horseradish, together with a insufficient iodine intake. In an animal study with rats, for GR doses up to 60 mg/kg no negative health effects could be found (Lai, Keck, Wallig, West, & Jeffery, 2008). Higher doses caused an inflammation of the cecum. This harmless dose would relate in humans (50 kg) to a dose of approximately 6900 μmol GR. In 100 g fresh broccoli concentrations in literature were found to be approximately 100 times lower (between 40 and 71 μmol) (Kushad et al., 1999; Verkerk et al., 2010).

For humans neither genotoxic nor goitrogenic effects could be observed after regular consumption of broccoli, which is rich in neoglucobrassicin compared to other *Brassica* vegetables (Latté et al., 2011). If the dosage used in the animal studies that showed genotoxic effects would be transferred to human diets, the dosage would be exceptional high. It can be concluded that negative health effects of GSs might be caused by very high concentrations of GSs, especially of neoglucobrassicin, and in the presence of active myrosinase, but with a normal diet including *Brassicaceae* these high concentrations will most probably not be reached. To estimate the safe daily intake dose of GSs in humans, more research is necessary.

6.4.3 Recommendations and future research

Besides the discussed health promoting effect of GS breakdown products, *Brassica* vegetables promote health by being low caloric, contain high contents of fibers, vitamins (tocopherol, ascorbic acid, folic acid), minerals (Cu, Zn, P, Mg, among others), and different groups of phytochemicals such as indole phytoalexins, carotenoids and phenolics (Jahangir, Kim, Choi, & Verpoorte, 2009).

To compare different *Brassica* cultivars, it would be useful to have an index for the healthiness of *Brassica* vegetable. An index for antioxidant nutritional quality was proposed in literature for tomatoes (Frusciante et al., 2007). For the antioxidant potential, lycopene, beta-carotene, other carotenoids, tocopherol, flavonoids, phenolic acid, ascorbic acid and dry matter as indicator of fibers were considered. The index (I_{QUAN}) was calculated by building a product of the measured component concentration (C_x) times a weighing factor (K_x) and dividing it by an optimum concentration (C_{Opt}) that was arbitrarily defined as the average literature concentration (C_a) increased by 50%. The sum of all considered components led to an index of nutritional quality for the species of tomatoes. The weighing factor was assigned

considering the following points: first, the contribution that tomato gives for the intake of that particular nutrient in the diet, second, the scientific evidence for a health effect of that component, and third, the bioavailability of the compound.

A similar indicator could be used for each *Brassica* vegetable, to access the healthiness of *Brassica* vegetables. For broccoli, for instance, the considered components could be ascorbic acid, α- and β-carotene, xanthophylls, tocopherol, selenium, quercetin, kaempferol, 2-caffeoyl-quinic acid derivatives and cinnamic acid derivatives and GSs, especially glucoraphanin and glucobrassicin (precursor of indole-3-carbinole) (Jahangir et al., 2009; Latté et al., 2011; Mahn & Reyes, 2012).

Only components with significant concentrations related to their health effect should be considered for each indicator. A possible way to calculate an indicator for the overall healthiness of *Brassicaceae* (I_{OHB}) is proposed in equation 1. For each component, the difference between the optimal concentration (C_{Opt}) and the real concentration found in the vegetable (C_x) is set in relation to C_{Opt} and multiplied by the weighing factor K_x. These equations for all components are summed and divided by the number of components considered for that vegetable (n).

$$I_{OHB} = \sum \left(\frac{|C_{Opt} - C_x|}{C_{Opt}} K_x \right) / n \qquad \text{(Eq. 1)}$$

As C_{Opt} for minerals and vitamins, an average recommended daily intake dose could be used, as, for instance, the DACH reference values for nutrient intake (Germany, Austria and Switzerland) or recommended daily allowance (RDA for USA and Canada), which is then multiplied with the percentage that the *Brassica* vegetable accounts for the average component intake (e.g., calculated from national nutritional surveys like the German national dietary study 2 (Max-Rubner-Institut, 2013)) and corrected for 100 g fresh weight. For components for which no daily recommendations are established, the C_{Opt} as proposed for the I_{QUAN} could be used.

The weighing factor K_x, as proposed for the I_{QUAN} for tomatoes, is the most important modulator for the healthiness indicator. For the I_{QUAN} a weighing factor between 5 and 20 was chosen. The weight of the K_x of the I_{QUAN} was partly determined by scientific evidence. This would be also very important for the I_{OHB}, as especially in the

field of phytonutrients a lot of research is still necessary to correlate health benefits with specific compounds. Thus, even knowledge from small studies can be taken into account, by giving it less weight than correlations proven by epidemiological studies. The bioavailability of that compound had a further impact on the I_{QUAN}. Bioavailability of most phytonutrients is not known and can change in different life conditions, it is, for instance, known that iron bioavailability increases during pregnancy (Barrett, Whittaker, Williams, & Lind, 1994) or at a low supply level (Hallberg & Hulthén, 2002). Even if the bioavailability of a health component is known, a high bioavailability will get a high weight. Ascorbic acid, for instance, has a high bioavailability and would get a high weight. But because it has a high bioavailability and is common in our diet, it can be ingested easily with other foods, such as citric fruits, cabbage and potatoes. Instead of bioavailability, bioaccessibility of components could be considered. The bioaccessibility would on the one hand exclude the intra-individual differences due to their life conditions, but on the other hand include the interaction of some food components that increase or decrease the bioavailability. Examples are carotenes, which are more bioaccessible if fat is ingested with the vegetable as well. In contrast, phytates, GSs and phenols are known to reduce the bioaccessibility of some minerals by forming complexes (Matthäus & Angelini, 2005).

The synergistic effect that GSs, selenium and flavonoids might have on cancer prevention (Mahn & Reyes, 2012), cannot be taken into account, as too little is known about this.

Up to now research about the desired intake levels of phytochemicals is scarce and the weight of the weighing factor highly debatable, why such an indicator could not be used in the near future.

6.5 Main conclusion

This research described the fate of glucosinolates from *Brassica* vegetables from the raw vegetable, during thermal processing and digestion till the gut wall. It could be shown that such an approach is possible, and different models were useful for different stages. This chain approach allows studying one food component, as a quality attribute of GSs containing foods, during different stages of processing and digestion. The gained knowledge can be used on the one hand to decide, which stages

are of interest for further investigation, and on the other hand to improve further epidemiological studies, by correcting for the effect of processing and preparation on GSs.

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Summary

Background

Glucosinolates (GSs) are secondary plant metabolites especially rich in our diet in *Brassicaceae*, such as Brussels sprouts, cabbage and broccoli. If the tissue of *Brassica* vegetable is disrupted, e.g., by cutting or chewing, the differently located endogenous enzyme myrosinase can get in contact with the GSs and hydrolyze their substrates into nitriles, epithionitriles, thiocyanates or isothiocyanates, depending on the environmental conditions. Isothiocyanates are believed to reduce the risk of several cancers. The most well-known isothiocyanate is sulforaphane (SF), one of the breakdown products of glucoraphanin. The concentration of GSs and isothiocyanates, and the activity of myrosinase in a meal can be influenced by industrial or domestic processing. After ingestion, the bioaccessibility of isothiocyanates and GSs could be affected by digestion fluids or food components.

Aim

The aim of this research was to investigate the fate of GSs from processing to digestion in *Brassicaceae*. For this aim, different models were used as tools: a mathematical model to investigate the fate of GSs during thermal processing, an *in vitro* digestion study to investigate the bioaccessibility of GSs and its breakdown products during digestion, and an *in vivo* study to examine the effect of chewing behavior of five subjects on GSs degradation.

Results

A mathematical model of the fate of GSs during thermal processing was described in **chapter 2**. For the fate of GSs from *Brassica* vegetables during thermal processing, three mechanisms were important and described as mathematical equations: enzymatic hydrolysis by myrosinase, thermal degradation of GSs and myrosinase and leaching of GSs and myrosinase into the cooking water. In **chapter 3** this model was used to estimate and compare reaction rate constants and activation energies of individual GSs during cooking of Brussels sprouts, red cabbage, white cabbage and broccoli. Because during cooking myrosinase was not active any more, the equations for enzyme activity were omitted. In comparison between the parameter of the different *Brassica* vegetable

not only a difference between the thermostability of several GSs in one vegetable could be found, but also between the same GSs in different vegetable tissues. The environment in the cells or cell components of the different cultivars is likely to have an influence on the thermostability of GSs. Furthermore, thermostability of GSs varied in different media, such as the intact vegetable tissues or the cooking water. This mathematical model and the estimated reaction rate constants and activation energies can be used to simulate the different GS contents in prepared foods, considering the processing method. This should be a useful tool to make predictions about the nutritional quality of foods and could be used by the industry to optimize the health related quality attributes in the food. The model could be used by future epidemiological studies as well, as instead of using the GS content of unprocessed foods or mean values for processed foods, the GS content could be corrected for the effect of processing individually. It was shown that processing influences the GS content in food highly (Dekker & Verkerk, 2003) and different processing methods will lead to a high variability of GS content in the food. With the modelled values for individual GS intake the sensitivity of the study could be enhanced by decreasing the variability of GS intake between subjects.

In an *in vitro* digestion study (**chapter 4**) with raw and steamed broccoli, the influence of meal composition and thermal processing on the fate of glucoraphanin (GR) was investigated. The addition of meal components, such as bovine serum albumine and olive oil, did not affect the bioaccessibility of GR, SF or sulforaphane nitrile (SFN). The main formation of SF and SFN took place in the oral phase. In the gastric phase myrosinase was inactivated irreversibly due to the low pH and no more GR was hydrolyzed in further digestion steps. In raw and 1-min steamed broccoli with active myrosinase (100% and 14%, respectively), up to 10-times higher SF concentrations could be found after the oral phase than in longer steamed broccoli containing very low or no detectable myrosinase activity. Because the myrosinase activity was determined by the thermal treatment, steaming time was the main influence on GR hydrolysis and SF production.

In an *in vivo* chewing study with raw and steamed broccoli, the influence of chewing time and steaming time on the fate of GR in the mouth were investigated (**chapter 5**). To determine the effect of chewing time, five subjects chewed raw and steamed broccoli florets for different times, with the same frequency (one bite per second).

During chewing of raw and shortly steamed broccoli (up to 1 min), the longer the chewing time was, the more GR was hydrolyzed by the endogenous enzyme myrosinase. Longer steaming times (2 min and 3 min), with only very little or without active myrosinase, were not affected by longer chewing times. In a second study, five subjects were asked to chew raw and steamed broccoli for the same time, with the same frequency. It could be shown that steaming time influenced the hydrolysis rate of GR, with the highest hydrolysis rate of GR in 0.5-min steamed broccoli. The highest production of the health beneficial compound SF after chewing was found in 2-min steamed broccoli samples, even though the myrosinase activity was very little in these samples compared to raw broccoli. This shows that myrosinase is present in excess in broccoli, and even if only very low amounts of myrosinase are still active (2.5% of initial activity in 2-min steamed broccoli), SF can be formed in significant amounts.

Conclusion

A mathematical model for the fate of GSs during thermal treatment was proposed and with it were the rate constants and activation energies of red cabbage, white cabbage, Brussels sprouts and broccoli estimated (with the exception of the activation energy of GSs in cooking water). The thermostability of GSs was found to be not only different between individual GSs, but also for the same GSs in different vegetable or in different media (vegetable or water).

Heat treatment had also a significant effect on the fate of GR after ingestion. In an *in vivo* chewing study it was found that chewing time as well as steaming time had an influence on GR hydrolysis and formation of the heath promoting SF. SF concentrations in the *in vivo* chewing study as well as in the *in vitro* digestion study were found to be highest for shortly steamed broccoli, even though these samples contained only low amounts of myrosinase. The *in vitro* digestion showed furthermore that, if the food still contained active myrosinase, the main GR hydrolysis took place during the oral phase and that myrosinase was inactivated in the gastric phase. Because bovine serum albumine and olive oil had no effect on the GR hydrolysis or bioaccessibility of GR, SF or SFN, it was concluded that the meal preparation is more important for the fate of GR than the meal composition.

In this research it could be shown that an interdisciplinary chain-approach is possible, and that different models can be useful for different stages during processing and digestion. This chain-approach enables the study of the fate of GSs, as an example of a food quality attribute, from the vegetable to the gut wall. The findings could identify key stages of the hydrolysis of GSs during digestion, could be used by the industry to improve their products in terms of GSs and help to improve further epidemiological studies. Furthermore, the possibility to monitor and enhance food quality attributes with such models could be shown.

Samenvatting

Achtergrond

Glucosinolaten (GSn) zijn fytochemicalien, die met name in Brassicaceae groenten aanwezig zijn, zoals spruitjes, koolsoorten of broccoli. Als het weefsel van de groente wordt beschadigd, zoals door snijden of kauwen, kan het endogene enzym myrosinase, dat zich in de cellen gescheiden van de GSn bevindt, een hydrolyse reactie aangaan. De producten die hierbij gevormd worden zijn nitrillen, epithionitrillen, thiocyanaten of isothiocyanaten, afhankelijk van de omstandigheden van de reactie. Isothiocyanaten worden gezien als stoffen, die het risico op verschillende kankers kunnen reduceren. Het meest bestudeerde isothiocyanaat is sulforaphane (SF), een van de afbraak producten van de glucosinolaat glucoraphanine (GR). De hoeveelheid van GSn, isothiocyanaten en de activiteit van myrosinase in een maaltijd is afhankelijk van industriële of huishoudelijke verwerking- en bereidingsprocessen van de Brassica groenten. Na de consumptie kan de biologische beschikbaarheid van de isothiocyanaten en GSn worden beïnvloed door spijsverteringssappen of door interactie met andere voedselcomponenten.

Doel van dit proefschrift

Het doel van dit onderzoek was het bestuderen van het lot van GSn in Brasssica groenten vanaf verwerking tot aan de voedselopname. Voor dit doel werden verschillende modellen gebruikt: een wiskundig model, om het lot van GSn tijdens thermische verwerking van de groenten na te gaan, een *in vitro* spijsverteringsmodel, om de biologische beschikbaarheid van GSn en afbraakproducten te onderzoeken en een *in vivo* model, om de vorming van afbraakproducten tijdens het kauwen van de groenten bij vijf proefpersonen te onderzoeken.

Resultaten

Een realistisch wiskundig model van het lot van GSn tijdens de thermische verwerking wordt beschreven in **hoofdstuk 2**. Voor het beschrijven van het lot van de GSn in Brassica groenten tijdens thermische verwerking, zijn drie mechanismes belangrijk. Deze mechanismes werden in wiskundige vergelijkingen uitgedrukt: enzymatische hydrolyse door myrosinase, thermische afbraak van GSn en inactivering van myrosinase en het uitlekken van GSn en myrosinase in het kookvocht. Dit model

wordt toegepast in **hoofdstuk 3** om de parameters te schatten tijdens het koken van spruitjes, rode kool, witte kool en broccoli. Omdat tijdens het koken myrosinase snel wordt geïnactiveerd, worden de vergelijkingen voor de enzym activiteit niet meegenomen in de modelbeschrijving. Bij het vergelijken van de verschillende parameters wordt vastgesteld dat niet alleen de thermische stabiliteit van verschillende GSn in een groente verschillend zijn, maar ook die van dezelfde GSn in verschillende groenten. De cellulaire omgeving of cel componenten van de verschillende cultivars beïnvloeden waarschijnlijk de thermische stabiliteit. Verder varieerde de thermische stabiliteit van GSn in verschillende media zoals bijvoorbeeld tussen het onbeschadigde groenteweefsel of het kookvocht. Dit wiskundige model en de geschatte parameters kan gebruikt worden om de GS concentraties in bereid voedsel met verschillende bereidingsmethoden en condities te simuleren. Deze simulatie studies kunnen een nuttig hulpmiddel zijn bij voedingsonderzoek voor de voorspelling van de nutritionele kwaliteit van voedsel en voor de industrie teneinde de gezondheidswaarde van het verwerkte product te optimaliseren.

In een *in vitro* studie (**hoofdstuk 4**) met rauwe en gestoomde broccoli, werd de invloed van de samenstelling van de maaltijd en de thermische behandeling op het lot van de GSn onderzocht. De toevoeging van het eiwit bovine serum albumine of olijfolie hadden geen effect op de biologische beschikbaarheid van GR, SF of sulforaphane nitril (SFN). De vorming van SF en SFN vond vooral plaats in de mond tijdens het kauwen van de groenten (orale fase). In de maag fase werd myrosinase door de lage pH irreversibel geïnactiveerd waardoor er geen GR in de volgende verteringsstappen gehydrolyseerd wordt. In rauwe en 1 minuut gestoomde broccoli met actief myrosinase (100% en 14%), werden na de orale fase tot 10 keer hogere SF concentraties gemeten dan in langer gestoomde broccoli met geen of bijna geen aantoonbare myrosinase activiteit. Omdat de myrosinase activiteit door de thermische behandeling wordt beïnvloed, had de stoomtijd van broccoli de grootste invloed op de mate van GR hydrolyse en SF vorming.

In een *in vivo* kauwstudie met rauwe en gestoomde broccoli (**hoofdstuk 5**) werd de invloed van de kauwtijd en de stoomtijd op het lot van GR in de mond onderzocht. Om het effect van de kauwtijd te bepalen, kauwden vijf proefpersonen rauwe en gestoomde broccoliroosjes voor verschillende tijden met dezelfde frequentie (één beet per seconde). Tijdens het kauwen van rauwe en kort gestoomde broccoli (tot 1

minuut), werd meer GR door het endogene enzym myrosinase gehydrolyseerd bij een langere kauwtijd. Langere stoomtijden (2 en 3 minuut), zonder actief myrosinase, worden door langere kauwtijden niet beïnvloed. In een tweede studie kauwden vijf proefpersonen rauwe en gestoomde broccoli voor dezelfde tijdsduur en frequentie.

De stoomtijd bleek de snelheid van hydrolyse van GR te beïnvloeden, de meeste hydrolyse van GR trad op in 0.5 minuut gestoomde broccoli. De hoogste productie van de gezondheidsbevorderende stof SF na het kauwen werd gevonden in 2 minuten gestoomde broccoli, ondanks dat de myrosinase activiteit heel laag was in dit monster in vergelijking met rauwe broccoli. Dit maakt duidelijk dat myrosinase in overmaat aanwezig is in broccoli en ook als de myrosinase activiteit sterk gedaald is (tot 2.5% van de oorspronkelijke activiteit in het 2 minuten gestoomde monster), kan SF nog steeds gevormd worden.

Conclusie

Er is een wiskundig model ontwikkeld dat het lot beschrijft van de GSn tijdens thermische verwerking van Brassica groenten, waarbij de parameters (snelheidsconstanten en hun activering energieën) van spruitjes, rode kool, witte kool en broccoli zijn geschat (met uitzondering van de activering energieën van GSn in het kookvocht). Hierbij is de thermische stabiliteit van de verschillende GSn in dezelfde groente verschillend gebleken, maar ook die van dezelfde GS in verschillende groenten en in verschillende media (groente of water).

De *in vitro* spijsverteringsstudie toonde aan dat de GR hydrolyse voornamelijk in de mond plaatsvindt en dat myrosinase in de maag irreversibel geïnactiveerd wordt. Omdat de toevoeging van bovine serum albumine en olijfolie geen effect op de GR hydrolyse of biologische beschikbaarheid van GR, SF of SFN had, kan worden geconcludeerd dat de maaltijdbereiding belangrijker is voor de vorming van SF uit GR dan de maaltijdsamenstelling.

Acknowledgements

Now that I have written my thesis, I would like to thank all the people who supported me on my way to get to this point.

I would like to thank especially my supervisors Ruud Verkerk, Matthijs Dekker and Tiny van Boekel. Thanks for trusting me with this PhD project and supporting me to learn the things needed. Thanks for many hours of discussion, for often letting me have my way, but telling me also clearly when I went in the wrong direction. Ruud, you were always approachable and understanding, even if I asked you again for some money for the GC-workshop. Most problems could be solved over a coffee and a chat in the doorframe. Matthijs, you are able to make the difficult look so easy and were able to explain the sometimes mysterious ways of Athena to me, again over a coffee, but then in front of the screen. Furthermore you were easy travelling mates: to Paris (souvenir shopping for Sinterklaas), Girona (Ruud, on the Eiffel-bridge), with all GSs girls in Lisbon (the food!) and in Nantes (rewriting a paper with Matthijs over a coffee break).Tiny, you explained the essentials and so many possibilities of mathematical modeling to me. You were my backup that I knew I could use if needed.

Thanks to Hans Bouwmeester, Peter Hollman, Evelien Kramer from Rikilt for a nice collaboration on in vitro digestion. It was a pleasure to work with you! Nathalie Perrot and Sebastien Gaucel from INRA Paris, it was great fun working with you. We did speak different languages (not French/Dutch, but mathematical modeling/food processing), but managed with a bit of humor to understand each other.

Michelle van der Klauw, Ilse-Maarte de Leeuw and Ilona de Kok thanks for your work on your part of my project. You were dedicated and I enjoyed to mentor you.

Kristin Hennig, Teresa Oliviero, Radhika Bongoni, what should I have done without you GSs-girls! The quick help (how to do this again in excel?), the solution for every-day problems (where is the red room?), the scientific and non-scientific discussions and your friendship: Thanks!

Geert Meijer, Charlotte van Twisk, Xandra Bakker de Haan and Frans Lettink, thanks for your help in the lab. If it was pipette tips missing or new columns to try, you were always helpful. Geert, it was nice working with you on the GC-MS and fun trying to find out together why it stopped working or what the beeping meant.

And then there are so many other people I met in Wageningen, to whom I owe gratitude: Kasia and Bobby, Daylan and Victor, Nicolas, Elsa, Etske, Liya, Dieuwerke, Van-Anh, Penny, Klementina, Sarn, Lu Jing, Marielle, Lina, Fahui, Jenneke, Grace, Marine, Annika, Georgios, Mary, Harry, Elke. It was great to learn Dutch together or try to understand Dutch behavior, to have a beer in de Vlaamsche Reus or sushi in Arnhem, to go climbing or dancing to the wii and over all to have a laugh.

Many thanks also to Gerrit for the great design my thesis cover.

Vielen Dank an meine Familie und Freunde. Danke fuer eure Unterstuetzung und ein offenes Ohr.

And thank you Anand, for your understanding and continuous support.

About the author

Irmela Sarvan (maiden name Kruse) was born on 21st of April 1982 in Hamburg, Germany. She grew up near Hamburg and later Bonn, and finished high school in 2001. After a two year course in furniture restauration, she started her studies in nutrition sciences in 2003. During her studies, Irmela completed a two-month internship at Max-Rubner Institut (Federal research institute of nutrition and food), Karlsruhe, Germany. She worked in the Department of Physiology and Biochemistry of Nutrition and measured gluthatione peroxidase and superoxide dismutase activity in human blood cells as biomarkers of oxidative stress. For her Diplom thesis (equivalent to a MSc) with the title "Preparing and characterising emulsions with labeled molecules for bioavailability studies", she worked as a trainee for 7 months at Unilever R&D, Colworth, UK. She tested the behavior of two different emulsions in a static and dynamic (in collaboration with the Institute of Food Research, Norwhich, UK) *in vitro* gastro-intestinal tract as well as in an *in vivo* study (in collaboration with the University of Nottingham, UK).After her graduation, she started an internship at Unilever R&D, Vlaardingen, Netherlands, in June 2009 to work for 4 months on an *in vitro* model of fat digestion with added plant proteins. On 1st of December 2009, Irmela started her PhD at the University of Wageningen in the Food Quality and Design group (FQD). Her project included the determination of the fate of glucosinolates and especially glucoraphanin from processing to bioaccessibility during digestion. During her PhD she joined the educational program of the VLAG graduate school, joined the VLAG PhD student board, participated in teaching activities and supervised BSc and MSc students.

List of publications

Publication in peer-reviewed journals

Sarvan, I., Verkerk, R., van Boekel, M. A. J. S., Dekker, M. (2014) Comparison of the degradation and leaching kinetics of glucosinolates during processing of four Brassicaceae (broccoli, red cabbage, white cabbage, Brussels sprouts) *Innovative Food Science and Emerging Technologies* 25, 58-66

Sarvan, I., Valerio, F., Lonigro, L. S., de Candia, S., Verkerk, R., Dekker, M., Lavermicocca, P. (2013) Glucosinolate content of blanched cabbage (Brassica oleracea var. capitata) fermented by the probiotic strain Lactobacillus paracasei LMG-P22043 *Food Research International 54,* 706-710

Sarvan, I., Verkerk, R., Dekker, M. (2012) Modelling the fate of glucosinolates during thermal processing of Brassica vegetables *Food Science and Technology 49,* 178-183

Hussein, M. O., Hoad, C. L., Stephenson, M. C., Cox, E. F., Placidi, E., Pritchard, S. E., Costigan, C., Ribeiro, H., Ciampi, E., Rayment, P., Nandi, A., Hedges, N., Sanderson, P., Peters, H. P. F., Kruse (now Sarvan), I., Marciani, L., Spiller R. C., Gowland, P. A. (2015) Magnetic resonance spectroscopy measurements of intragastric fat fraction of oil emulsions in humans *European Journal of Lipid Science and Technology 117,* 31-36

Submitted publications

Sarvan, I., van der Klauw, M., Oliviero, T., Dekker, M., Verkerk, R. The effect of chewing on oral glucoraphanin hydrolysis in raw and steamed broccoli

Sarvan, I., Kramer, E., Bouwmeester, H., Dekker, M., Verkerk, R. Sulforaphane formation and bioaccessibility is more affected by steaming time than meal composition during *in vitro* digestion of broccoli

Overview of completed training activities

Training activities	Year
Discipline specific activities	
Courses	
Training in usage of scala at INRA, Paris, France	2010
Reaction kinetics in food sciences (7th edition), Wageningen	2012
Kwalitatief werken met de Trace DSQ, Interscience, Breda	2012
Advanced food analysis (4th edition), Wageningen	2013
International congresses	
DREAM annual meeting 2010 (oral), Girona, Spain	2010
International Horticultural Congress (poster), Lisbon, Portugal	2010
Berlin FOOD 2010 (oral), Berlin, Germany	2010
DREAM annual meeting 2011 (oral), Ljubiljana, Slovenia	2011
DREAM annual meeting 2012, Budapest, Hungary	2012
DREAM annual meeting 2013 (oral), Nantes, France	2013
EuroFoodChem 2013 (oral), Istanbul, Turkey	2013
General courses	
VLAG PhD week	2010
PhD competence assessment, Wageningen	2010
Design of experiments, Wageningen	2010
Statistics for life sciences, Wageningen	2010
Techniques for writing and presenting a scientific paper, Wageningen	2011
Project planning and time management, Wageningen	2012
Applied statistics for food technologists, Wageningen	2012
Optionals	
Preparation PhD research proposal	2010
Predicting food quality, MSc course of Wageningen University	2010
PhD trip to UK	2012

The research leading to these results has received funding from the European Community's Seventh Framework Program (FP7/ 2007e2013) under the grant agreement n° FP7-222 654-DREAM.

Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

Cover design: Gerrit Gnad, gerrit.gnad@web.de
Printed by: Gildeprint Drukkerijen, Enschede (NL), www.gildeprint.nl