Evaluation of glucosinolate levels throughout the production chain of *Brassica* vegetables

Towards a novel predictive modelling approach

Promotor:	Prof. dr. W.M.F. Jongen Hoogleraar Productontwerpen en Kwaliteitskunde met bijzondere aandacht voor ketenaspecten Wageningen Universiteit
Co-promotor:	Dr. ir. M. Dekker Universitair docent, leerstoelgroep Productontwerpen en Kwaliteitskunde, Wageningen Universiteit
Promotiecommissie:	Prof. dr. ir. P. Folstar (Wageningen Universiteit) Prof. dr. ir. D. Kromhout (Wageningen Universiteit/ RIVM, Bilthoven) Prof. dr. O. van Kooten (Wageningen Universiteit) Prof. dr. I.T. Johnson (Institute of Food Research, Norwich, United Kingdom)

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Ruud Verkerk

Proefschrift

Ter verkrijging van de graad van doctor
op gezag van de rector magnificus
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Stellingen

1. Voor het ontwikkelen van producten met een constante samenstelling van inhoudsstoffen is een ketenbenadering cruciaal.
   Dit proefschrift

2. Voorspellend modelleren is een onmisbaar gereedschap voor een gezonde ontwikkeling van de markt voor 'functional foods'.
   Dit proefschrift

3. Naast een substantieel gehalte aan glucosinolaten in een product is de aanwezigheid van een actief myrosinase enzym van belang voor de hoogte van het potentiële gezondheidsbeschermend effect.
   Dit proefschrift

4. Magnetronbereiding van kool verhoogt het beschikbare glucosinolaatgehalte.
   Dit proefschrift

5. *Brassica* groenten uit conserven dragen nauwelijks bij aan de inname van glucosinolaten bij de mens.

6. In tegenstelling tot wat Getahun en collega's beweren zijn het niet de isothiocyanaten maar de glucosinolaten die als voornaamste componenten voorkomen in *Brassica* groenten.
   Getahun et al. (1999) Cancer Epidemiol. Biomark. Prev. 8, 447-451

7. 'Functional foods' kunnen gezond zijn voor consument en economie aangezien ze bijdragen aan de verrijking van zowel het levensmiddel als de betrokken marktpartijen.

8. De kracht van epidemiologische bewijsvoering kan sterk vergroot worden door gebruik te maken van technologische kennis.

9. Gezondheid verkoopt, echter niet aan kinderen.

Stellingen behorend bij het proefschrift

Evaluation of glucosinolate levels throughout the production chain of Brassica vegetables; towards a novel predictive modelling approach

Ruud Verkerk
Wageningen, 17 mei 2002

Abstract

Evaluation of glucosinolate levels throughout the production chain of *Brassica* vegetables; towards a novel predictive modelling approach

PhD thesis by Ruud Verkerk, Product Design and Quality Management Group, Department of Agrotechnology and Food Sciences, Wageningen University, the Netherlands. May 17 2002.

Glucosinolates are a group of plant secondary metabolites, that can have important implications for human health. Vegetables of the *Brassica* genus, including cabbage, Brussels sprouts, broccoli, cauliflower and kohlrabi contribute almost exclusively to our intake of glucosinolates. Their added value towards vegetable quality can be ascribed to their health promoting properties by a role in the prevention of various cancers. The research described in this thesis was done to evaluate how levels of glucosinolates and their health-protective breakdown products are affected by various factors within the production chain of *Brassica* vegetables towards a better understanding of the alleged health effects of glucosinolates in *Brassica* vegetables. The research focused specifically on the effects of processing, namely chopping and cooking, on the content of glucosinolates.

It was demonstrated that chopping of raw *Brassica* vegetables resulted in unexpected, increased levels of indolyl glucosinolates after chopping and storage of cabbage and broccoli under ambient conditions. In white cabbage a 15-fold increase of 4-methoxy- and 1-methoxy-3-indolylmethyl glucosinolates was noted after 48 h of storage of chopped cabbage. Chopping and storage of broccoli vegetables resulted in a strong reduction of most glucosinolates, except for 4-hydroxy- and 4-methoxy-3-indolylmethyl glucosinolates, which increased 3.5- and 2-fold respectively. In this study we showed that the well-known and accepted breakdown mechanism of glucosinolates (hydrolysis by the endogenous enzyme myrosinase) appeared to be counteracted by a yet unknown mechanism causing an increase of some indolyl glucosinolates. It is postulated that chopping, by mimicking pest damage, triggers a defence mechanism in harvested *Brassica* vegetables.

Microwave cooking of red cabbage showed to be an interesting alternative for conventional cooking. In general, high total glucosinolate levels were observed for various microwave treatments due to the absence of leaching of glucosinolates into cooking water that takes place in conventional cooked vegetables. An increase in glucosinolate levels appeared to be associated with the time/energy input applied resulting in levels exceeding the total glucosinolate content of the untreated cabbage. This was probably caused by an increased extractability of glucosinolates from the vegetable matrix after the microwave treatment. Furthermore, at low (180 Watt) and intermediate microwave powers (540 Watt) substantial myrosinase activity was retained in cabbage. Thus, microwave prepared *Brassica* vegetables can offer a higher retention of glucosinolates and controllable amounts of active myrosinase, thereby increasing the health-promoting potential of the product.

Overall it was demonstrated that many steps in the food production chain of *Brassica* vegetables or vegetable products can have a large impact on the glucosinolate content and thus affect the final intake of health-protective glucosinolates and breakdown products for humans. A novel predictive modelling approach is proposed (and elaborated in a case study

on cooking) to handle the variations in the production chain and to provide a tool that can be used to assist product and process development. This model provides us with more insight in the behaviour and fate of glucosinolates and protective derivatives and may lead to options for improvement of investigations aimed at understanding the role of dietary glucosinolates and breakdown products in the protection against various cancers. Furthermore, predictive modelling can be helpful in enhancing the sensitivity of epidemiological studies and eventually provide solid evidence for assessment of the risks and benefits of glucosinolate consumption.

Contents

Chapter 1

General introduction

Introduction

In the past few years research from different fields has demonstrated that a broad range of non-nutritive, bioactive compounds in foods can play an important role in human health and well being. Examples are the groups of flavonoids, carotenoids, phyto-oestrogens, folates, and glucosinolates that are ingested via vegetables and fruit. The added value can be ascribed to their important role in the prevention of various diseases, most importantly ageing diseases like cancer and coronary heart diseases. Consequently, with regard to the health-protective properties of these products, product nutritional quality has to be defined not only by presence of essential nutritive compounds, and absence of undesirable compounds but also by the presence of so-called health-protective or promoting phytochemicals.

Food technology and health

The potential role of fruits and vegetables and derived products in promoting health and preventing diseases is studied basically at four levels. In this respect, epidemiology looks at associations between nutritional intake and incidence of (malignant) diseases. Animal studies investigate the protective mechanisms in controlled experiments. Molecular mechanistic studies, usually carried out *in vitro,* also aim at the elucidation of mechanisms. The fourth level consists of human intervention studies, where people are given certain amounts of (non)nutrients or fruits and/or vegetables and the effects on biomarkers in e.g. human serum are measured as indicators for various diseases. In this respect, the field of food technology can be seen as an essential input for investigating health benefits from food products. Because people eat food products and not ingredients, food technology can study the effects of the manufacture of foods on health-protective ingredients. Accurate information on the (bio)-availability of protective nutrients and non-nutrients to humans is needed in order to devise food-based strategies for optimising health and improving quality of live. Moreover, based on acquired knowledge, food technology can provide tools to design foods with measurable health benefits.

Glucosinolates in *Brassica* vegetables

Nowadays there is a growing amount of evidence for the protective effects of different phytochemicals. Food scientists, nutritionists and toxicologists investigate promising individual compounds, while epidemiologists focus on certain groups of vegetables. Mechanistic studies have shown various health protective effects of a large number of compounds. Evidence from animal experiments and epidemiological studies suggests that, among other vegetable families, the *Brassica* vegetables play a role in the prevention of cancer. *Brassica* vegetables comprise a large number of

vegetables such as different types of cabbage, cauliflower, broccoli, and Brussels sprouts. The protective effect of *Brassica* vegetables against cancer has been suggested to be partly due to their relatively high content of glucosinolates, which distinguishes them from other vegetables.

The research described in this thesis has focussed on this group of glucosinolates, a class of more than 100 plant secondary metabolites abundantly present in the *Brassicacea* (Rosa et al., 1997; Verkerk et al., 1998). Glucosinolates themselves exhibit minimal anticancer activity, however upon cell damage they undergo hydrolysis by the endogenous enzyme myrosinase to yield, amongst others, the biological active groups of isothiocyanates and indoles (Fenwick & Heaney, 1983). Currently, the role of glucosinolate breakdown products in the prevention of carcinogenesis is still under investigation and different protective mechanisms are hypothesised. Two distinct protective mechanisms are identified based on inhibition of enzymatic activation (phase I) of procarcinogens and induction of enzymes (phase II) that deactivate carcinogens (Zhang et al., 1992) and the suppression of tumour development via deletion of damaged cells from colonic mucosal crypts by induction of programmed cell death (Lund et al., 2001).

Despite the fact that different epidemiological studies indicate that a diet rich in *Brassica* vegetables can reduce the risk from a number of cancers (Verhoeven et al., 1996; Voorrips et al., 2000; Zeegers et al., 2001), epidemiology cannot reproducibly correlate protection against certain cancers or other diseases with specific vegetables, subgroups or individual components. Differences in bioavailability of glucosinolates and their breakdown products from foods may partly explain the inconsistency between vegetable intake and disease incidence. Therefore, knowledge of their bioavailability to human tissues, and in particular of the site and rate of production following ingestion of glucosinolates, becomes more crucial (Elfoul et al., 2001). Another plausible explanation can be the lack of realistic intake data of phytochemicals. To get a realistic estimation of the effect of bio-active compounds from foods, a quantitative insight in the processes that take place throughout the entire production chain is an absolute requirement. In other words, assessment of accurate dietary intake of glucosinolates and their breakdown products can play a crucial role in evaluating the protective effects of *Brassica* vegetables.

Outline of this thesis

The objective of this thesis is to quantify glucosinolate levels and to evaluate their variability throughout the production chain of *Brassica* vegetables with special emphasis on the effects of processing of the vegetables on glucosinolate content. The large number of parameters that affect the glucosinolate level in each step of the

production chain hampers the quantification and optimisation of the dietary intake of bioactive glucosinolates. Therefore a predictive modelling concept is proposed, which describes the fate of glucosinolates and its breakdown products in the production chain of *Brassica* vegetables.

In Chapter 2 an overview of the various aspects of glucosinolates is given in which the versatility of this group of plant metabolites is demonstrated as well as the complexity of the glucosinolate-myrosinase system. In Chapter 3 the effects of chopping and storage of cabbage are described by which the myrosinase-mediated hydrolysis of glucosinolates appears to be counteracted by a yet unknown mechanism causing an increase of some indolyl glucosinolates. The fate of glucosinolates and effects on myrosinase activity during microwave cooking of red cabbage as alternative for conventional cooking is described in Chapter 4. Subsequently, in Chapter 5 an impression is given of the variation of glucosinolate levels throughout the entire production chain of *Brassica* vegetables. Furthermore, in this chapter a novel predictive modelling approach is proposed to handle these variations in the production chain and to provide a tool that can be used to assist product and process development. This concept is further elaborated in Chapter 6 with a case study on predictive modelling of the glucosinolate-myrosinase system during the cooking of red cabbage. Simulation studies were carried out for conventional and microwave cooking of cabbage. The results and possible implications of this research are discussed in Chapter 7.

The research described in this thesis provides more insight in the behaviour and fate of glucosinolates and protective derivatives and may lead to options for improvement of investigations aimed at understanding the role of dietary glucosinolates and breakdown products in the protection against cancers.

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Chapter 2

Glucosinolates in *Brassica* vegetables
The nutritional significance, biosynthesis and bioavailability

Based on

Mithen R, Dekker M, Verkerk R, Rabot S, Johnson IT. (2000) The nutritional significance, biosynthesis and bioavailability of glucosinolates in human foods. Review; *Journal of the Science of Food and Agriculture* 80: 967-984.

Verkerk R, Dekker M, Jongen WMF. (1998) Glucosinolates. in: Natural Toxicants in Food. Ed: DH Watson, Sheffield Academic Press Sheffield, UK: 29-53.

Introduction

Fruit and vegetables are rich sources of micronutrients and dietary fibre, but they also contain an immense variety of biologically active secondary metabolites which provide the plant with colour, flavour and sometimes antinutritional or toxic properties. Amongst the most important classes of such substances are the carotenoids, flavonoids, saponins, phytosterols and the glucosinolates. This review is concerned with the last of these, a large group of sulphur-containing compounds that are found in all the economically important varieties of *Brassica* vegetables. Currently more than 120 different glucosinolates have been identified, of which only a few have been investigated thoroughly.

Glucosinolates and their breakdown products are of particular interest in food research, because of their nutritive and antinutritional properties (Fenwick et al., 1983b), the adverse effects of some glucosinolates on health, but also because of their anticarcinogenic properties and finally because they are responsible for the characteristic flavour and odour of many *Brassica* vegetables (van Doorn et al., 1998). The versatility of these compounds is also demonstrated by the fact that in particular some breakdown products are quite toxic to some insects, and therefore could be included as one of many natural pesticides. However, a small number of insects such as the cabbage aphids use glucosinolates to locate their favourite plants to use as a feed and to find a suitable environment to deposit their eggs (Harborne, 1989). Furthermore glucosinolates show antifungal and antibacterial properties (Chew, 1988).

Historical overview

Five stages of research activities can be identified stretching back over almost two centuries. (1) The classical structural studies of Gadamer at the end of the 19th century; (2) the natural product chemical investigations by Kjaer and co-workers over the period 1950-80; (3) the interdisciplinary studies responding to the importance of rapeseed as an oilseed of commerce and of its defatted meal as a protein-rich animal feedstuff and (4) the role and mechanisms of glucosinolates and their breakdown products in protecting plants against fungal and insect attack. The growing interest for the protective properties of glucosinolates and breakdown products against biological processes associated with cellular damage and cancer development in humans can be seen as a fifth stage of investigation.

The glucosinolate sinalbin was isolated from *Sinapis alba* seeds (white mustard) as early as in 1831 (Robiquet and Boutron, 1831). Subsequently Bussy (1840) isolated a related compound, sinigrin, from the seeds of black mustard (*B. Nigra* Koch). The first general, although incorrect, structure for these compounds was proposed at the

end of the nineteenth century by Gadamer (1897), who concluded that the side chain was linked to the nitrogen rather than the carbon atom of the "NCS" group. This was generally assumed to be correct until 1956, when Ettlinger and Lundeen (1956) pointed out the inadequacies of the Gadamer structure and proposed the now correct structure (Figure 2.1) and described the first chemical synthesis of a glucosinolate.

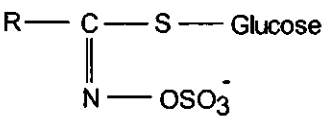

Figure 2.1 General structure of glucosinolates

The newly proposed structure contained a β-thioglucose group, side chain R and sulphonated oxime moiety. Because of the introduction of more sophisticated techniques of analysis and also the pioneering work of Kjaer and coworkers in Denmark (Kjaer, 1974; 1976) it was possible to identify more members of the group of glucosinolates.

Recent developments

The enormous amount of knowledge that is gathered over the many years of intensive research is now of great value for the more interdisciplinary approaches for elucidating the of recent interest health-protective effect of glucosinolates and their breakdown products for humans. Different theories of protective mechanisms are hypothesised and investigated intensively. Fahey (2001) has reviewed several cancer-preventive potentials of glucosinolate metabolites.

Glucosinolates appear to have little biological impact themselves, but are converted to biologically active products such as isothiocyanates, indoles, organic cyanides, oxazolidinethiones, and ionic thiocyanate upon enzymatic degradation by myrosinase in the presence of water. The key-role of the hydrolytic enzyme myrosinase is elaborated further on in this chapter. The anticarcinogenic mechanisms by which some of the breakdown products may act include the induction of detoxification enzymes and the inhibition of activation of promutagens/procarcinogens (Dragsted et al., 1993; Wattenberg, 1992; Jongen, 1996). The health-protective properties of *Brassica* vegetables are substantiated with compelling evidence obtained over the past 20 years linking increased consumption of *Brassica* vegetables to reduced incidence of many types of cancer (Steinmetz and Potter 1991, 1996; Verhoeven et al., 1996).

Glucosinolate distribution among plants

The occurrence of glucosinolates is limited to dicotyledonous angiosperms. They are present in at least 16 different families including Capparaceae, Brassicaceae, Moringaceae, Resedaceae containing the largest number of glucosinolates. Among the Brassicaceae, the genus *Brassica* contains a large number of the commonly consumed species. These species are cultivated mainly as vegetables, seasonings and sources of oil and feed. A considerable amount of data on levels of total and individual glucosinolates is now available. Generally, plant species contain four to six different glucosinolates in significant amounts. The highest concentrations are usually found in the seeds, except for indol-3-ylmethyl and N-methoxyindol-3-ylmethyl glucosinolates, which are rarely found in seeds (Tookey et al., 1980).

Several reports have given the results of the glucosinolate composition of cabbages and rapeseed varieties. These reports have been reviewed extensively by Rosa et al. (1997). The data are summarised in Table 2.1. The differences in the levels of these substances are large, even in the same studies, and even larger in different studies. Reasons for these different findings are the use of different varieties, growing conditions and the analytical methods used.

Table 2.1 Principal glucosinolates occurring in the main *Brassica* vegetables (From Rosa et al., 1997).

		Concentration (μmol/100 g of fresh weight)		
Species	Glucosinolate	Average	Range	Reference
White cabbage				
(*B. oleracea* L., Capitata group)				
	2-Propenyl	36.3	4.3-147.4	VanEtten et al. (1976)
		26.4	8.8-148.6	VanEtten et al. (1980)
		57.2	18.6-104.3	Sones et al. (1984a)
		66.2	18.6-162.7	Sones et al. (1984c)
	3-Methylsulfinylpropyl	34.7	13.0-70.9	VanEtten et al. (1976)
		28.3	10.0-58.6	VanEtten et al. (1980)
		72.7	5.0-193.1	Sones et al. (1984a)
		97.6	5.0-279.8	Sones et al. (1984c),
	Indole glucosinolates	49.4	28.0-106.4	VanEtten et al. (1976)
		31.2	10.5-104.9	VanEtten et al. (1980)
	Indol-3-ylmethyl	39.3	9.3-129.8	Sones et al. (1984a)
		60.7	9.3-200.0	Sones et al. (1984c),
	Total	143.8	66.4-236.7	VanEtten et al. (1976)
		117.3	57.5-234.5	VanEtten et al. (1980)
		68.6	17.7-112.8	Mullin and Sahasrabudhe (1977)
		200.9	93.8-348.2	Sones et al. (1984a)
		238.3	78.8-602.6	Sones et al. (1984c)

Table 2.1 (continued)

Species	Glucosinolate	Concentration (μmol/100 g of fresh weight) Average	Range	Reference
Savoy cabbage				
(*B.oleracea* L., Sabauda group)				
	2-Propenyl	-	35.8	VanEtten et al. (1976)
		14.2	0.1-39.7	VanEtten et al. (1980)
		93.2	31.5-162.7	Sones et al. (1984a)
	3-Methylsulfinylpropyl	-	100.7	VanEtten et al. (1976)
		46.7	15.2-91.1	VanEtten et al. (1980)
		169.8	72.5-2 79.8	Sones et al. (1984a)
	Indole glucosinolates	-	111.3	VanEtten et al. (1976)
		80.5	61.7-108.2	VanEtten et al. (1980)
	Indol-3-ylmethyl	123.0	70.2-199.8	Sones et al. (1984a)
	2-Hydroxybut-3-enyl	-	1.6	VanEtten et al. (1976)
		0.5	0.0-1.3	VanEtten et al. (1980)
		13.8	5.6-29.5	Sones et al. (1984a)
	Total	-	275.6	VanEtten et al. (1976)
		164.5	100.4-265.0	VanEtten et al. (1980)
		461.3	267.1-653.4	Sones et al. (1984a)
Red cabbage				
(*B. oleracea* L., Capitata group)				
	2-Propenyl	12.6	11.1-14.1	VanEtten et al. (1976)
		10.5	1.5-25.7	VanEtten et al. (1980)
	3-Methylsulfinylpropyl	16.1	12.4-19.7	VanEtten et al. (1976)
		14.5	4.8-31.0	VanEtten et al. (1980)
	4-Methylsulfinylbutyl	56.8	46.7-66.9	VanEtten et al. (1976)
		52.3	31.6-82.1	VanEtten et al. (1980)
	Indole glucosinolates	72.8	42.6-102.9	VanEtten et al. (1976)
		-	31.9-67.9	VanEtten et al. (1980)
	But-3-enyl	15.1	13.9-16.3	VanEtten et al. (1976)
		9.9	4.6-15.6	VanEtten et al. (1980)
	2-Hydroxybut-3-enyl	12.2	10.1-14.3	VanEtten et al. (1976)
		8.3	4.4-5.5	VanEtten et al. (1980)
	Total	204.3	150.5-258.1	VanEtten et al. (1976)
		163.4	88.2-234.4	VanEtten et al. (1980)
		68.8	34.4-98.9	Mullin and Sahasrabudhe (1977)
Brussels sprouts				
(*B. oleracea* L., Gemmifera group)				
	2-Propenyl	136.0	27.7-392.9	Heaney and Fenwick (1980)
		10.7	3.9-22.7	Carlson et al. (1987a)
		112.1	4.0-280.6	Sones et al. (1984c)
	3-Methylsulfinylpropyl	76.6	0.0-154.2	Sones et al. (1984c)
		11.8	2.4-18.9	Carlson et al. (1987a)

Table 2.1 (continued)

Species	Glucosinolate	Concentration (μmol/100 g of fresh weight) Average	Range	Reference
Brussels sprouts (cont.)				
	4-Methylsulfinylbutyl	8.2	0.4-22.6	Carlson et al. (1987a)
	Indol-3-ylmethyl	113.2	45.3-228.4	Heaney and Fenwick (1980a)
		128.4	54.3-326.3	Sones et al. (1984c)
		391.8	327.8-469.4	Carlson et al. (1987a)
	1-Methoxyindol-3-ylmethyl	21.3	1.9-34.3	Sones et al. (1984c)
	But-3-enyl	36.5	7.3-121.7	Heaney and Fenwick (1980a)
		61.3	6.1-221.2	Sones et al. (1984c)
		4.2	0.5-12.2	Carlson et al. (1987a)
	2-Hydroxybut-3-enyl	67.9	93.7-231.9	Heaney and Fenwick (1980a)
		111.9	29.3-303.5	Sones et al. (1984c)
		8.3	1.0-25.4	Carlson et al. (1987a)
	Total	367.2	330.3-406.5	Mullin and Sahasrabudhe (1977)
		461.9	138.6-900.7	Heaney and Fenwick (1980a)
		495.0	318.4-861.9	Sones et al. (1984c)
		553.0	465.6-600.6	Carlson et al. (1987a)
Collards				
(*B. oleracea* L., Acephala group)				
	2-Propenyl	20.7	12.6-28.7	Carlson et al. (1987a)
	3-Methylsulfinylpropyl	38.6	8.4-69.3	Carlson et al. (1987a)
	Indol-3-ylmethyl	55.5	44.2-69.5	Carlson et al. (1987a)
		47.3	-	VanEtten and Tookey (1979)
	Total	220.4	64.4-306.7	Carlson et al. (1987a)
Kale				
(*B. oleracea* L., Acephala group)				
	2-Propenyl	97.0	62.5-197.3	Carlson et al. (1987a)
	3-Methylsulfinylpropyl	11.7	0.0-49.9	Carlson et al. (1987a)
	Indol-3-ylmethyl	107.5	67.2-165.3	Carlson et al. (1987a)
	But-3-enyl	21.3	5.8-38.1	Carlson et al. (1987a)
	2-Hydroxybut-3-enyl	70.1	16.8-130.3	Carlson et al. (1987a)
	Total	439.1	316.1-600.0	Carlson et al. (1987a)

Table 2.1 (continued)

Species	Glucosinolate	Concentration (μmol/100 g of fresh weight) Average	Range	Reference
Broccoli				
(*B.oleracea* L., Italica group)				
	3-Methylsulfinylpropyl	74.1	0-327.2	Lewis et al. (1991)
	4-Methylsulfinylbutyl	63.9	28.9-88.3	Carlson et al. (1987a)
		97.5	54.0-190.2	Lewis et al. (1991)
	Indol-3-ylmethyl	59.4	42.2-71.7	Carlson et al. (1987a)
		56.0	22.8-101.0	Lewis et al. (1991)
	1-Methoxyindol-3-ylmethyl	8.6	2.4-18.4	Lewis et al. (1991)
	Total	161.9	98.5-323.9	Mullin and Sahasrabudhe (1977)
		188.2	102.2-262.7	Carlson et al. (1987a)
		248.4	152.2-448.6	Lewis et al. (1991)
Cauliflower				
(*B.oleracea* L., Botrytis group)				
	2-Propenyl	37.8	1.3-157.9	Sones et al. (1984b)
		35.8	1.3-157.9	Sones et al. (1984c)
		10.0	2.9-16.5	Carlson et al. (1987a)
	4-Methylsulfinylbutyl	63.8	1.8-190.1	Lewis et al. (1991)
	3-Methylsulfinylpropyl	41.0	0.0-90.9	Sones et al. (1984b)
		37.5	1.3-90.9	Sones et al. (1984c)
		5.2	0.0-22.8	Carlson et al. (1987a)
		51.0	0.0-327.2	Lewis et al. (1991)
	Indol-3-ylmethyl	50.0	14.8-162.3	Sones et al. (1984b)
		46.7	13.6-162.3	Sones et al. (1984c)
		60.6	18.8-104.7	Carlson et al. (1987a)
		42.1	21.0-101.0	Lewis et al. (1991)
	1-Methoxyindol-3-ylmethyl	10.0	1.1-32.0	Sones et al. (1984b)
		9.3	1.2-32.0	Sones et al. (1984c)
		7.3	2.3-17.4	Lewis et al. (1991)
	Total	105.0	59.1-180.6	Mullin and Sahasrabudhe (1977)
		161.9	30.2-520.4	Sones et al. (1984b)
		135.7	30.2-455.8	Sones et al. (1984c)
		94.6	41.1-160.6	Carlson et al. (1987a)
		178.2	57.1-448.6	Lewis et al. (1991)
Turnip tops				
(*B. campestris* L. and *B. rapa* L., Rapifera group)				
	But-3-enyl	-	294.0	Carlson et al. (1981)
		103.0	38.0-181.0	Carlson et al. (1987b)
	Pent-4-enyl	-	151.0	Carlson et al. (1981)
		58.0	20.0-112.0	Carlson et al. (1987b)

Table 2.1 (continued)

Species	Glucosinolate	Concentration (μmol/100 g of fresh weight) Average	Range	Reference
Turnip tops (cont.)				
Total		-	586.0	Carlson et al. (1981)
		186.0	80.0-292.0	Carlson et al. (1987b)
Rapeseed				
(*B. napus* L.)				
	But-3-enyl	3,187	-	Fenwick et al. (1983a)
	2-Hydroxybut-3-enyl	10,937	-	Fenwick et al. (1983a)
	Pent-4-enyl	824	-	Fenwick et al. (1983a)
	2-Hydroxypent-4-enyl	522	-	Fenwick et al. (1983a)
	Total			
	(Summer rape)	8,031	8,425-17,002	Fenwick et al. (1983a)
	(Spring rape)	-	8,140-12,582	Fenwick et al. (1983a)
		2,175	1,000-2,700	Sang and Salisbury (1988)
Rapeseed				
(*B. campestris* L.)				
	But-3-enyl	13,455	10,706-16,107	Fenwick et al. (1983a)
		3,863	1,302-10,281	Sang and Salisbury (1988)
		14,207	11,960-15,698	Sang and Salisbury (1988)
		23,450	-	Davis et al. (1991)
	2-Hydroxybut-3-enyl	1,836	1,050-2,387	Sang and Salisbury (1988)
		209	0.0-520	Sang and Salisbury (1988)
		250	-	Davis et al. (1991)
	Pent-4-enyl	1,704	1,092-2,941	Sang and Salisbury (1988)
		240	0-334	Sang and Salisbury (1988)
	2-Hydroxypent-4-enyl	322	234-385	Sang and Salisbury (1988)
		161	0-334	Sang and Salisbury (1988)
	4-Hydroxyindol-3-ylmethyl	396	294-475	Sang and Salisbury (1988)
		261	0-474	Sang and Salisbury (1988)

Chemical structure

All the glucosinolates possess a common basic structure (Figure 2.1) comprising of a ß-D-thioglucose group, a sulphonated oxime moiety and a variable side-chain derived from methionine, tryptophan, phenylalanine and some branched-chain amino acids. Glucosinolate side-chains are characterised by a wide variety of chemical structures. The side chains can be divided into three main groups: aliphatic (alkyl or alkenyl group), heterocyclic (indolyl) and aromatic chains. Table 2.2 gives an overview of the glucosinolates commonly found in Brassica vegetables. The most numerous glucosinolates are those containing either straight or branched carbon chains. Many of these compounds also contain double bonds (olefins), hydroxyl or carbonyl groups or sulphur linkages. The largest single group (one-third of all glucosinolates) contain a sulphur atom in various states of oxidation (e.g. methylthioalkyl-, methylsulphinylalkyl-, or methylsulphonylalkyl). The side chain of the glucosinolates is the basis for the structural heterogeneity and for the biological activity of the enzymatic and chemical breakdown products.

Table 2.2 Glucosinolates commonly found in *Brassica* vegetables.

Trivial name	Chemical name (side chain R)
Aliphatic glucosinolates	
glucoiberin	3-methylsulphinylpropyl
progoitrin	2-hydroxy-3-butenyl
sinigrin	2-propenyl
gluconapoleiferin	2-hydroxy-4-pentenyl
glucoraphanin	4-methylsulphinylbutyl
glucoalyssin	5-methylsulphinylpentyl
glucobrassicanapin	4-pentenyl
glucocheirolin	3-methylsulphonylpropyl
glucoiberverin	3-methylthiopropyl
gluconapin	3-butenyl
Indolyl glucosinolates	
4-hydroxyglucobrassicin	4-hydroxy-3-indolylmethyl
glucobrassicin	3-indolylmethyl
4-methoxyglucobrassicin	4-methoxy-3-indolylmethyl
neoglucobrassicin	1-methoxy-3-indolylmethyl
Aromatic glucosinolates	
glucosinalbin	p-hydroxybenzyl
glucotropaeolin	benzyl
gluconasturtiin	2-phenethyl

Synthesis of glucosinolates

Biosynthesis

Kjaer and Conti (1954) suggested that amino acids might be natural precursors of the aglycone moiety of glucosinolates based on the similarities between the carbon skeletons of some amino acids and the glucosinolates. This hypothesis was confirmed by studies for the different biosynthetic stages. Most of these studies have involved the administration of variously labelled compounds (^{3}H, ^{14}C, ^{15}N or ^{35}S) to plants and the assessment of their relative efficiencies as precursors on the basis of the extent of incorporation of isotope into the glucosinolate. Glucosinolate biosynthesis can be classified in three stages i) amino acid chain elongation, ii) synthesis of the glucosinolate from the amino acid and iii) chain modifications (see Figure 2.2).

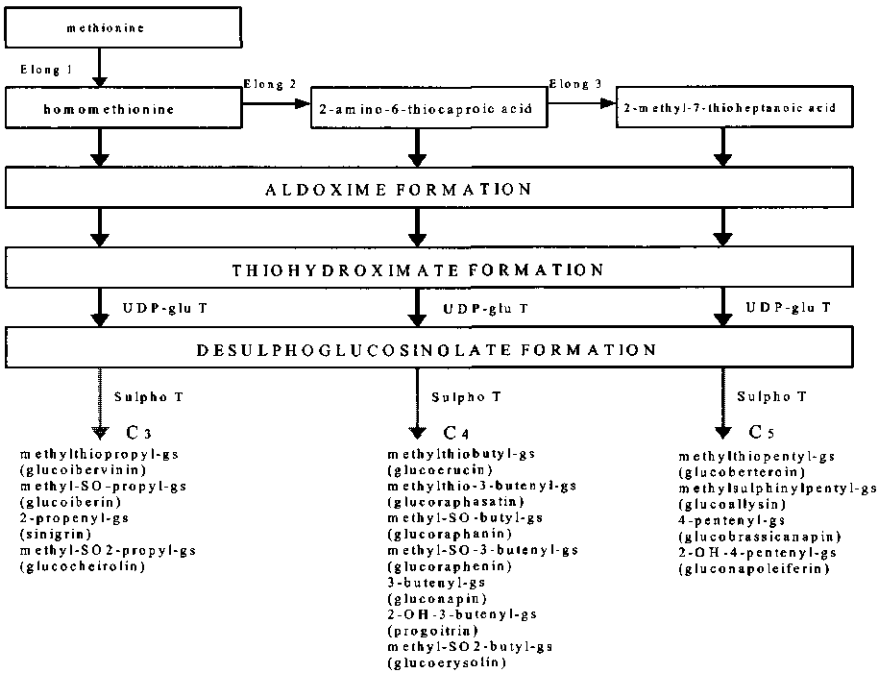

Figure 2.2 Model for the biosynthesis of methionine derived aliphatic glucosinolates in *Brassica* vegetables. Abbreviations: Elong = methionine elongation enzyme, UDP-glu T = UDP-glucose: thiohydroximate glucosyltransferase, Sulpho T = 3'PAPS-5'-phosphosulphate: desulphoglucosinolate sulphotransferase, gs = glucosinolate (Source: van Doorn et al., 1999).

i) side-chain elongation

The biosynthesis of glucosinolates from amino acids starts with modification of the amino acids (or chain extended derivatives of amino acids) via an aldoxime intermediate. Kutácek et al. (1962) demonstrated that [3-^{14}C]tryptophan was converted into 3-indolylmethylglucosinolate (glucobrassicin). Similarly Underhill et

al. (1962) and Benn (1962) showed that phenylalanine was incorporated into the benzylglucosinolate (glucotropaeolin) with great efficiency. The origin of glucosinolates can be divided into two groups: those derived from common amino acids and those from modified amino acids. Not all of the common amino acids lead to corresponding glucosinolates being found in nature. For instance the glucosinolate from glycine is probably chemically unstable and has therefore never been found in plants (Kjaer, 1976). The glucosinolate from alanine (methyl glucosinolate) is apparently absent in *Brassicas*, but is the most widely distributed glucosinolate within the *Capparaceae*. Valine and isoleucine glucosinolates (isopropyl and sec-butyl glucosinolates) are widely distributed in *Brassicas*. Phenylalanine and tyrosine can be transformed into the aromatic benzyl- and p-hydroxybenzyl glucosinolates respectively. The glucosinolate from tryptophan (indol-3-ylmethyl glucosinolate) appears to occur only in seedlings and young vegetative tissue in various families, including the *Brassicaceae*.

The modification of the common amino acids is mainly in the form of side chain elongation. A general route for this is proposed by Kjaer (1976). Various enzymes are involved in these steps. The elongation of the side chain from the amino acid methionine gives rise to a large family of glucosinolates that occur in *Brassicas*. Their side chains can be generally expressed as $MeS(CH_2)_n$ with n ranging from 3 to 11 (Dawson et al., 1993). In a later stage these glucosinolates can be oxidized to the corresponding sulfoxides or sulfones. Aromatic amino acids may also undergo homologization, for example, phenylalanine being metabolized into 2-phenethyl glucosinolate (Dawson et al., 1993).

The same modifications also take place in the biosynthesis route of other plant toxins: the cyanogenic glycosides. However the co-occurrence of glucosinolates and cyanogenic glycosides in the same plant is very rare (the exception is *Carica papaya*). The biosynthesis of the cyanogenic glycosides has been recently elucidated in more detail by Halkier et al. (1991) and by Koch et al. (1992). Following the formation of the aldoxime, the glucosinolate is formed by S-insertion, glucosylation and sulphatation. Further modification of the side chain can occur in the formed glucosinolate by for example oxidation and/or elimination reactions. The different steps in the synthesis is further discussed in some more detail.

ii) Aldoxime formation

Aldoxime formation systems have been elucidated in cassava and sorghum (Halkier and Lindberg-Møller, 1991; Koch et al., 1992). In these plants the aldoxime formation is involved in the synthesis of cyanogenic glycosides. In Chinese cabbage the formation of indole-3-aldoxime is by another system (Ludwig-Muller and Hilgenberg, 1988). Both systems are membrane bound. The most recent pathway for

aldoxime biosynthesis has been proposed by Koch et al. (1992). Dawson et al. (1993) have shown homophenylalanine to be effectively converted to 3-phenylpropanaldoxime when added to rapeseed (*Brassica napus*) leaf microsomal preparations. Lykkesfeldt and Lindberg- Møller (1993) have observed extracts of *Tropaeolum majus* to inhibit glucosinolate formation in microsomal systems. The extracts also inhibited the cyanogenic glucoside formation in a sorghum microsomal preparation. The authors suggested benzyl isothiocyanate to be the (partly) inhibiting compound.

iii) Glucosylation and sulphation

Formation of the aldoxime is followed by conjugation reactions which introduce sulphur to form thiohydroximic acid. Sulphur from cysteine is most effectively incorporated (Underhill et al., 1973). The subsequent S-glucosylation is catalyzed by thiohydroximate S-glucosyltransferase. This enzyme has been purified and characterized by GrootWassink et al. (1994). Sulphation of the desulphoglucosinolate is catalyzed by 3'-phosphoadenosine-5'-phosphosulfate. After this formation of the overall glucosinolate skeleton modification can occur like stereo-specific insertion of oxygen and elimination of methylthiols.

Chemical synthesis

Although several methods for the synthesis of a number of glucosinolates (e.g. benzyl-, methyl-, phenethyl-, 2-propenyl- and indolyl-) were reported over the past four decades, it is clear that these compounds were not routinely synthesised (Ettlinger and Lundeen, 1957; Benn and Ettlinger, 1965; Gil and MacLeod, 1980). Synthetic routes to naturally occurring indolyl glucosinolates have been developed by Rollin and colleagues (Viaud and Rolin, 1990; Viaud et al., 1992; Chevolleau et al., 1993).

Most recently Cassel et al. (1998) have reported a new approach to the synthesis of glucosinolate precursors that may broaden the range of synthetically accessible compounds. Glucoraphanin, the most interesting type of glucosinolate from a health-protective standpoint, has not yet been synthesised. However, controlled oxidation of glucoerucin to yield glucoraphanin and corresponding isothiocyanate sulphoraphane has been recently reported by Iori et al. (1999).

Degradation of glucosinolates

Myrosinase

Myrosinase (thioglucoside glucohydrolase EC 3.2.3.1) is the trivial name for the enzyme (or group of enzymes) responsible for the hydrolysis of glucosinolates. It is located in cellular compartments separate from glucosinolates in the plant and is

released when plant cells are damaged upon wounding of the plant, mastication of fresh plants (vegetables) or by tissue damage caused by post-harvest treatments or processing (Fenwick & Heany, 1983; Rosa et al., 1997).

The glucosinolate/myrosinase system may have several functions in the plant; (i) plant defence against fungal diseases and pest infestation, (ii) sulphur and nitrogen metabolism and (iii) growth regulation. Myrosinase has been found in seed, leaf, stem, and roots of glucosinolate-containing plants and the activity, which is dependent on the species, cultivar and plant organ, appears to be highest in young tissues of the plant (Bones, 1990). The complexity of the myrosinase-glucosinolate system indicates an important role in cruciferous plants (Bones & Rossiter, 1996).

Plant breeding strategies over the past decades have concentrated on reducing the glucosinolate content of rapeseed to improve the acceptability (reduce toxicity) of rapeseed meal and meet the increasingly stringent requirements of the processing industry. One approach to reduce undesired breakdown products of glucosinolates would be to change the amount of myrosinase available for hydrolysis of the glucosinolates.

Myrosinase exists in multiple forms in many plants. By analytical gel electrophoresis various studies have demonstrated the presence of several myrosinase isoenzymes (MacGibbon and Allison, 1970; Buchwaldt et al., 1986). Different patterns were found depending on whether the extracts were made from leaf, stem, root or seed. No direct correlation between myrosinase activity and glucosinolate content has been found (Bones, 1990). Several reports have described the isolation and physico-chemical characterisation of myrosinase in different Brassicaceae species (Durham and Poulton, 1990; Björkman, and Janson, 1972).

All plant myrosinases characterised are glycosylated, although to different extents for different isoforms. Little is known about the substrate specificity of myrosinase isoenzymes. Two myrosinases isolated by James and Rossiter (1991) degraded different glucosinolates at different rates. However, both isoenzymes show highest activity against aliphatic glucosinolates and least activity against indole glucosinolates. They concluded that members of a given class of glucosinolates are degraded at approximately the same rate in vitro. It is also possible that the specificity is affected by associated factors like epithiospecifier protein, myrosinase-binding protein or other myrosinase associated proteins or components.

Factors affecting myrosinase activity

The rate of hydrolysis of glucosinolates is determined to large extent by the activity of the enzyme myrosinase. The activity depends on the amount of substrate and the enzyme concentration, but also on some intrinsic (metal ions, ascorbic acid, pH) and extrinsic (temperature) factors.

As for all enzymes, the temperature plays a dual role in the myrosinase activity. The activity increases with increasing temperatures, while at high temperature inactivation will take place by denaturation of the enzyme. Different temperature optima are described probably depending on the different sources of myrosinase. Optimal myrosinase activity on sinigrin from radish roots (*Raphanus Sativus*) was 37°C and complete deactivation of the enzyme was achieved over 45°C (Jwanny et al., 1995). Temperature optima for white and red cabbage is 60°C (Yen and Wei, 1993) and for Brussels sprouts 50°C (Springett and Adams, 1989). The optimal pH for myrosinase activity is strongly dependent on its origin. The optimal pH for broccoli, both in the absence and presence of enzyme activators, was situated between 6.5 and 7, corresponding to the natural pH of fresh broccoli juice (Ludikhuyze et al., 2000). Myrosinase from white and red cabbage was characterised by a pH optimum of 8 (Yen and Wei, 1993), while white mustard and rapeseed myrosinase exerted maximal activity in the pH range 4.5 to 4.9 (Bjorkman and Janson, 1972). For myrosinase from Brussels sprouts on the other hand, two pH optima were found, 6.0 to 6.5 and 8.0 (Springett and Adams, 1989).

Ascorbic acid has been shown to modulate myrosinase activity in some species, it inhibits at high concentrations and activates at low concentrations. The activation appears to be due to a conformational change in the protein structure, leading to an enhanced reaction rate when the effector binding sites are occupied (Ohtsuru and Hata, 1973). Metal ions are known to affect the enzyme myrosinase either showing an increase or decrease on the activity as well as affecting the course of the reaction and the ratio of products formed (Jwanny et al., 1995; MacLeod and Rossiter, 1987). A combination of $MgCl_2$ and ascorbic acid was found to enhance enzyme activity, while it was observed that $MgCl_2$ in itself could not be used as enzyme activator (Ludikhuyze et al., 2000).

Hydrolysis products

Hydrolysis products of glucosinolates contribute significantly to the typical flavour of *Brassica* vegetables. The enzyme myrosinase catalyses the hydrolysis of glucosinolates by splitting off the glucose, the unstable aglucone (thiohydroxymate-O-sulphonate) then eliminates sulphate by a Lossen rearrangement (Figure 2.3). The resulting products can be either nitriles, isothiocyanates, indoles, amines, epithionitriles, thiocyanates, oxazolidine-2-thiones or other less prevalent products. The structure of the resulting products depends on a variety of factors. Whether isothiocyanates or nitriles are formed depends on the specific glucosinolates, the part of the plant where they are located, the treatment of plant material before the hydrolysis of glucosinolates and conditions, especially pH, during hydrolysis.

Isothiocyanates are usually produced at neutral pH while nitrile production occurs at lower pH. Indole glucosinolates such as glucobrassicin undergo enzyme hydrolysis to give 3-indolemethanol, 3-indoleacetonitrile and 3,3'-diindolylmethane (Labague et al., 1991). Hanley et al. (1990) isolated an indole isothiocyanate from neoglucobrassicin degradation under specific experimental conditions. Less volatile compounds such as epithionitriles and oxazolidine-2-thiones are formed from glucosinolates with an hydroxyl group at the 2-position of the side chain.

Only three glucosinolates produce thiocyanates during hydrolysis. These are allyl-, benzyl- and 4-methylthiobutyl glucosinolates. Epithioalkanes are produced from the hydrolysis of alkenyl glucosinolates when myrosinase co-occurs with a small labile protein known as epithiospecifier protein.

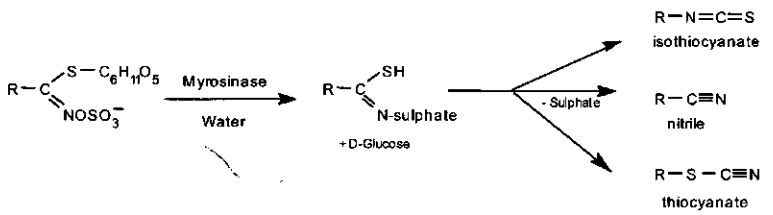

Figure 2.3 Hydrolysis of alkenyl glucosinolates and their breakdown products formed.

Biological effects of glucosinolates

The consumption of vegetables and fruit has always been seen as health promoting. The protective effect of *Brassica* vegetables against cancer has been suggested to be partly due to their relatively high content of glucosinolates, which distinguishes them from other vegetables.

Besides the beneficial health effects some glucosinolates and breakdown products show toxicological effects as well. The toxicity of glucosinolates has been described in many studies. Such effects are determined by the aglucones.

Anticarcinogenicity

In the largest and most detailed review of diet and cancer yet published, The World Cancer Research Fund (WCRF) concluded that diets rich in cruciferous vegetables are very likely to protect human beings specifically against cancers of the colon, rectum and thyroid. Also when consumed as part of diet high in other types of vegetable, they are assumed to give protection against cancer at other sites. This epidemiological evidence is consistent with many experimental studies, which from the 1960s onwards

have indicated that glucosinolate breakdown products exert anticarcinogenic activity in experimental animal models (Verhoeven et al., 1997; Hecht, 1999).

Carcinogenesis is a multistage process in which at least three distinct phases can be recognised: the initiation phase, the promotion phase and the progression phase. At each stage of the carcinogenic process a possibility of intervention exists. Wattenberg proposed a system of classification of dietary anticarcinogens based on the stage of carcinogenesis at which they act (Wattenberg, 1983; 1985).

Anticarcinogens can then be divided in three major classes. The first class consists of compounds that *prevent* the formation of carcinogens from precursor substances. The second are called "*blocking* agents" and have been found to be effective when given immediately before or during treatment with chemical carcinogens, while the third class, called "*suppressing* agents", are thought to act by preventing the progression of initiated cells to fully transformed tumour cells.

There has been much interest in isothiocyanates that can exert high protective, anticarcinogenic effects. Isothiocyanates arise in plants as a result of enzymatic cleavage of glucosinolates by the endogenous enzyme myrosinase. These compounds are attracting increasing attention as chemical and dietary protectors against cancer. Their anticarcinogenic activities have been demonstrated in rodents (mice and rats) with a wide variety of chemical carcinogens (Table 2.3). The anticarcinogenic effects of isothiocyanates can be explained by two different mechanisms. The first, a "blocking" effect, involves induction of Phase II enzymes, including quinone reductase in the small intestinal mucosa and liver (Zhang et al., 1992a, Talalay & Zhang, 1996). These enzymes are involved in the detoxification of xenobiotics. Increased activity will therefore block exposure of target tissues to DNA damage. The second mechanism, a "suppressing" effect, involves suppression of tumour development via deletion of damaged cells from colonic mucosal crypts by induction of programmed cell death (apoptosis). Smith et al. (1996) showed that dietary supplementation with the glucosinolate sinigrin, or its breakdown product allyl isothiocyanate, can protect against chemical induced colorectal carcinogenisis by stimulation of apoptosis. Another important example is sulphoraphane, an isothiocyanate present in high levels in broccoli, which acts as a strong inducer of phase II enzymes in vitro (Talalay et al., 1995). Other isothiocyanates derived from common *Brassica* vegetables may well exert comparable levels of biological activity (Chung et al., 1996).

The evidence for anticarcinogenic effects of *Brassica* vegetables in humans is strongly supported by evidence obtained with experimental animals. In a review by Steinmetz & Potter (1991) the overall conclusion from an analysis of 115 case-control studies was that a relatively high consumption of *Brassica* vegetables was associated with a reduction in risk of cancer at many sites. For Broccoli consumption in

particular, there was a uniform protective effect, with no contrary evidence in any study. Consumption of *Brassicas*, which might be expected to yield high levels of indoles and isothiocyanates was particularly strongly associated with a lower risk of colon cancer.

Table 2.3 Protection by a variety of isothiocyanates and glucosinolates against chemical carcinogenesis in rat and mouse organs.

Carcinogens
3'-Methyl-4-dimethylaminoazobenzene
4-Dimethylaminoazobenzene
N-2-Fluorenylacetamide, acetylaminofluorene
7,12-Dimethylbenz[a]anthracene (DMBA)
Benzo[a]pyrene
Methylazoxymethanol acetate
N-Nitrosodiethylamine
4-(Methylnitroamino)-1-(3-pyridyl)-1-butanone (NNK)
N-Nitrosobenzylmethylamine (NBMA)
N-Butyl-*N*-(4-hydroxybutyl)nitrosamine
Protective isothiocyanates
α-Naphthyl-NCS, β- naphthyl-NCS
Phenyl-$[CH_2]_n$-NCS, where n = 0, 1, 2, 3, 4, 5, 6, 8, 10
$PhCH(Ph)CH_2$-NCS, $PhCH_2CH(Ph)$-NCS
$CH_3[CH]_n$-NCS, where n = 5,11
$CH_3[CH_2]_3CH(CH_3)$-NCS
Sulphoraphane, CH3S(O)[CH2]4-NCS
2-Acetylnorbornyl-NCS (3 isomers)
Protective glucosinolates
Indolylmethyl glucosinolate (glucobrassicin)
Benzyl glucosinolate (glucotropaeolin)
4-Hydroxybenzyl glucosinolate (glucosinalbin)
Tumour target organs
Rat: liver, lung, mammary gland, bladder, small intestine/colon, oesophagus
Mouse: lung, forestomach

Source: P. Talalay and Y. Zhang (1996)

Toxicity

Vegetables and seeds of the Cruciferea family, such as crambe, kale, mustard, rape, cabbage, turnips, etc., are rich in glucosinolates. Many feeds containing a high concentration of rape or crambe seed meal have been shown to decrease feed intake and growth rate and to cause goitrogenity, enlarged livers, kidneys, thyroid and adrenal glands among different animal species. These adverse effects were attributed to the high content of glucosinolates and their derivatives, which include goitrin, isothiocyanates and nitriles. In humans, epidemiological surveys show a correlation

between endemic goitre and consumption of cruciferous vegetables whereas experimental studies are unambiguous. Langer et al. (1971) demonstrated the goitrogenic properties of cruciferous vegetables and purified glucosinolates and their derivatives but the findings of McMillan et al. (1986) are not in agreement with these results.

Among the problems associated with the consumption of these compounds, those affecting the thyroid have been studied most extensively (van Etten, 1969). The breakdown product 5-vinyl- oxazolidine-2-thione (OZT) was found to be the predominant product from the heat-treated rapemeal regardless of the source of myrosinase enzyme, which may explain the ability of rapeseed to induce thyroid hypertrophy (McKinnon and Bowland, 1979). In certain parts of the world the consumption of excessive amounts of *Brassicas* may contribute to hypothyroidism, particularly when natural iodine in the diet is limited. Furthermore the extent to which the thyroid function is impaired by glucosinolates is related to species, intake, duration of feeding and the nature of the compound. Also the mechanism involved seemed to be different. Thiocyanate ions are considered to behave as iodine competitors and, therefore cause goitrogenicity only in cases of iodine deficiency, while oxazolidine-2-thiones interfere with thyroxine synthesis and therefore, will be goitrogenic irrespective of the iodine status (Fenwick et al., 1983). In addition, isothiocyanates of the parent glucosinolates sinigrin, glucocheirolin, glucotropaeolin and nitriles have shown as well goitrogenic effects also depending on the iodine content of the diet.

Most of the studies on the physiological properties of glucosinolates and their breakdown products have been carried out with feeding experiment using rapeseed and *Crambe abyssinica*. These studies showed considerable enlargement of thyroid, adrenal gland, kidney and liver. The levels ingested by humans are usually not a problem, but animals can suffer if they are fed too much rapeseed meal, which is used as a protein supplement in livestock and poultry feeds. Unfortunately the use of this meal for feeding purposes can result in various manifestations of toxicity. These problems have led to the introduction of "double zero" varieties of rape which are low in both erucic acid (less than 2% of the total fatty acids) and glucosinolates (less than 1% w/w). These "double zero" varieties are common in Canada, and are given the general name Canola (Bell, 1993).

During seed processing most glucosinolate breakdown products are formed by which the degree of degradation depends on seed properties and processing conditions such as moisture level, pressure, or temperature. Reduction in glucosinolate content can be obtained by autoclaving meal for 1.5 hr (Mansour et al., 1993), treatment of meal with Cu^{2+} (Schöne et al., 1990) and use of ammonia in conjugation with other processing (Keith and Bell, 1982).

The use of rapeseed meal containing glucosinolates as feedstuff and their antinutritional effects has been extensively studied. Although there is some controversy about the quantity of glucosinolates that is tolerated by various animal species, threshold levels for glucosinolates in diets have been suggested (Hill, 1991; Bell, 1993; Mawson et al., 1993).

Food quality and glucosinolates

The typical flavour and odour of *Brassica* vegetables is largely due to glucosinolate-derived volatiles (isothiocyanates, thiocyanates, nitriles). It has been shown that the glucosinolates sinigrin and progoitrin are involved in the bitterness observed in Brussels sprouts (Fenwick et al., 1983b). Van Doorn et al. (1997) confirmed the role of sinigrin and progoitrin in taste preference by using taste trials with samples of Brussels sprouts. It appeared that consumers preferred Brussels sprouts with a low sinigrin and progoitrin content. In cabbage sinigrin is an abundant glucosinolate which gives a pungent and bitter flavour. The stronger flavour in the heart of the cabbage is in agreement with the presence of higher amounts of sinigrin found in the cabbage heads. Low levels of 2-propenyl isothiocyanates formed from sinigrin, result in a flat and dull product (Rosa et al., 1997). Pungency and bitterness caused by glucosinolate breakdown products play a role in the taste preference of consumers and are therefore important quality factors for *Brassicas*.

Improvement of flavour and nutritional properties in *Brassica* can be achieved by use of molecular markers in selection of specific glucosinolate lines in breeding programmes (Campos-De Quiroz and Mithen, 1996). Developing *Brassicas* less susceptible to diseases, less attractive to insects and with desirable agronomic storage and sensory characteristics by manipulating the glucosinolate levels can result in crops with higher commercial value (Borek et al., 1994; Brown & Morra, 1995).

The positive effects against cancer can be considered as another notable quality factor of glucosinolates and their derivatives. Brassicaceous vegetables or glucosinolate derivatives have been shown to modify endogenous detoxification processes and, thus, may interfere in a positive way with the metabolism of chemical carcinogens (see §6.1; McDanell et al. 1988; Jongen, 1996). Enhancement of these effects by increasing the levels of specific glucosinolates is of importance for obtaining protective effects at normal consumption levels

Responses to stress factors

Glucosinolates and their breakdown products are considered to function as part of the plant's defence against insects attack and to act as phagostimulants (Fenwick et al., 1983; Chew, 1988). There is now considerable information on the importance of

glucosinolates in insect-plant interactions. However less is known about the influence of biotic factors on glucosinolates metabolism in plants. It has been demonstrated in different studies that attack by insects, including aphids (Lammerink et al., 1984), root flies (Birch et al., 1990), flea beetles (Koritsas et al., 1991), changes both the total concentration of glucosinolates on different plant tissues and the relative proportions of aliphatic and aromatic compounds. Other examples of stress-induced increases in levels of glucosinolates are mechanical wounding and infestation (Koritsas et al., 1991), methyl jasmonate exposure (Doughty et al., 1995), grazing (Macfarlane Smith et al., 1991) for intact plants or UV-irradiation (Monde et al., 1991) and chopping (Verkerk et al., 2001; Chapter 3 of this thesis) for post-harvest vegetables. Apparently, besides the breakdown mechanism of glucosinolates also an induction mechanism of glucosinolate biosynthesis by stress factors is present in *Brassica* vegetables.

Effects of processing

The effects of processing on glucosinolate levels in vegetables have been reviewed by De Vos and Blijleven (1988). Processes like chopping for raw consumption, cooking and fermentation damages plant cells and brings myrosinase in contact with glucosinolates which influence the levels of glucosinolates, the extent of hydrolysis and the composition, flavour and aroma of the final products. Also low-temperature storage like freezing and refrigerating can alter the content of glucosinolates. Freezing without previous inactivation of myrosinase results in an almost complete glucosinolate decomposition after thawing (Quinsac et al., 1994). During the sauerkraut fermentation of white cabbage all glucosinolates were hydrolyzed within 2 weeks according to Daxenbichler et al. (1980). The breakdown products investigated were the thiocyanate ion, isothiocyanates, goitrin and the nitriles 1-cyano-3-methylsulfinylpropane (from glucoiberin) and 1-cyano-2,3-epithiopropane (from sinigrin). Isothiocyanates, goitrin, and cyano-2,3-epithiopropane were not detectable throughout fermantation. The effect of cooking on glucosinolates has received a relatively large amount of attention. Cooking reduces glucosinolate levels by approximately 30% - 60%, depending on the type of compound. Also thermal degradation and washing out occurs, leading to large losses of intact glucosinolates. Degradation products apparently are hardly detectable after cooking, with the exception of the thiocyanate ion and ascorbigen (McLeod & McLeod, 1968; McMillan et al., 1986). Pulping of plant tissues results in the complete breakdown of glucosinolates by autolysis.

Prior to many processing steps chopping of the vegetable is necessary. Chopping of fresh plant tissues creates optimal conditions for myrosinase and a high degree of

glucosinolate hydrolysis can be expected. In contrast to these expectations and reported findings Verkerk et al. (2001) observed, after chopping and storage to air of different kinds of *Brassica* vegetables, elevated levels of indolyl glucosinolates. In white cabbage the largest increase they found was for 4-methoxyglucobrassicin which increased 15-fold. These data are further elaborated in chapter 3. Increasing the amount of (indolyl)glucosinolates can have large influences on quality factors as flavour and anticarcinogenicity of *Brassica* vegetables. These findings will change our ideas about the fate of glucosinolates and the consequences for estimation of intake levels from processed *Brassica* vegetables.

Bioavailability

It is now clear that our understanding of anticarcinogenic properties of isothiocyanates has improved considerably over recent years. Therefore, our knowledge of their bioavailability to human tissues, and in particular of their site and rate of production following ingestion of glucosinolates, becomes more crucial. The structural diversity and chemical reactivity of glucosinolate breakdown products, as well as the complexities of the environment from which they have to be isolated, have long inhibited progress in this field. Improvements in analytical methods for detecting and quantifying isothiocyanates and other metabolites are now changing this situation. Current evidence suggests that when plant myrosinase is active, glucosinolates are rapidly hydrolysed in the food or in the proximal gut. If the enzyme myrosinase is deactivated, for example by cooking the vegetables prior to consumption, the intact glucosinolates may be able to reach the distal gut where they could be metabolised by the resident microflora. This hypothesis was confirmed by studies with gnotobiotic rats in which the introduction of a whole faecal flora from rats or humans into initially germfree rats resulted in the disappearance of intact glucosinolates in the cecal and colonic contents, coupled with the emergence of systemic effects reflecting glucosinolate hydrolysis. It appears that the ability to degrade glucosinolates is widely distributed among intestinal bacteria (Oginski et al., 1965), and Rabot et al. (1995) have isolated from human faeces representatives of various genera (e.g. *Bacteroides, Peptostreptococcus, Enterococcus, Escherichia, Proteus*) which are able to carry out the degradation of progoitrin and sinigrin *in vitro*. However little is known of the nature of glucosinolate breakdown products released by human colonic bacteria. Isothiocyanates have been detected upon *in vitro* incubation of human faeces with cooked watercress juice (Getahun and Chung, 1999).

The contribution of the digestive microflora to the production of isothiocyanates *in vivo*, in the distal gut, has been recently ascertained; following gavage with 50 µmol sinigrin, substantial amounts of allyl isothiocyanate (up to 100 nmol 12 h after dosing)

were measured in the cecal and colonic contents of gnotobiotic rats harbouring a human digestive strain of *Bacteroides*, while no allyl cyanide could be detected (Elfoul et al., 1999). The formation of other derivatives, eg desulphoglucosinolates or thiocyanates, has scarcely been investigated, and studies are often not conclusive, mainly because of analytical impediments. Nevertheless, the versatility of microbial enzymatic activities could lead to a wider array of metabolites than those so far identified.

Analytical methods

The large amount of different glucosinolates and the fact that each glucosinolate can produce different breakdown products makes the analysis very complicated. The analytical methodology was extensively reviewed by McGregor et al. (1983). A brief overview is presented here.

The analysis can be divided into methods for total glucosinolates, individual glucosinolates and the breakdown products. Over the past 4 decades, increased knowledge of the diversity of the glucosinolates, their enzymatically released products and factors influencing their release have led to a multiplicity of analytical methods. Glucosinolates coexist with myrosinase in the plant, and processes like grinding or cutting of fresh tissue in presence of water will initiate a rapid hydrolysis of these compounds. For analysis of intact glucosinolates inhibition of myrosinase activity is essential. Before disruption of the material, samples should be completely dry by freeze-drying or frozen in liquid nitrogen. The use of aqueous methanol for extraction, in combination with high temperatures, also denatures myrosinase (Heaney & Fenwick, 1993).

Total glucosinolates

Glucosinolates yield equimolar amounts of glucose upon hydrolysis with myrosinase. This is true for almost all glucosinolates, and methods based on the measurement enzymatically released glucose proved to be relatively rapid and simple to apply (Heaney et al., 1988). Therefore the total glucosinolate content of a food sample can be measured by determining the quantity of glucose released after treatment with the enzyme, but this takes no account of endogenous glucose. Alternatively, extraction of glucosinolates can be performed followed by selective cleanup that eliminates free glucose and other interfering compounds, after which controlled enzymatic release of bound glucose is possible.

Myrosinase hydrolysis of glucosinolates gives rise to an unstable aglucone, which after a Lossen rearrangement produce an equimolar amount of bisulfate. Several methods have been described for the quantification of this bisulfate ion using titrimetric and gravimetric methods. Schnug (1987) has described a method in which

the bisulfate liberated after sulfation, is precipitated with bariumchloride and residual barium is measured by X-ray emission spectroscopy.

Individual glucosinolates

Gas liquid chromatography (GLC) of derivatized glucosinolates is the traditional method for the identification and quantification of individual glucosinolates (Underhill & Kirkland, 1971). Initially glucosinolates were extracted with boiling water, derivatized and separated by isothermal chromatography. Substantial improvements have been subsequently made by Thies (1976). Ion exchange purification of glucosinolate extracts to remove carbohydrates and other impurities before derivatization increased the sensitivity. A major breakthrough in glucosinolate analysis has been achieved with the introduction of enzymatic on-column desulfatation using aryl sulfatase. The introduction of a desulfation step before derivatization was performed to eliminate sulfate that interfered with GC analysis. Desulfation was elegantly carried out on the ion exchange column, using a commercially available sulfatase isolated from an edible snail (*Helix pomatia*). Free sulfate in the glucosinolate extract, which could inhibit the sulfatase, was precipitated by addition of barium acetate and removed by centrifugation before addition of the extract to the ion exchange column.

Some glucosinolates (indoles) are thermally unstable, therefore HPLC has become a more preferred method. High performance liquid chromatography (HPLC) has the advantage of direct determination of (desulpho)glucosinolates. The first successful application of the technique was described by Helboe et al. (1980). Glucosinolates were purified and desulfated on-column and then separated by ion-exchange (Olsen & Sørensen, 1979) chromatography or reverse phase ion-pairing chromatography using a C_{18} Nucleosil column with gradient elution using acetonitril-water mixtures as the mobile phase and tetraoctylammonium bromide as the source of counter ion. By avoiding the use of buffer solutions and ion-pairing reagents, glucosinolates could be collected in a pure form suitable for identification by mass spectrometry. With the aid of this method, 2 new glucosinolates were separated and identified, 4-hydroxy-3-indolylmethyl glucosinolate and 4-methoxy-3-indolylmethyl glucosinolate (Truscott et al., 1982).

Several mass spectrometric techniques have been investigated for structure elucidation of the various (desulpho-)glucosinolates e.g. direct probing electron impact, chemical ionization, and fast atom bombardment. Considerable structural information can be obtained with these techniques.

One of the major problems in the analysis of glucosinolates has been the lack of suitable standards. The only commercial available glucosinolates are benzylglucosinolate (glucotropaeolin) and 2-propenylglucosinolate (sinigrin). Sinigrin

is not a suitable internal standard because of the presence of this compound in most brassicacious plants. Glucotropaeolin is not normally present in *Brassica* and has been frequently used as internal standard.

Breakdown products

The application of HPLC to the investigation of glucosinolate breakdown products has been limited due to the volatility of many compounds. Furthermore, thiocyanates and nitriles are not detectable spectrometrically. Isothiocyanates and nitriles can be analyzed by GLC. HPLC with UV detection may be used for analysis of oxazolidinethiones and indoles. Quinsac et al. (1992) developed a method for analyzing oxazolidinethiones in biological fluids with a high degree of selectivity. However HPLC finds most use in the analysis of intact glucosinolates or desulfoglucosinolates. For identification and confirmation of structures, both techniques can be coupled to mass spectrometry (MS). Mass spectroscopy has proved to be an invaluable tool in the identification and structural elucidation of glucosinolates and their breakdown products. Positive ion fast atom bombardment mass spectrometry (FAB) (Fenwick et al., 1982) has yielded mass spectra characterized by abundant protonated and cationized molecular ions with relatively little fragmentation. In the negative ion mode, FAB produces an abundant molecular ion (of the glucosinolate anion). This proved especially advantageous in the analysis of crude plant extracts and mixtures of purified glucosinolates.

Zhang et al. (1992b) developed a spectroscopic quantitation of organic isothiocyanates. Under mild conditions nearly all organic isothiocyanates (R-NCS) react quantitatively with an excess of vicinal dithiols to give rise to five-membered cyclic condensation products with release of the corresponding free amines (R-NH2). The method can be used to measure 1 nmol or less of pure isothiocyanates or isothiocyanates in crude mixtures.

Summary and conclusions

Glucosinolate research is expanding from toxicological areas into more health-promoting areas. Much research is nowadays focussed on the potential effects of glucosinolates and their breakdown products against biological processes associated with cellular damage and cancer development. Also the role and mechanisms of glucosinolates and their products in protecting plants against fungal and insect attack and the allelochemical effects on behaviour, health and growth of other species is an important area of research.

It is clear that factors inducing and directing indole glucosinolate metabolism in plants need to be studied in much greater detail as evidence increases on the biological activities of these compounds to man.

There is still much to be learned from *in vitro* studies about the mechanisms of interaction between glucosinolate breakdown products and their target tissues.

Priorities for future research must be in vivo studies with human volunteers. Such studies should preferably be conducted with well-characterised *Brassica* vegetables, and employ protocols which enable the dose-response relationship for both beneficial and adverse effects to be properly quantified.

Further investigations are needed to provide a comprehensive evaluation of glucosinolate bioavailability in the context of the whole diet. In particular, it must be stressed that many factors may influence the digestive and post-absorptive metabolism of glucosinolates and derivatives, and consequently their tissue disposition and excretion. For example, dietary fibre and minerals have been suspected to modulate the microbial metabolism of glucosinolates in the hindgut (Roland et al., 1996), pointing strongly to the role of the complex dietary environment as a determinant of the digestive fate of these compounds.

Finally, as with all phytochemicals, any exploitation of their beneficial effects depends upon a full understanding of their behaviour within the changing human food chain. The fate of glucosinolates in fresh materials during food production is extremely complex since it depends on numerous different processes and several mechanisms of degradation and biosynthesis, which seem to occur simultaneously. The development of a robust predictive model to quantify the effects of these phenomena, and the integration with it of models describing the bioavailability and biological activity of the most important glucosinolates in humans, should be the ultimate goal for future research in this area.

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Chapter 3

Post-harvest increase of indolyl glucosinolates in response to chopping and storage of *Brassica* vegetables

Ruud Verkerk, Matthijs Dekker and Wim M.F. Jongen (2001) *Journal of the Science of Food and Agriculture* 81: 953-958.

Abstract

Brassica vegetables contain high amounts of glucosinolates, which contribute to the beneficial health effects of their consumption. Processing of such vegetables in domestic food preparation or industrial processing will influence levels of glucosinolates considerably and thus affect their health-protective capacity. This study demonstrates the effects of chopping of raw *Brassica* vegetables on their glucosinolate composition. Limited breakdown of aliphatic glucosinolates in cabbage was found, whereas unexpected increased levels of indolyl glucosinolates were detected after chopping and storage of cabbage and broccoli under ambient conditions. In chopped white cabbage a 15-fold increase of 4-methoxy- and 1-methoxy-3-indolylmethyl glucosinolates was noted after 48 h of storage. Chopping and storage of broccoli resulted in a strong reduction of most glucosinolates, except for 4-hydroxy- and 4-methoxy-3-indolylmethyl glucosinolates, which increased 3.5- and 2-fold respectively. The myrosinase-mediated hydrolysis of glucosinolates appears to be counteracted by a post-harvest increase of some indolyl glucosinolates. In this chapter we propose a mechanism of stress-induced increase of glucosinolates, which plays an important role besides the well-known breakdown mechanism.

Introduction

Brassica vegetables such as cabbage, Brussels sprouts, broccoli and cauliflower are an important dietary source for a group of secondary plant metabolites known as glucosinolates. The sulphur-containing glucosinolates are present as glucosides in *Brassica* vegetables and can be hydrolysed by the endogenous plant enzyme myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1). Myrosinase and the glucosinolates are physically separated from each other in the plant cell and therefore hydrolysis can only take place when cells are damaged, e.g. by cutting or chewing. This hydrolysis generally results in the further breakdown of glucosinolates into isothiocyanates, nitriles, thiocyanates, indoles and oxazolidinethiones. Glucosinolate breakdown products contribute to the characteristic flavour and taste of *Brassica* vegetables (Van Doorn et al., 1998; Vaughn et al., 1976). More important are the health-protective effects ascribed to the isothiocyanates and some indolic compounds. Glucosinolates and their biological effects have been reviewed in detail (Rosa et al., 1997; Verkerk et al, 1998). During the last decade, intensified research on glucosinolates in *Brassica* vegetables has increased the knowledge of their anti-carcinogenic effects. Among the different protective mechanisms that have been proposed, the induction of phase II enzymes, including glutathione S-transferase and quinone reductase, is most extensively investigated (Sparnins et al., 1982; Tawfiq et al., 1995). These enzymes, present in the small intestinal mucosa, liver and colon, are involved in the detoxification of carcinogens. Increased phase II enzyme activity will therefore block the exposure of target tissues to DNA damage. Different studies have shown that the breakdown products sulphoraphane and indole-3-carbinol, released by the glucosinolates glucoraphanin and glucobrassicin respectively, are strong phase II enzyme inducers (Zhang et al., 1992; Hecht, 1995; Talalay and Zhang, 1996).

The level of glucosinolates ingested by humans depends on a variety of factors within the overall production chain of *Brassica* vegetables (Dekker et al., 2000). The glucosinolate content in *Brassica* vegetables can vary depending on the variety, cultivation conditions, harvest time and climate (Rosa et al., 1996; Kushad et al., 1999). Storage and processing of the vegetables can also greatly affect the glucosinolate content. Processes such as chopping, cooking and freezing influence the extent of hydrolysis of glucosinolates and the composition of the final products. As most vegetables are processed in some way before consumption, the effects of processing should be taken into account in order to know what the intake of these compounds will be. Some processes have been studied and reviewed by de Vos and Blijleven (1988), who concluded that, in general, processing gives rise to a certain degree of glucosinolate breakdown caused by myrosinase hydrolysis.

Chopping of the *Brassica* vegetables is a common step in food preparation prior to further processing (industrial or domestic). Little is known about the effects of physical damage (chopping or slicing) of *Brassica* vegetables on the levels of glucosinolates. Disruption of the tissue caused by chopping releases the enzyme myrosinase, which is then able to hydrolyse the glucosinolates (Figure 3.1). This hydrolysis is a very efficient process, especially under moist conditions. When vegetables are pulped or when water is added to chopped vegetables, the glucosinolates are, in a short period of time, enzymatically converted to their breakdown products (de Vos and Blijleven, 1988).

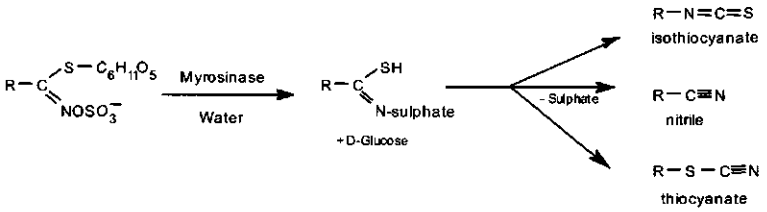

Figure 3.1 Schematic diagram of hydrolysis of glucosinolates. R can represent an aliphate, aromate or indolyl group.

There are a number of reports describing the effects of storage of broccoli on the glucosinolate content. Hansen et al. (1995) reported the behaviour of glucosinolates in broccoli stored under controlled atmosphere (CA) conditions. The CA treatment and storage time appeared to have no significant effect on the relative content of the most important glucosinolates in broccoli. Rodrigues and Rosa (1999) studied the effects of post-harvest treatments on the levels of glucosinolates in broccoli. They observed a decrease of most glucosinolates after 5 days of storage at room temperature. However, their data show an unexplainable increase of 4-hydroxy-3-indolylmethyl glucosinolate.

Few data are currently available on the effects of chopping and storage of *Brassica* vegetables under less moist conditions as apply for pre-chopped vegetables in the vegetable-processing industry. An investigation was therefore conducted to examine the effect of chopping and storage of different *Brassica* vegetables on their glucosinolate content.

Experimental

Plant material and sample preparation

White cabbage (cv Marathon) and red cabbage (cv Reliant) were supplied by Novartis Seed BV (Enkhuizen, The Netherlands). The vegetables were grown in the open air on loam soil. After harvesting they were stored at 4°C until further

processing. Broccoli was purchased from local groceries and used immediately. The outer leaves of the cabbage were removed and complete cabbages were used for the experiments. Florets of different broccoli crops were combined to make a composite sample. The vegetables were chopped into pieces of approximately 1 cm^2 and mixed thoroughly, and portions of 100g were stored in open containers at room temperature for different times. Homogenisation (pulping) of white cabbage was carried out in a food blender. After storage the material was frozen with liquid nitrogen, ground in a Waring blender (Model 34BL99, Dynamics Corpn of America, New Hartford, Connecticut, USA) and freeze-dried for at least 48 h.

Glucosinolate analysis

Freeze-dried plant tissue (0.5 g) was extracted in 10 ml of boiling methanol (70%) in a water bath at 75°C for 20 minutes. The supernatant was collected after centrifugation (5000 x g, 10 min, RT). The pellet was re-extracted twice in the same way. The glucosinolate glucotropaeolin was absent from all tissues and therefore, a known quantity (1.0 ml 3.0 mM) was added to each sample during extraction as internal standard for subsequent high-performance liquid chromatography (HPLC) analysis. Part of the supernatant (2.0 ml) was loaded onto ion exchange mini-columns (DEAE Sephadex A-25) and the glucosinolates were desulphated on-column (Helboe et al., 1980). The desulphoglucosinolates were eluted with water and separated by gradient system HPLC (Spectra Physics) using a Nova Pak C18 (5 µm) reverse phase column (3.9mm x 159mm; Waters Corpn, Milford, MA, USA) with a flow rate of 1 ml/min. The mobile phase used was water (A) vs acetonitril/water (20:80 v/v, B), the total running time was 31 min and the gradient was changed as follows; 100% A/0% B for 1 min, then in 20 min to 0% A/100% B, and in 5 min to 100% A/0% B. Afterward the column was equilibrated at 100% A/0% B for 5 min. An UV detector was used at a wavelength of 229 nm.

The desulphoglucosinolates were quantified against the internal standard glucotropaeolin and expressed as µmol/g dry matter. The peaks were identified by comparison with standard glucosinolates (sinigrin, glucoraphanin, glucotropaeolin, gluconasturtiin) and with data obtained from the literature (Minchinton et al., 1982). Glucosinolates were analysed for significant differences by analysis of variance performed using Anova tests.

Results

HPLC analysis of red and white cabbage revealed a total of nine different glucosinolates (GS), namely five aliphatic and four indolyl glucosinolates. Although the glucosinolate pattern is similar in the different cabbage types, the individual levels vary strongly (Table 3.1). Differences in absolute glucosinolate levels compared to the literature (Kushad et al., 1999) are considered to be primarily due to differences in cultivars and growing conditions.

Table 3.1 Glucosinolates concentrations of the fresh *Brassica* vegetables (concentration in μmol/100g dry weight)

Structure of R group	Trivial name	White cabbage	Red cabbage	Broccoli
3-Methylsulphinylpropyl	Glucoiberin	542±98	103±12	165±30
2-Hydroxy-3-butenyl	Progoitrin (PROG)	113±5	99±1	254±17
2-Propenyl	Sinigrin (SIN)	961±262	81±14	0±0
4-Methylsulphinylbutyl	Glucoraphanin (RAPH)	4.0±0.6	425±29	735±121
3-Butenyl	Gluconapin	127±7	300±2	26±10
4-Hydroxy-3-indolylmethyl	4-OH-Glucobrassicin (4-OHGB)	50±6	151±29	74±22
3-Indolylmethyl	Glucobrassicin (GB)	294±3	175±3	350±29
4-Methoxy-3-indolylmethyl	4-Methoxy-glucobrassicin (4-MeGB)	45±5	134±17	26±1
1-Methoxy-3-indolylmethyl	Neoglucobrassicin (NeoGB)	8±4	2.2±0.1	96±6
Total glucosinolates		2144±390	1470.2±67	1726±210

The glucosinolates 3-methylsulphinylpropyl (25%) and 2-propenyl (45%) dominate in white cabbage, whereas in red cabbage 4-methylsulphinylbutyl is the major glucosinolate (29% of total GS). The indolyl glucosinolates (particularly 3-indolylmethyl) represent about 19 % and 31 % of the total glucosinolates in white and red cabbage respectively. The glucosinolate content in broccoli is mainly represented by 4-methylsulphinylbutyl (43%) and the indolyl glucosinolates (32%). This is in agreement with other reports (Kushas et al., 1999; Rodrigues and Rosa, 1999) and makes broccoli the most important source for 4-methylsulphinylbutyl glucosinolate, which is the precursor for the anticarcinogenic isothiocyanate sulphoraphane. Storage of chopped white cabbage under ambient conditions did not affect the aliphatic glucosinolate content in a significant manner. The levels of aliphatic glucosinolates, as shown for 2-hydroxy-3-butenyl (PROG) and 2-propenyl (SIN), remained relative constant even after 48 h of storage (Figure 3.2).

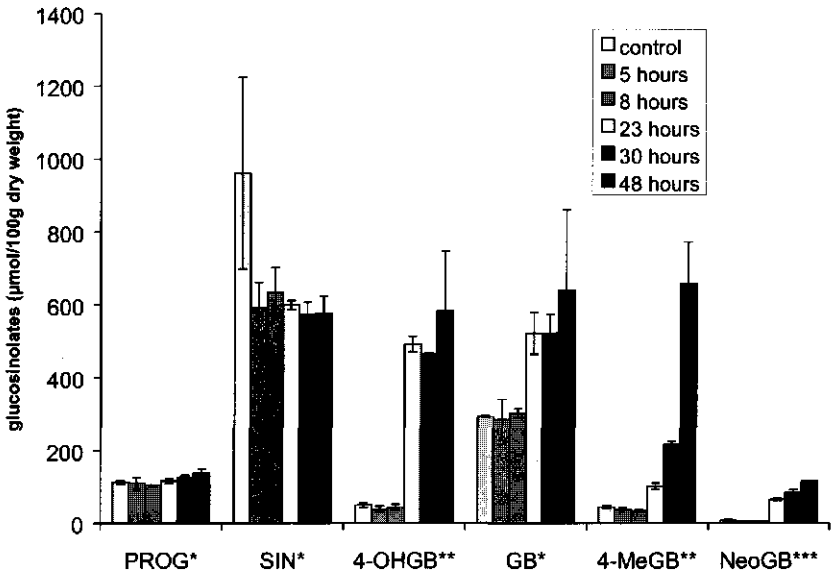

Figure 3.2 Effects of chopping and storage of white cabbage on glucosinolate content (see Table 3.1 for abbreviations). Vertical bars are standard errors of the mean (n=2). Significance of 48h storage for the difference in individual glucosinolates: *not significant, $p>0.05$; **$p<0.05$; ***$p<0.01$.

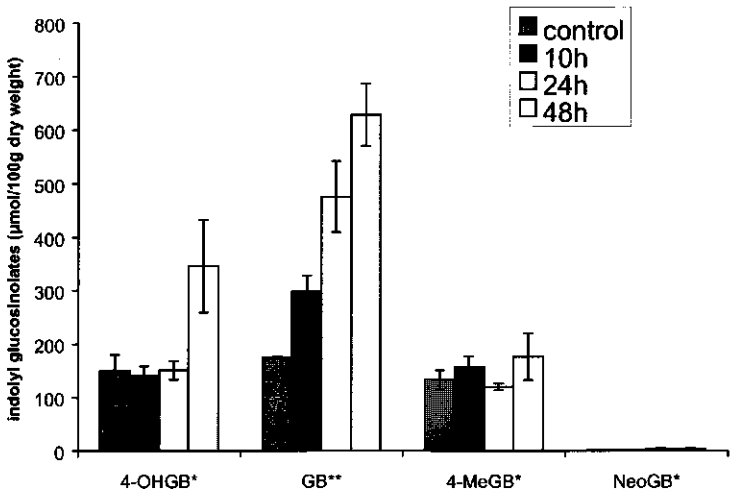

Figure 3.3 Effects of chopping and storage of red cabbage on indolyl glucosinolate content (see table 3.1 for abbreviations). Vertical bars are standard errors of the mean (n=2). Significance of 48h storage for the difference in individual glucosinolates: *not significant, $p>0.05$; **$p<0.01$.

However, the indolyl glucosinolates are strongly affected by chopping and storage of white cabbage. The total level of indolyl glucosinolates increased up to 3-fold after 23 hours ($P<0.01$) and 5-fold (2 mmol/100g DW) after 48 hours ($P<0.05$). This substantial increase is the result of the increase of all four individual indolyl glucosinolates, of which 4-methoxy- (4-MeGB) and 1-methoxy-3-indolylmethyl (NeoGB) glucosinolate both showed a 15-fold rise in concentration after 48 h (Figure 3.2). The increase changed the glucosinolate pattern dramatically, with the indolyls as major glucosinolates representing 61% of the total amount of glucosinolates (versus 19% before treatment).

In Figure 3.3 the levels of the individual indolyl glucosinolates are shown after chopping and storage of red cabbage. A gradual increase in the level of 3-indolylmethyl glucosinolate (GB) was detected, resulting in a 3.5-fold rise after 48 h ($P<0.01$). 4-Hydroxy-3-indolylmethyl glucosinolate (4-OHGB) showed no increase after 24 h, though it seemed to increase after 48 h of storage (not significant). The levels of the other indolyl glucosinolates remained unchanged even after 48 h. Similarly as for white cabbage, the aliphatic glucosinolates remain unaffected in red cabbage after storage (not shown).

Chopping and storage of broccoli affected the glucosinolates differently (Figure 3.4). Significant decreases ($P<0.001$) were noted for the aliphatic glucosinolates 2-hydroxy-3-butenyl (PROG) and 4-methylsulphinylbutyl (RAPH) after 48 h of storage of chopped broccoli.

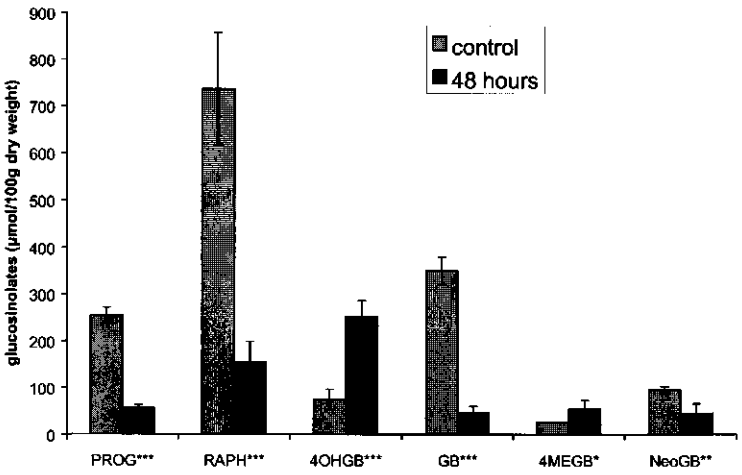

Figure 3.4 Effects of chopping and storage of broccoli on glucosinolate content. (see table 3.1 for abbreviations). Vertical bars are standard errors of the mean (n=3). Significance of 48h storage for the difference in individual glucosinolates: *p<0.05; **p<0.01; ***P<0.001.

Among the indolyl glucosinolates the 4-hydroxy-3-indolyl glucosinolate showed a substantial increased (3.5-fold, $P<0.001$) and 4-methoxy-3-indolylmethyl glucosinolate a smaller increase (2-fold, $P<0.05$), whereas the concentration of 3-indolylmethyl glucosinolate declined 7.6-fold. As a consequence, 4-hydroxy-3-indolylmethyl glucosinolate became the major glucosinolate in the treated broccoli (41 % of total GS).

In a comparable experiment, hydrolysis of white cabbage was challenged with increasing tissue damage carried out by homogenisation of the cabbage to moist pulp. This treatment did not result in increases of any of the glucosinolates; instead, a decline of approximately 70% of aliphatic and indolyl glucosinolates was observed after 24 h of storage.

Discussion

The breakdown of glucosinolates by the hydrolytic plant enzyme myrosinase is usually a very rapid event. However, the rate of hydrolysis is influenced by conditions such as humidity, temperature and the presence of co-factors and pH may affect the breakdown pathway. The prerequisites for hydrolysis are the release of both constituents, glucosinolates and myrosinase, from the plant cells, but also the presence of free water.

Our data show that when cabbage is chopped or sliced, hydrolysis of glucosinolates is limited, while thorough homogenisation (pulping) of cabbage results in a high degree of glucosinolate degradation. The extent of physical damage and therefore the release of the constituents can explain these differences. Hydrolysis will occur only at the cut surfaces. A rough calculation predicts that this will account for a few percent of cell damage possibly explaining the limited glucosinolate hydrolysis. Increasing cell damage by pulping or juicing of the cabbage will release more myrosinase and glucosinolates, resulting in a higher amount of breakdown as shown for white cabbage. Broccoli, on the other hand, is a much more perishable vegetable in which senescence (visible as yellowing of the florets) can cause more severe cell damage, resulting in strong hydrolysis (autolysis) of the glucosinolates (Figure 3.4) by the endogenous enzyme myrosinase.

Besides limited amount of hydrolysis of aliphatic glucosinolates in cabbage, elevated levels of some indolyl glucosinolates were observed after chopping and storage under ambient conditions of the different types of *Brassica* vegetables. This remarkable stress-induced post-harvest increase of glucosinolates in *Brassica* crops has not been described in the literature before.

In plants in the field, stress-induced increases of indolyl glucosinolates have been found in swede and kale caused by infestation of turnip root fly (Birch et al., 1992) or

of cabbage stem flea-beetle (Koritsas et al., 1991). Bodnaryk (1992) demonstrated the correlation of mechanically wounding, mimicking pest damage, and induction of 3-indolylmethyl and 4-hydroxy-3-indolylmethyl glucosinolates. In those experiments, cotyledons of *B napus* were damaged by needle puncturing, which resulted in a 3-fold increase of 3-Indolylmethyl glucosinolate after 24 h. Exposure of leaves to methyl jasmonate was found to cause selective induction of glucosinolates in oilseed rape (Doughty et al., 1995). These examples of stress-induced increase of indolyl glucosinolates are related to intact plants. In our research we describe changes of the glucosinolate levels in post-harvest vegetables. A recent study of post-harvest broccoli described results that are in agreement with our observations. Rodrigues and Rosa (1999) investigated glucosinolate levels in the primary and secondary inflorescence of fresh broccoli after different treatments. They observed a strong decline of most of the glucosinolates when the fresh material was left at room temperature for 5 days. Their data however, show an almost 5-fold increase of 4-hydroxy-3-indolylmethyl glucosinolate and 2-fold increase of 4-methoxy-3-indolylmethyl glucosinolate but a large decrease of 3-indolylmethyl glucosinolate in the secondary inflorescences of broccoli stored for 5 days. No explanation was given for this increase of glucosinolates.

Previous studies on the effects of vegetable processing have always focussed on the loss of total glucosinolates. Processes such as steaming, blanching and cooking mostly resulted in a decrease of all types of glucosinolates (Slominski and Campbell, 1989; de Vos and Blijleven, 1988). This can be explained by breakdown of glucosinolates by myrosinase or high temperatures, and leaching of glucosinolates into the cooking water. In our study we showed that the opposite, i.e. an increase of some indolyl glucosinolates, could also take place during processing. In this respect we can argue that measuring total amounts of glucosinolates in (processed) vegetables can mask the behaviour of less abundant individual glucosinolates. These findings will change our ideas about the fate of glucosinolates and the consequences for estimation of intake levels from processed *Brassica* vegetables.

Conclusions

Apparently, mechanical damaging, i.e. chopping of post-harvest *Brassica* crops induced physiological changes markedly affecting the levels of individual glucosinolates. We propose that the total glucosinolate content of chopped cabbage is possibly a reflection of two opposing mechanisms, namely breakdown of glucosinolates by myrosinase and formation of some indolyl glucosinolates by a so far unknown mechanism. What types of indolyl glucosinolates are inducible depends on the species of *Brassica*.

It is clear that factors inducing and regulating indolyl glucosinolates in plant metabolism need to be studied in much greater detail as evidence increases on the biological activities of these compounds in humans. The possibility of increasing the amounts of some indolyl glucosinolates by chopping, as found in this study, opens up new ways of improving the potential health benefits of *Brassica* vegetables.

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Chapter 4

Fate of glucosinolates during microwave treatment of red cabbage (*Brassica oleracea* L. var. *Capitata* f. *rubra* DC.)

Ruud Verkerk and Matthijs Dekker, submitted for publication.

Abstract

The concentrations of individual and total glucosinolates (GS) were measured in red cabbage after different microwave treatments varying in time and intensity of the treatments. Furthermore the myrosinase enzyme activity of the microwave-heated vegetables is determined. The retention of glucosinolates in the cabbage and the residual activity of the hydrolytic enzyme as a result of microwave preparation were compared with untreated cabbage. In general, high total glucosinolate levels were observed for all the applied microwave treatments. This high retention probably reflects the absence of leaching of glucosinolates into cooking water that takes place in conventional cooked vegetables.

It is striking that many of the time/energy input combinations result in levels exceeding the total GS-content of the untreated cabbage material. Moreover the increase in levels seems to be associated with the energy input applied. A possible explanation for this behaviour is an increased extractability of GS from heat-treated cabbage as compared to raw cabbage. The activity of myrosinase was affected differently when vegetables were microwave cooked with varying powers. Substantial myrosinase activity was retained in cabbage at low (24 min 180 Watt) and intermediate microwave powers (8 min 540 Watt) while microwave cooking for 4.8 minutes at 900 W (259.2 kJ energy input) resulted in complete loss of hydrolytic activity. In this respect, differences in observed temperature profiles of the various microwave treatments play an important role. Higher retention of glucosinolates and controllable amounts of active myrosinase can offer increasing health-promoting properties of microwave prepared *Brassica* vegetables.

Introduction

Glucosinolates are a group of secondary plant metabolites that occur in crops belonging to the family of Brassicaceae. The widely cultivated, economically important vegetables such as broccoli, cauliflower, cabbage and Brussels sprouts, are the major sources of glucosinolates in the human diet. In the past few years *Brassica* vegetables are receiving more attention due to the health-promoting properties ascribed to the glucosinolates. The particular interest in glucosinolates for the food research is based on their anticarcinogenic properties and also because of their contribution to the characteristic flavour and odour of many *Brassica* vegetables. Specific hydrolysis products of glucosinolates are responsible for these important properties. When the plant tissue is damaged by food preparation or mastication of the vegetables the glucosinolates are brought into contact with, and hydrolyzed by, the endogenous plant enzyme myrosinase (thioglucoside glucohydrolase EC 3.2.3.1), releasing a broad range of breakdown products including isothiocyanates and indoles.

The level of glucosinolates ingested by humans depends on a variety of factors along the complete production chain of *Brassica* vegetables (Dekker et al., 2000).

Most likely, processing and food preparation of the vegetables affect mostly the glucosinolate content and consequently determine the final intake levels of health-protective compounds. Processes such as chopping, cooking or freezing influence the extent of hydrolysis of glucosinolates and the composition of the final hydrolysis products. As most vegetables are processed in some way before consumption, the effects of processing should be taken into account in order to make accurate estimates of dietary intake of these protective compounds (Dekker et al., 2000). Hence, control of glucosinolate levels and myrosinase activity in *Brassica* vegetables is highly desirable.

A large number of *Brassica* vegetables are consumed after cooking. Various studies on different phytochemicals have shown that conventional cooking can lower their contents in foods. Examples as folate in spinach (Leichter et al., 1978) and in broccoli (Klein et al., 1979; 1981) show large losses caused by leaching of the protective compounds in the cooking water.

Also considerable reductions of glucosinolates levels are demonstrated in different studies (Mullin and Sahasrabudhe, 1978; Ciska and Kozlowska, 2001; Sones et al., 1984). Rosa and Heaney (1993) analysed the effects of cooking of different cabbage types and measured individual and total glucosinolate levels in the cooked leaves and the cooking water. It appeared that the glucosinolate content of the cabbages was reduced by more than 50%, mostly ascribed to leaching of the glucosinolates into the cooking water. However, the effects of cooking of *Brassica* vegetables on the hydrolytic activity of the enzyme myrosinase are hardly studied. Besides the

glucosinolates, the presence of active myrosinase is a prerequisite for formation of protective breakdown products.

Microwave cooking is an interesting alternative way of cooking with little or no water needed for preparation of the vegetables. Therefore leakage of glucosinolates is limited and higher retention of glucosinolates and breakdown products in the *Brassica* vegetables can be expected. Microwaves generated by a magnetron are absorbed by food; alignment of dipole molecules of the medium (mainly water) with the microwave field creates friction among molecules, which results in heating of the product. The temperature rise in the food depends on the duration of heating, the location in the food, convective heat transfer at the surface, and the extent of evaporation of water inside the food and at its surface. Typically, microwave food processing uses the 2 frequencies of 2450 and 915 MHz. Of these two, the 2450 MHz frequency is used for home ovens and both are used in industrial heating (Knutson et al., 1987). Use of microwave energy rather than conventional methods to cook food results in savings in energy and time, improved acceptability of some foods by consumers and improved nutritive quality of many foods. Research has considered the convenience and consumer satisfaction of microwave-heated foods and inactivation of spoilage and pathogenic micro-organisms by microwave energy. Special interest goes to the high retention of nutrients in microwave prepared foods. One of the main claimed advantages is that the speed of the heat treatment produces less degradation of the nutritional value resulting in a product of high quality.

The aim of this study was to investigate the behaviour of the glucosinolate/myrosinase system in red cabbage during a broad range of microwave treatments varying in time and power (energy input). Glucosinolate content and the hydrolytic activity of myrosinase were measured in red cabbage samples treated at high power and short heating times in comparison with treatments at low power and long heating times. The overall effects of microwave cooking on the protective capacity of cabbage are discussed in respect to intake of glucosinolates and health-protective breakdown products as compared to conventional cooking methods.

Materials & Methods

Sample preparation. Red cabbage (*Brassica* oleracea L., Capitata group) material was purchased from local supermarkets (Wageningen, The netherlands). The outer leaves of the heads were removed and complete cabbage heads were used for the experiments. The cabbage was chopped into pieces of approximately 1 cm and mixed thoroughly. For glucosinolate analysis of the fresh cabbage the chopped material was directly frozen with liquid nitrogen. The frozen material was ground in a Waring

Blender (Model 34BL99, Dynamics Corp. of America, New Hartford, Connecticut, USA) and stored at -30°C until further analysis.

Preparation of cabbage juice for the analysis of myrosinase activity. Red cabbage material was chopped and juice was prepared with a commercial juice centrifuge (Braun, type 4290). After the microwave treatment 200 g cabbage was cooled down on ice until 23°C and juiced. The obtained juice was sieved to remove the larger parts. Subsequently the juice was incubated for 1 hour at 40°C in an oven to hydrolyse the endogenous glucosinolates present in the cabbage. The obtained batch of glucosinolate-free juice was considered as a crude myrosinase extract in which different cabbage components are present that could affect the myrosinase activity (e.g. ascorbic acid). Part of the juice was incubated for 15 minutes at 100°C in order to inactivate the enzyme myrosinase. This juice was used for dilution purposes in the activity assays.

Experimental set-up

Microwave cooking. Approximately 2 kg of red cabbage was chopped (1 cm^2) and divided into portions of 300 g each. Each portion was placed in a 500 ml beaker and cooked in a microwave oven (Daewoo, Model KOC-87-T, Korea) at 2450 MHz according to the scheme in Table 4.1. After the microwave treatment, a subsample of 100 g was taken for analysis of desulphated glucosinolates. The vegetables were frozen with liquid nitrogen, ground in a Waring Blender and stored at -20°C until analysis. The remaining 200 g of cabbage was used for the preparation of juice for the analysis of the hydrolytic myrosinase activity.

Separate experiments were carried out for the temperature registration of cabbage samples during microwave cooking. The temperature of cabbage samples was measured in a Whirlpool microwave (type m506, 750W output) using a glassfibre probe (Takaoka, Type FTP3-3003 s/n 31888). The probe was inserted, via an opening in the microwave, in the middle of chopped portions of red cabbage. The obtained data (time/temperature profiles) were used for the development of a predictive temperature model.

Analysis

Glucosinolate analysis. The glucosinolates were analysed in the fresh cabbage or cabbage juice using high performance liquid chromatography (HPLC) following on-column desulphation as described by Verkerk et al. (2001).

Determination of myrosinase activity. The activity of the enzyme myrosinase present in the juice is measured by hydrolysis of a known amount of sinigrin added to

the juice. To 5.0 g of cabbage juice 1 ml of 6 mM sinigrin was added and incubated at 40°C for 0, 5, 10 and 20 minutes. The reaction was stopped by adding 12 ml of 100% hot methanol (for 10 min at 75°C). The juices were centrifuged (5000 x g, 10 min, RT) and remaining sinigrin was isolated from the collected supernatant and analysed by HPLC.

Table 4.1 Heating scheme of red cabbage samples according to different powers and heating times.

Treatment	Energy input (kJ)	180W	540W	900W
A	32.4	3 min	1 min	36 s
B	64.8	6 min	2 min	1 min 12s
C	129.6	12 min	4 min	2 min 24s
D	194.4	18 min	6 min	3 min 36s
E	259.2	24 min	8 min	4 min 48s

Modelling of the temperature in cabbage during microwave heating

Time-temperature profiles within a food product are influenced by both internal heat generation due to absorption of electrical energy from the microwave field and heat transfer by conduction, convection and evaporation (Komolprasert and Ofoli, 1989). Microwave heating is complicated and not easily modelled because the rate of energy absorption and energy distribution is controlled by the physical, thermal and electrical properties of the product and the variation with temperature during radiation. In this study modelling of the cabbage temperature was simplified with the assumption that the $T_{c,\,max}$=100 °C. The specific food properties (e.g. density, specific heat) were not characterised individually but were lumped in the energy conversion coefficients. During the process of microwave heating the cabbage will also transfer heat to the surroundings which subsequently warms up.

The model was based on the energy input, cabbage weight and heat transfer to the surroundings (equations 1 and 2).

$$\frac{dT_c}{dt} = k_1 \cdot \frac{P}{m} - k_2(T_c - T_{sur}),\ T_c \le T_{c,max} \qquad (1)$$

$$\frac{dT_{sur}}{dt} = k_3 \cdot (T_c - T_{sur}),\qquad T_{sur} \le T_{sur,max} \qquad (2)$$

in which *Tc* is the temperature of the cabbage (°C) and *Tsur* the temperature of the surroundings increasing in time *t* (s) and *P* (Watt) refers to the applied microwave power on the mass *m* (g) of the cabbage. k_1 is an energy conversion coefficient ($°C \cdot g \cdot J^{-1}$) and k_2, k_3 are heat transfer coefficients (s^{-1}).

The parameters in equation 1 and 2 were estimated from experimental data obtained from microwave treatments of 100g, 200g and 300g red cabbage upon heating at 150, 450 and 750 Watt for varying times.

Data analysis. Statistical analysis of the data was performed on the original data by one-way analysis of variance (ANOVA) using the statistical package from Microsoft Excel software. Fitting of the model equations on the experimental data has been done by minimising the sum of squares of the relative errors between model prediction and measured data using non-linear regression with the "solver" routine of Microsoft Excel 97.

Results

Temperature profiles. The measured temperatures of the cabbage microwave heated at different power inputs were fitted to model equations 1 and 2 (Figure 4.1).

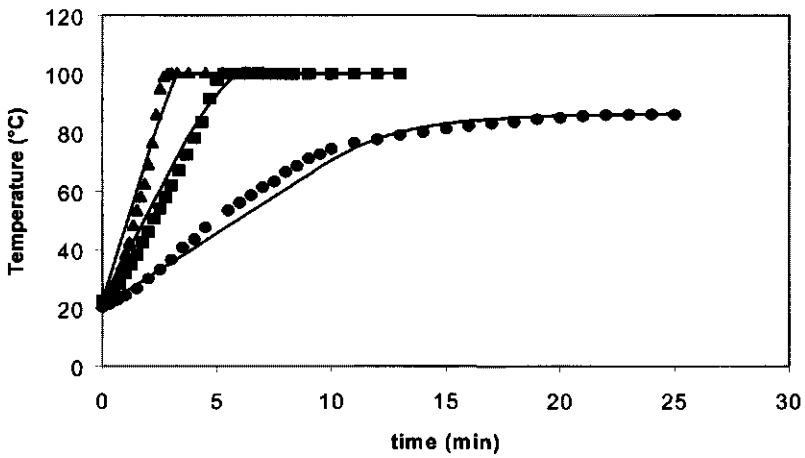

Figure 4.1 Temperature profiles of 300 g red cabbage microwave heated at 150W (●), 450W (■) and 750W (▲), solid lines are the model fits.

Microwave cooking at 450 and 750W resulted in a temperature of the cabbage of 100°C reached within 5 and 3 minutes respectively and remained constant after that time. On the other hand, the cabbage microwave treated at 150W raised in temperature considerably slower and did not reach higher than 86°C after 25 minutes

of exposure (Figure 4.1). With use of the developed model temperature profiles of different powers can be predicted. The obtained model parameters are presented in Table 4.2.

Table 4.2 Estimation of heat transfer parameters.

Parameters	
k_1	10.7 °C·g·J^{-1}
k_2	0.26 s^{-1}
k_3	4.0 s^{-1}

Note: Parameters k_2 and k_3 are fitted for a cabbage weight of 300g. These parameters can deviate when using different weights.

Total and individual glucosinolates. The main glucosinolates identified in the red cabbage are listed in Table 4.3. The glucosinolates 2-Propenyl and 4-Methylsulphinylbutyl (MSB) are representing together 70 % of the total amount of glucosinolates. These two types of glucosinolates are responsible for the important characteristics of flavour (sinigrin) and health-protection (glucoraphanin) of red cabbage, respectively.

Table 4.3 Average levels (μmol·100g^{-1} fresh weight) of the main glucosinolates identified by HPLC in untreated (fresh) red cabbage.

Structure of R group	Trivial name	[C]*	SD
2-propenyl	Sinigrin	33.0	2.2
4-Methylsulphinylbutyl	Glucoraphanin	20.8	2.7
4-Hydroxy-3-indolylmethyl	4-Hydroxyglucobrassicin	5.4	0.8
3-Indolylmethyl	Glucobrassicin	8.6	1.3
4-Methoxy-3-indolylmethyl	4-Methoxyglucobrassicin	8.8	0.9
	Total	76.6	7.9

* Mean concentrations of the five different batches (A-E) of red cabbage.

We used for the different energy inputs A, B, C, D and E different batches of red cabbage bought at the local supermarket. This caused some variation in the individual and total glucosinolate levels between batches (Table 4.3). Substantial variations in glucosinolate content between and within *Brassica* groups have been reported earlier (Kushad et al., 1999; Carlson et al., 1987). Genetic and environmental factors are supposed to contribute both significantly to the variation in levels of glucosinolates.

The total energy input of a microwave treatment (Joules) is the result of applied power multiplied by the time of exposure (seconds).

Effect of microwave treatments on GS content. The total glucosinolate contents (GS) in red cabbage after the different microwave treatments are presented in Table 4.4. The relative change in GS-content was calculated for each cabbage batch. In general, high total glucosinolate levels were observed for all the applied microwave treatments. It is striking that many of the time/energy input combinations resulted in levels exceeding the total GS-content of the untreated cabbage material. Moreover the increase in levels seems to be associated with the energy input applied (Figure 4.2). Total glucosinolate content of red cabbage microwave treated for 3 minutes at 180W (32.4 kJ) increased from 74.4 ± 4.3 μmol/100g fresh weight to 128.0 ± 5.2 μmol/100g fresh weight when cooked for 4 min 48 s at 900W (259.2 kJ). This latter more intense microwave treatment resulted in 178% increase of total glucosinolate content compared to the control ($P<0.01$).

Table 4.4 Mean concentration of total amount of glucosinolates in red cabbage after different microwave treatments[a] (concentration in μmol·100g^{-1} fresh weight; concentration relative to untreated cabbage (expressed as %) is indicated in parentheses). Significant difference: *P<0.05; **P<0.01.

Treat-ment	Energy Input (kJ)	Untreated		180W		540W		900W	
		[C]	SD	[C]	SD	[C]	SD	[C]	SD
A	32.4	72.2	11.1	74.4 (103)	4.3	73.1 (101)	1.7	80.5 (111)	15.0
B	64.8	81.0	12.3	77.9 (96)	6.6	71.0 (88)	14.5	85.0 (105)	11.9
C	129.6	83.6	15.4	85.1 (102)	14.1	**119** (142)	5.3	**113** (135)	1.1
D	194.4	74.0	9.8	**129** (174)	7.9	**103** (139)	1.4	**100** (134)	5.2
E	259.2	71.9	9.0	**115** (160)	1.8	**103** (143)	4.9	**128** (178)	5.2

[a]Treatments were performed in duplicate ([C] ±SD).

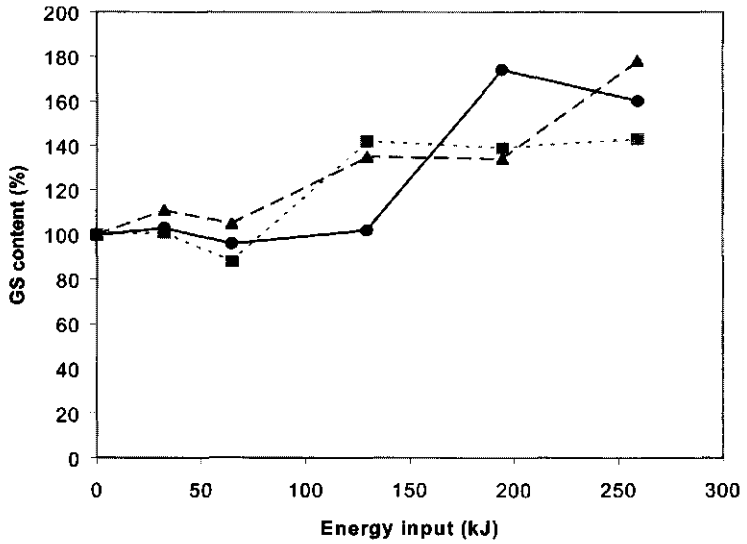

Figure 4.2 The effect of different microwave energy inputs on the total glucosinolate content in red cabbage. 180 W (●), 540W (■), 900 W (▲).

Table 4.5 Mean concentration of 2-Propenyl glucosinolates in red cabbage after different microwave treatments[a] (concentration in μmol·100g^{-1} fresh weight; concentration relative to untreated cabbage (expressed as %) is indicated in parentheses). Significant difference: *P<0.05; **P<0.01.

Treat-ment	Energy Input (kJ)	Untreated		180W		540W		900W	
		[C]	SD	[C]	SD	[C]	SD	[C]	SD
A	32.4	31.2	*1.2*	**40.6*** **(130)**	*2.5*	33.9 (109)	*1.2*	41.0 (132)	*8.2*
B	64.8	35.5	*4.3*	38.8 (110)	*5.2*	26.5 (75)	*3.0*	**37.4*** **(105)**	*1.4*
C	129.6	41.0	*0.0*	47.3 (115)	*16.4*	**58.6**** **(143)**	*0.1*	**56.8**** **(139)**	*1.3*
D	194.4	29.0	*5.3*	**64.6*** **(223)**	*5.9*	40.4 (139)	*4.8*	**42.2*** **(146)**	*1.8*
E	259.2	28.2	*0.0*	**56.9*** **(202)**	*3.8*	**47.9**** **(170)**	*1.7*	**66.7**** **(237)**	*3.7*

[a]Treatments were performed in duplicate ([C] ±SD).

Despite the large increase of total glucosinolate content, there are remarkable differences in behaviour of individual glucosinolates for the different microwave treatments. As the glucosinolates 2-Propenyl and 4-Methylsulphinylbutyl represent most of the glucosinolates in red cabbage they determine mainly the course of the

total GS-content. The glucosinolate 2-Propenyl (Sinigrin) presented in Table 4.5 showed a substantial increase especially at higher energy inputs (treatment E). The 2-Propenyl concentration was 202%, 170% and 237% higher for treatments E180 ($P<0.05$) E540 ($P<0.01$) and E900 ($P<0.01$) respectively as compared to the untreated samples of the same cabbage batch. However, 4-Methylsulphinylbutyl (MSB, Glucoraphanin) glucosinolate levels decrease at lower energy inputs (treatments A and B) after which some treatments showed a small increase at higher energy inputs (Table 4.6). However, as a whole the different microwave treatments do not show a notable effect on MSB glucosinolates.

Table 4.6 Mean concentration of 4-Methylsulphinylbutyl glucosinolates in red cabbage after different microwave treatments[a] (concentration in $\mu mol \cdot 100g^{-1}$ fresh weight; concentration relative to untreated cabbage (expressed as %) is indicated in parentheses). Significant difference:*P<0.05; **P<0.01.

Treat-ment	Energy Input (kJ)	Untreated		180W		540W		900W	
		[C]	SD	[C]	SD	[C]	SD	[C]	SD
A	32.4	20.0	*0.3*	**14.2** (71)**	*0.4*	17.9 (89)	*1.9*	19.4 (97)	*1.8*
B	64.8	21.5	*0.4*	**15.9** (74)**	*0.4*	**17.7* (82)**	*0.9*	18.2 (85)	*2.2*
C	129.6	23.3	*0.0*	**17.1** (73)**	*0.3*	**28.1* (121)**	*1.2*	22.0 (95)	*0.6*
D	194.4	21.1	*9.3*	**23.8* (113)**	*0.7*	27.0 (128)	*0.0*	20.6 (98)	*0.7*
E	259.2	18.1	*3.3*	**17.6* (98)**	*0.5*	20.4 (113)	*1.3*	21.1 (117)	*0.8*

[a]Treatments were performed in duplicate ([C] ±SD).

Glucosinolate levels of the three 3-Indolylmethyl glucosinolates generally also showed an increase with increasing energy inputs (Table 4.7-4.9, Figure 4.3-4.5). After an initial decrease, significant increases were noted in 4-Hydroxy-3-indolylmethyl glucosinolate content for treatments D (194.4 kJ) and E (259.2 kJ) compared to untreated cabbage samples (Table 4.7). There is a similar trend for 4-Methoxy-3-indolylmethyl glucosinolates although the values of D900 and E540 deviate from this having lower levels (Table 4.9). In the case of 3-Indolylmethyl glucosinolates no decrease was observed; instead all treatments (except for B180) resulted in levels exceeding the untreated samples. However large variation in the measurements caused not all of the levels to be significantly increased (Table 4.8).

One remarkable observation in this respect is the trend of highest increase at the intermediate microwave treatments C540 [129.6 kJ] and C900 [129.6 kJ] and the subsequent decline at more intense treatments E540 [259.2 kJ] and E900 [259.2 kJ].

Table 4.7 Mean concentration of 4-Hydroxy-3-indolylmethyl glucosinolates in red cabbage after different microwave treatments[a] (concentration in $\mu mol \cdot 100g^{-1}$ fresh weight; concentration relative to untreated cabbage (expressed as %) is indicated in parentheses). Significant difference:*P<0.05; **P<0.01.

Treat-ment	Energy Input (kJ)	Untreated		180W		540W		900W	
		[C]	SD	[C]	SD	[C]	SD	[C]	SD
A	32.4	4.63	*0.12*	**3.18*** **(69)**	*0.20*	3.52 (76)	*0.37*	3.30 (71)	*0.80*
B	64.8	6.38	*0.46*	4.69 (74)	*0.60*	**5.53*** **(87)**	*0.18*	4.96 (78)	*1.21*
C	129.6	5.47	*0.27*	**3.61**** **(66)**	*0.06*	**6.77**** **(124)**	*0.15*	**5.89*** **(108)**	*0.37*
D	194.4	5.63	*2.88*	**7.04*** **(125)**	*0.61*	**6.68**** **(119)**	*0.10*	**7.43**** **(132)**	*0.39*
E	259.2	5.08	*0.15*	**8.05**** **(159)**	*0.08*	**6.69**** **(132)**	*0.13*	**9.03**** **(178)**	*0.07*

[a]Treatments were performed in duplicate ([C] ±SD).

Table 4.8 Mean concentration of 3-Indolylmethyl glucosinolates in red cabbage after different microwave treatments[a] (concentration in $\mu mol \cdot 100g^{-1}$ fresh weight; concentration relative to untreated cabbage (expressed as %) is indicated in parentheses). Significant difference:*P<0.05; **P<0.01.

Treat-ment	Energy Input (kJ)	Untreated		180W		540W		900W	
		[C]	SD	[C]	SD	[C]	SD	[C]	SD
A	32.4	9.64	*0.31*	11.19 (116)	*2.05*	**12.37*** **(128)**	*0.83*	10.89 (113)	*3.06*
B	64.8	8.29	*0.02*	8.18 (99)	*0.09*	14.65 (177)	*0.00*	14.26 (172)	*5.44*
C	129.6	5.47	*0.08*	9.83 (180)	*1.56*	16.25 (297)	*3.37*	**18.2**** **(333)**	*1.17*
D	194.4	9.20	*5.03*	**18.05*** **(196)**	*1.23*	17.72 (193)	*0.00*	**20.7**** **(225)**	*1.39*
E	259.2	10.55	*1.18*	**17.55*** **(166)**	*1.29*	18.58 (176)	*3.38*	**18.4**** **(174)**	*0.32*

[a]Treatments were performed in duplicate ([C] ±SD).

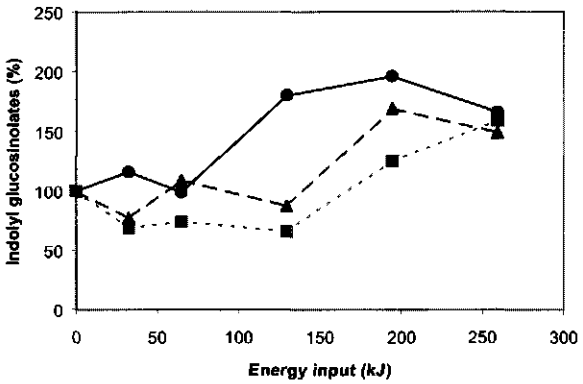

Figure 4.3 The effect of microwave energy inputs (180W) on various indolyl glucosinolate contents. Glucobrassicin (●); 4-Hydroxyglucobrassicin (■); 4-Methoxyglucobrassicin (▲).

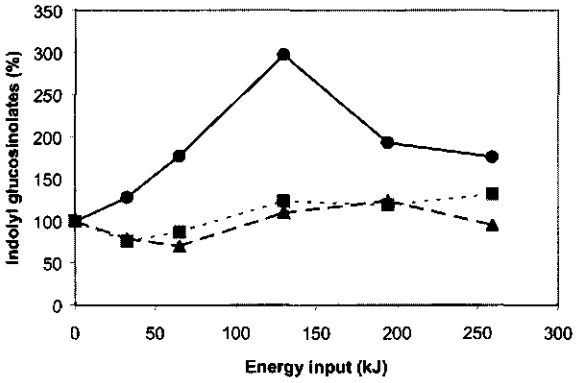

Figure 4.4 The effect of microwave energy inputs (540W) on various indolyl glucosinolate contents. Glucobrassicin (●); 4-Hydroxyglucobrassicin (■); 4-Methoxyglucobrassicin (▲).

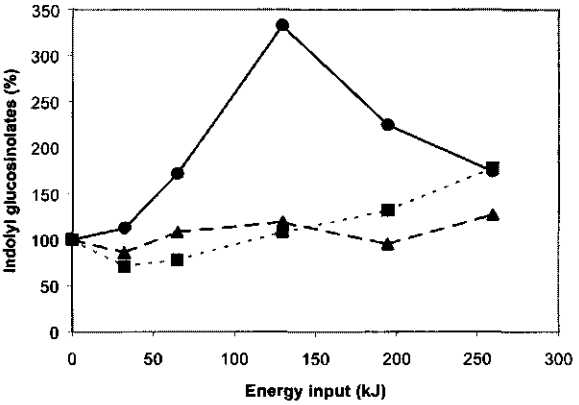

Figure 4.5 The effect of microwave energy inputs (900W) on various indolyl glucosinolate contents. Glucobrassicin (●); 4-Hydroxyglucobrassicin (■); 4-Methoxyglucobrassicin (▲).

Table 4.9 Mean concentration of 4-Methoxy-3-Indolylmethyl glucosinolates in red cabbage after different microwave treatments[a] (concentration in $\mu mol \cdot 100g^{-1}$ fresh weight; concentration relative to untreated cabbage (expressed as %) is indicated in parentheses). Significant difference:*P<0.05; **P<0.01.

Treat-ment	Energy Input (kJ)	Untreated		180W		540W		900W	
		[C]	SD	[C]	SD	[C]	SD	[C]	SD
A	32.4	6.78	*0.03*	**5.23**** **(77)**	*0.01*	**5.38**** **(79)**	*0.14*	5.85 (86)	*1.16*
B	64.8	9.45	*0.22*	**10.27*** **(109)**	*0.51*	**6.61*** **(70)**	*0.47*	10.23 (108)	*1.79*
C	129.6	8.39	*0.25*	7.29 (87)	*1.01*	**9.20*** **(110)**	*0.55*	**10.0**** **(119)**	*0.02*
D	194.4	9.09	*3.84*	**15.4**** **(169)**	*0.66*	**11.3**** **(124)**	*0.30*	8.63 (95)	*0.98*
E	259.2	10.06	*0.32*	**15.0*** **(149)**	*1.27*	9.51 (95)	*1.30*	**12.8**** **(127)**	*0.42*

[a]Treatments were performed in duplicate ([C] ±SD).

Effect of microwave treatments on myrosinase activity. Since the presence of the enzyme myrosinase is crucial in the production of health-protective breakdown products of glucosinolates it is important to assess the remaining hydrolytic activity in the cabbage after the various microwave treatments.

The activity of the enzyme myrosinase was determined in juices prepared from fresh and microwave treated red cabbage samples. Previous research (unpublished) showed that juicing of cabbage resulted in high myrosinase activity in the juice and little remained in the cabbage pulp. Since we are interested in the myrosinase activity at cellular conditions, measuring the activity in the juice of cabbage is preferred over the activity of the isolated enzyme, which is usually done (Yen and Wei, 1993; Ludikhuyze et al., 1999, 2000; Wilkinson et al., 1984). The presence of known (e.g. ascorbic acid, $MgCl_2$ and iron) and yet unknown components in the cabbage juice that are important for myrosinase activity gives a valuable advantage to this approach. After preparation of the juice the existing myrosinase was tested for its ability to hydrolyse pure sinigrin added to the juice sample. In Figure 4.6 the amounts of convertible sinigrin are presented after 20 minutes of exposure to the juiced samples. This figure shows that the activity of myrosinase diminished with increasing energy inputs. Cabbage microwave treated with the highest power (900W) and the highest energy inputs (C900, D900 and E900) (almost) completely lost hydrolytic capacity.

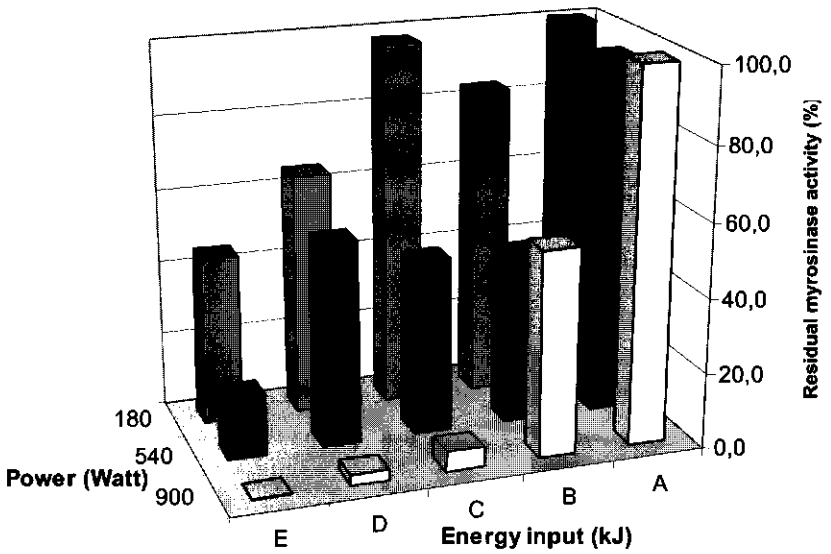

Figure 4.6 Residual myrosinase activity of red cabbage as a function of energy input to microwave. Energy inputs are A: 32.4 kJ, B: 64.8 kJ, C: 129.6 kJ, D: 194.4 kJ and E: 259.2 kJ.

The milder microwave treated cabbage at 540 Watt resulted in a reasonable amount of myrosinase residual activity capable to convert the exogenous sinigrin even at higher energy inputs (treatment D and E). Cabbage treated at lowest microwave powers (180W) retained the highest myrosinase activities.

Discussion

Different processes that can take place during microwave cooking determine the fate of glucosinolates. In this respect it is essential to realise that in intact cells of cabbage the membrane of the vacuole separates the enzyme from its substrate. First, hydrolysis will occur at the cutting surface of the chopped cabbage. Second, further membrane damage and cell rupture can be the result of increasing temperatures and microwave radiation. In the case of conventional cooking cell lysis will occur giving rise to a sudden increase in osmotic pressure difference over the vacuole membrane which will result in collapse of this membrane, resulting in a mixing of the glucosinolates and the myrosinase in the cooking water. Enzymatic degradation can then take place (see Chapter 6). Third, myrosinase activity increases with moderate heat at temperatures up to about 60°C, inactivation will occur at higher temperatures (Chapter 6).

Glucosinolates. In our study, microwave cooking of cabbage at low (180W), intermediate (540W) and high (900W) energy inputs did not result in losses in glucosinolate levels in the cabbage as occurs during conventional cooking in water. Unexpectedly, many microwave treatments with varying energy inputs revealed total GS-contents exceeding the levels present in untreated cabbage (Figure 4.2). This high retention probably reflects the absence of leaching of glucosinolates into cooking water that takes place in conventional cooked vegetables. The process of cell disruption probably will take place when temperatures are reached unfavourable for the hydrolytic enzyme myrosinase. Especially at high microwave power (900W) there is little opportunity for hydrolysis of glucosinolates taking place. During the different microwave treatments of the cabbage samples differences in behaviour can be recognised between the individual glucosinolates. The microwave treatments did not reveal a large affect on MSB glucosinolates, while 2-propenyl glucosinolate levels increased substantially.

Published data concerning the effects of microwave cooking on glucosinolate levels are scarce. In this respect, effects of microwave treatments on antioxidant compounds like ascorbic acid (AA) and β-carotene (β-C) were studied in more detail. Howard et al. (1999) reported no effects on the AA or β-C content after microwave cooking of broccoli for 8 min at 700W.

In our study, microwave cooking decreased the moisture content of cabbage by evaporation causing elevated glucosinolate levels in the samples. However a maximum weight loss of 20% after cooking was determined experimentally (not shown) and this could not explain the high increase of glucosinolates. A possible explanation for this phenomenon is an increase in chemical extractability of the glucosinolates after an intense heat treatment. Cooking has been reported to increase extractability of carotenoids. Hart & Scott (1995) showed in various green vegetables, peas and beans an average increase of 24% lutein and 38% β-carotene. These substantial increases of health-protective compounds after microwave cooking can have important consequences with respect to bioavailability of these compounds to humans.

Myrosinase. The activity of myrosinase is affected differently when vegetables are microwave cooked with varying time-power combinations though same total energy inputs. For example, substantial myrosinase activity was retained in cabbage after 24 minutes microwave cooking at 180W (259.2 kJ) while 4.8 min microwave cooking at 900 W (259.2 kJ) resulted in complete loss of hydrolytic activity (Figure 4.3). Similar profiles were observed with treatment C (129.6 kJ) and D (194.4 kJ). An explanation for these differences can possibly be found in the temperature profiles fitted for the different applied microwave treatments (Figure 4.7).

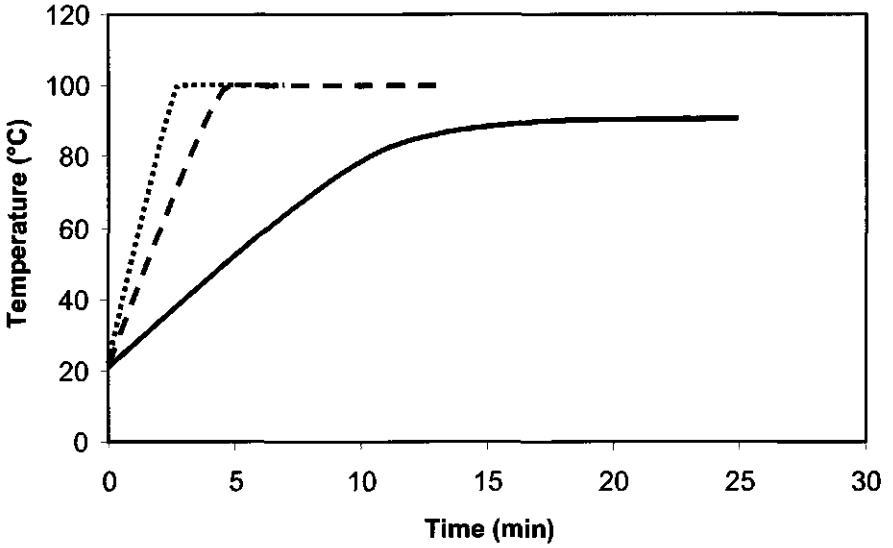

Figure 4.7 Temperature profile fits of 300 g red cabbage microwave heated at 180W (solid line), 540W (dashed line) and 900W (dotted line).

Microwave cooking at 900W resulted in a temperature of the cabbage of 100°C reached after 2.8 minutes and continued cooking for another 2 minutes. Apparently, the myrosinase enzyme was denatured at these conditions. However, red cabbage microwave treated at 180W raised in temperature considerably slower and did not reach higher than 90°C after 25 minutes of exposure (Figure 4.7). Under these conditions the more thermostable myrosinase (Yen and Wei, 1993) apparently can survive partly and maintain some hydrolytic activity. More difficult to explain were the myrosinase activities at the 540W microwave treated cabbage samples. Cabbage microwave treated at 540W reached a temperature of 100°C after 4.6 minutes and continued cooking for another 3.4 minutes. Striking in this treatment is the substantial remaining hydrolytic activity of 47%, 55% and 17% after 4, 6 and 8 minutes respectively. While cabbage treated at 900W retained only 5.4% and 2.7 % myrosinase activity after 3.6 and 4.8 minutes respectively. Certain discrepancies in activity differences can be imputed to the microwave principle of going on and off to regulate the power output (cycling). Furthermore, moisture content during microwave heating is known to affect enzyme inactivation and/or denaturation of proteins (Wang and Toledo, 1987).

The use of microwave energy with respect to enzyme inactivation in food systems has been investigated in a number of studies (Kermasha et al., 1993; Owusu-Ansah and Marianchuk, 1991). In these studies, mainly focussed on improving palatability and nutritional value of foods, high microwave powers are used, generally indicating that microwave enzyme inactivation appears to be more effective than conventional

heating. Our study shows a retention of substantial myrosinase activity at less intense microwave conditions, which is essential for the release of protective glucosinolate breakdown products during consumption.

Concluding remarks

The health benefits of vegetables are well recognised, but vegetable intake in Europe is below recommendations. Food preparation methods such as conventional cooking reduce intake of important potentially health-protective and promoting compounds as glucosinolates even more. In this study, microwave cooking appeared to show high retention of the glucosinolates even exceeding in some instances the levels in untreated cabbage material. Therefore microwave-cooked cabbage would result in a relatively higher intake of glucosinolates as compared to conventional cooked cabbage in water. The residual activity of the hydrolytic enzyme myrosinase as obtained at certain milder microwave conditions can possibly also cause conversion of glucosinolates into protective breakdown products during mastication of the vegetables. However it should be investigated to what degree myrosinase is able to exert hydrolysis when it is partially inactivated.

In conclusion, this study showed that microwave cooking of cabbage is an interesting alternative for conventional cooking. Higher retention of glucosinolates and controllable amounts of active myrosinase offers increasing health-promoting properties of microwave prepared *Brassica* vegetables.

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Chapter 5

Variation of glucosinolate levels throughout the production chain of *Brassica* vegetables

Adapted from

Matthijs Dekker, Ruud Verkerk and Wim M.F. Jongen (2000) Predictive modelling of health aspects in the food production chain: a case study on glucosinolates in cabbage. *Trends in Food Science and Technology* 11: 174-181.

Abstract

In this chapter it is demonstrated that many steps in the food production chain of *Brassica* vegetable products can have a large impact on the final intake of health protective glucosinolates. The large amount of variables for each step in the chain makes an experimental quantification of dietary intake of phytochemical extremely difficult. We present a concept of predictive modelling of health aspects in the production chain of vegetable products, which is intended to be used for the development of tools to facilitate both product and process development for health products as well as epidemiological input data for bioactive substances in the diet.

In the model, essential parameters that determine the health-promoting qualities of the food product need to be selected after which the most critical sub-processes for each parameter are investigated during the whole process. These sub-processes are translated to mathematical equations, usually (partial) differential equations in combination with mass balances, describing what happens to the health promoting quality during the process. In a case study the health protective glucosinolates present in *Brassica* vegetables are used to illustrate the value of such a predictive model. The described model provides a powerful tool for handling the variation of glucosinolate levels throughout the chain in a quantitative way. Product development, consumer advice and epidemiological research are important areas that can benefit enormously from this approach.

Introduction

There is a growing awareness of nutritionists, food scientists, the food industry, and, perhaps most importantly, of the consumers on the relation between diet and health. Nowadays there is an increasing amount of evidence for protective effects of compounds present in fruit and vegetables (phytochemicals) by epidemiology and mechanistic studies in vitro and in vivo or on human biomarkers. Some of the compounds that have been studied extensively are carotenoids, flavonoids, folates, and glucosinolates. It is thought that they play an important role in the prevention of various diseases, most importantly ageing diseases like cancer and coronary heart diseases. Research on this subject is focussed on promising individual compounds by food scientists or on certain fruit or vegetables by epidemiologists.

Mechanistic studies have shown various health protective effects of a large number of compounds. Epidemiological studies however have only shown associations between the total intake of fruit and vegetables and health protection with limited evidence linked to the individual components (Steinmetz and Potter, 1991). It is not yet possible to resolve whether these associations are to be attributed to Brassica vegetables per se or to vegetables in general.

The impact of food processing and storage on some micro-nutrients such as vitamins (especially vitamin C) and minerals are reasonably well known. Unfortunately, the stability and fate of phytochemicals such as e.g. flavonoids or glucosinolates in the production chain of fruits or vegetables have not been investigated to the same extent. The lack of knowledge on the effects of processing and storage has implications on the reliability of intake data used in epidemiological studies. Due to the lack of this quantitative information optimum intake levels for the majority of phytochemicals are not known. Despite this problem, there is a growing interest in enhancing levels of beneficial phytochemicals in crops by conventional breeding or genetic modification. With more knowledge on the effects of the complete production chain a more effective choice can be made on how to enhance, if desired, phytochemical levels in the final consumed product.

There is a growing amount of evidence directing to protective effects of certain phytochemicals in a dose-response relating way. The effective concentration-window of ingested phytochemicals can possibly play a crucial role for their protective mode.

For example, vitamin C, an essential constituent of the human diet, used widely throughout the food industry for its antioxidant properties. Epidemiologists recently reported that people who take 500 mg of vitamine C each day were two and a half (nonsmokers) to five (smokers) times more likely to suffer thickening of the carotid artery (Southon, 2000). While nobody disputes that vitamin C is an essential micronutrient needed to prevent disease and promote health the dose appears to be

crucial. Other examples emphasising the importance of intake balance are β-carotene (1994) and minerals like iron (Halliwell and Gutteridge, 1989). The dose-response relationship can make the difference between no effect, protective effects or harmful effects. Therefore, in order to get a realistic estimation of the effect of these bio-active compounds from foods, a quantitative approach throughout the entire production chain is an absolute requirement to obtain reliable intake data.

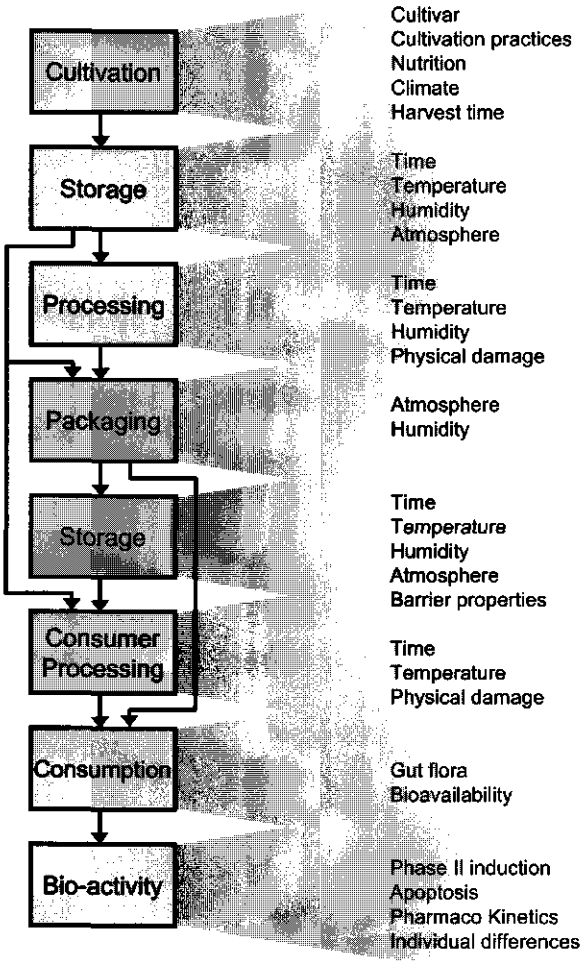

Figure 5.1 Schematic representation of a food production chain for *Brassica* vegetables and possible causes for variation in levels of bio-active compounds.

In Figure 5.1 a schematic representation of a food production chain of *Brassica* vegetables is given. It is indicated that all steps in this chain can have an effect on the

level of the phytochemicals, depending on several processes and conditions. In this chapter we describe a modelling concept which enables the quantitative prediction of the effects of these conditions and processes in the production chain on the phytochemical levels and potential health related benefits.

The production chain and possible effects on health

General

Healthiness of consumed products is determined by the level of bio-active components in the final product and by their bio-availability from the (digested) final product. There is scattered information about the effects of certain conditions at some steps in the production chain on the level of specific bio-active components (examples are on flavonoids from apple during juice production (Dekker et al., 1999) and on glucosinolates from *Brassica* vegetables during cultivation and processing (Verkerk et al., 1998; Mithen et al., 2000). Even less information is available on the effects of steps in the production chain on the bioavailability (a notable exception is the effect of processing on the bioavailability of carotenoids (Thane and Reddy, 1997). The effects of all steps in the production chain on health promoting components have not been systematically studied and because of the almost infinite amount of possible variation in all the factors they never will be. Due to this lack of information we feel that a predictive modelling approach can be a very effective tool to estimate the effects of variation in conditions and processes on healthiness of products. This modelling approach should then be based upon a sound mechanistic understanding of the most relevant conditions and processes within the entire production chain.

To illustrate this approach we have chosen glucosinolates as an example. Research on the protective effects of dietary glucosinolates from *Brassica* vegetables in human cancer development requires an accurate assessment of the dietary intake of these components.

General information on glucosinolates and health

Glucosinolates are an important group of phytochemicals that are widely distributed throughout the Cruciferae, a family that includes the *Brassica* vegetables such as cabbage, Brussels sprouts, broccoli and cauliflower. Detailed information on glucosinolates is given in chapter 2 of this thesis, however some aspects relevant for this chapter are summarised here. Glucosinolates co-exist with, but are physical separated from, the hydrolytic enzyme myrosinase in the intact *Brassica* plant. Upon mechanical injury of the tissue, the enzyme and substrate come into contact resulting in hydrolysis. The features of the hydrolysis environment such as pH, temperature and the presence of co-factors determine the proportion and nature of the various breakdown products. Intact glucosinolates can also be hydrolysed by the human gut

flora. There are however indications that the bioavailability of the breakdown products is lower from intact glucosinolates in the diet compared to pre-hydrolysed glucosinolates. Substantial evidence suggests that the hydrolysed glucosinolate products possess important protective properties against cancer. This protective effect against cancer has been attributed to the ability of some breakdown products, mainly isothiocyanates, to inhibit phase I enzymes that are responsible for the bio-activation of carcinogens (Guo et al., 1992) and to induce phase II detoxification enzymes (Sparnins et al., 1982; Wattenberg 1978; Zhang et al., 1992).

The next sections deal with the sources and the extent of variation of glucosinolate content within the production chain of *Brassica* vegetables.

Genetical and environmental variation

Comparative studies of glucosinolate distribution and variability between and within groups of the most widely consumed *Brassica* vegetables such as broccoli, cabbage and Brussels sprouts show large differences (Kushad et al., 1999). It is supposed that genetic factors and environmental factors contribute both significantly to the variation in levels of glucosinolates. An example of the variation within a variety is given in Figure 5.2 (for methods and materials see chapter 3). For seven different varieties of white cabbage the individual glucosinolates show substantial variation (up to 5-fold) in levels.

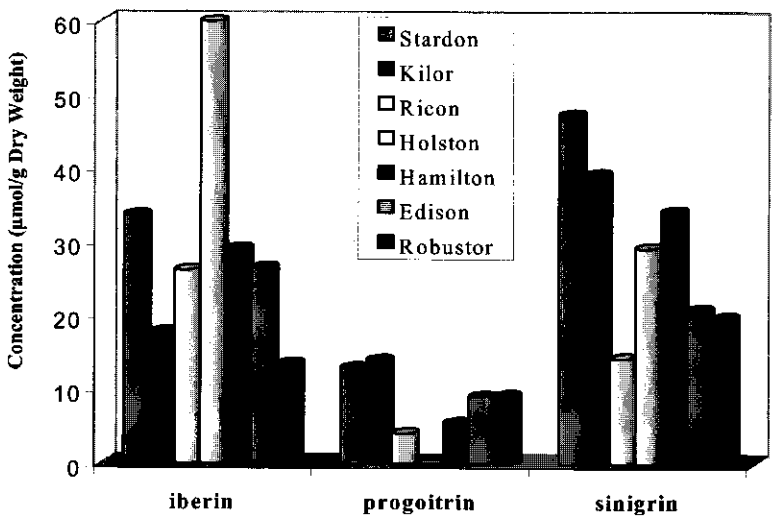

Figure 5.2 Variation in the level of individual glucosinolates in white cabbage varieties. The glucosinolates are 3-methylsulphinylpropyl (iberin), 2-hydroxy-3-butenyl (progoitrin) and 2-propenyl (sinigrin).

In some *Brassica* vegetables significant variations in glucosinolate levels are reported from year to year due to environmental conditions (Carlson et al., 1987; Rosa et al., 1994). Factors that may have contributed to this variation included growing sites, soil type, sulphate and nitrate fertilisation, climate and date of harvest.

The glucosinolate degradation products are partly responsible for the typical flavour of *Brassica* vegetables. It has been shown that the glucosinolates sinigrin and progoitrin are involved in bitterness in Brussels sprouts. Breeding and selection of cultivars for more desirable organoleptic properties have led to considerable variation in the glucosinolate profiles of vegetables such as cabbage and Brussels sprouts (Griffiths and Fenwick, 1984; van Doorn et al., 1998).

Storage and distribution

Other important steps in the production chain are post-harvest treatments of the vegetables. The variability rising from raw material storage depends on duration and conditions of handling and storage. Raw materials change with time and unless careful handling, transportation and storage procedures are used, the initial quality of the raw materials (such as phytochemical content) may be irreversible lost. It is known that in fresh vegetable products the greatest loss of vitamins occurs during the first 24 hrs and after that the level stays steady during normal storage time in appropriate circumstances. The transit of fresh produce takes approximately 7 to 14 days and post-harvest handling before commercial freezing is usually takes less than 12 h. The faster the cooling happens the smaller the loss. Until now there is little information about the stability of phytochemicals in general and glucosinolates in particular during storage, handling and distribution.

Some hydrolysis of glucosinolates can take place during harvest and storage caused by senescence. Obviously cabbage is more resistant to this than vegetables as broccoli and cauliflower that are highly perishable and must be cooled immediately after harvest. For broccoli, refrigeration at 4°C and freezing were shown to be the best preservation processes for maintaining high levels of glucosinolates (Rodrigues and Rosa, 1999). However freezing causes ice formation within the cells resulting in damage to cell structures (Reid, 1990). This becomes apparent upon thawing and can cause subsequent hydrolysis of glucosinolates.

An increase in total glucosinolate content was reported in broccoli when stored under air or under controlled atmosphere for 7 days, while the absence of O2 with a 20% CO2 concentration resulted in total glucosinolate loss (Hansen et al., 1995).

The gas conditions, humidity and temperature are also of importance during transport of the vegetables to the processor or supplier.

Processing

Fruits and vegetables are abundant sources of different phytochemicals and are consumed widely and in varying amounts. Both groups, but vegetables in particular, are subjected to different types of processing prior to consumption. Although consumption of minimal processed or even fresh unprocessed fruit and vegetables is widely advocated, it may not always be possible or even desirable. For reasons of costs, availability and edibility, processing is necessary or desired. Industrial processing may be minimal or more extensive, involving procedures as washing, cutting, blanching, addition of processing chemicals, drying, fermenting, freezing, canning and sterilising. Regarding the *Brassica* vegetables, any process that disrupts cellular integrity may result in some glucosinolate hydrolysis. However another mechanism has found to be induced by post harvest processing of *Brassica* vegetables. Verkerk et al. (2001) found that cutting of several *Brassica* vegetables and storage in air resulted in a remarkable increase of especially the indolyl glucosinolates. While other glucosinolates were found to be unaffected by this treatment, some indolyl glucosinolate increased up to 15-fold in concentration (in detail elucidated in chapter 3). Again these results show a large possible variation in levels depending on the type of treatment.

A large number of *Brassica* vegetables are consumed after cooking, and the amounts of glucosinolates are usually reduced considerably in cooked vegetables. Processes that can take place during cooking are the following;

- (Partial) inactivation of myrosinase;
- Heat degradation of glucosinolates and breakdown products;
- Enzymatic breakdown of glucosinolates;
- Loss of enzymatic co-factors (ascorbic acid, iron);
- Leaching of glucosinolates, breakdown products and myrosinase in cooking water.

Figure 5.3 illustrates the effect of the amount of cooking water and the condition at the start of the cooking process (addition of the vegetable to cold or to already boiling water) on the glucosinolate levels in broccoli. It appeared that the level of leakage of glucosinolates into the cooking water is strongly related with increasing amount of cooking water and to lesser extent with the cooking time or method.

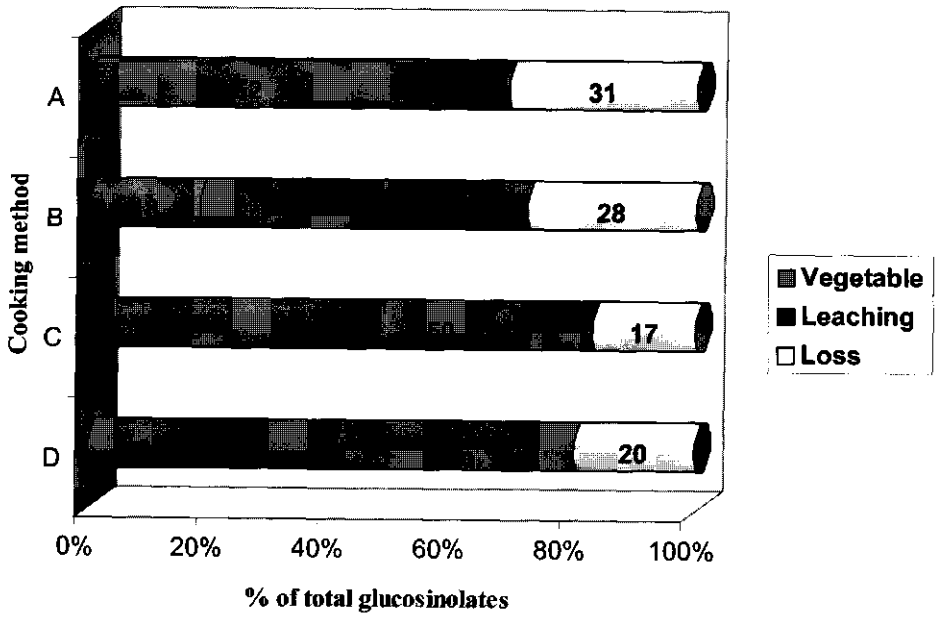

Figure 5.3 Effect of different conventional cooking methods of broccoli on the total glucosinolate content. A: hot start (1:1); B: hot start (1:4); C: cold start (1:2); D: cold start (1:8), indicating start of cooking with cold or hot water and ratio vegetable/cooking water.

When large amounts of water were used, cooking reduced the total glucosinolate content of the broccoli by more than 80%. Most of this loss was due to leaching of the glucosinolates into the cooking water. Also other studies show large effects of cooking on glucosinolate composition of several *Brassica* vegetables (Rosa and Heaney, 1993; Jiao et al., 1998). All these studies demonstrate the importance of assessing the intake of glucosinolates after processing. However processing is affecting the glucosinolate levels in a complex manner caused by the variation in process conditions. In the case of cooking of the vegetables, the temperature profile and amount of cooking water are of importance.

Overall variation as affected by the production chain

In conclusion we can state that the glucosinolate content of processed vegetables depends on:

- genetic and environmental factors (determining their quantity in the original raw foods);
- the extent and nature of processing (industrial and domestic);
- packaging and storage conditions.

As illustrated in the above mentioned examples the intake data of glucosinolates can exhibit large variation throughout the entire food production chain. It can be estimated that the levels at different steps in the production chain can easily have a variation of 5-10 fold in the raw material (e.g. cultivar differences), 5-10 fold variation caused by industrial processing and storage, 5-10 fold variation by household preparation (e.g cooking practices). These mentioned parts of the food production chain therefore result in an expected input variation of at least 100 fold between individual consumers (Figure 5.4).

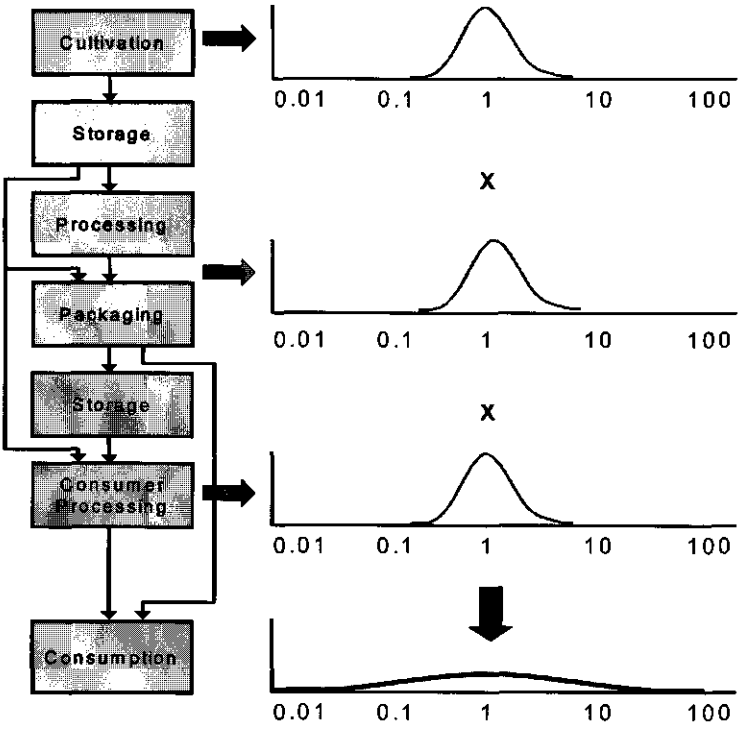

Figure 5.4 Variation in phytochemical (glucosinolate) levels at three levels in the food production chain and the resulting overall variation in intake levels at the consumer level.

This is of course a very undesirable situation for many actors in the food chain:
i) Industry for the development of functional food products with a guaranteed level of a certain component or a reproducible and reliable physiological impact;
ii) Government agencies giving advice regarding best practices and diets related to public health;
iii) Scientists who have to rely on intake data to study the relation between food and health (epidemiologists).

Moreover, it is conceivable that the variation of levels of bio-active compounds and lack of accurate intake data is responsible for the limited solid epidemiological evidence on the protective effect of certain groups of fruit or vegetables so far. Therefore we present a more integrated study of the dietary exposure of important phytochemicals in the food production chain.

Predictive modelling approach

We have demonstrated the large variability in levels of glucosinolates throughout the production chain of *Brassica* vegetables. Because of the almost infinite amount of possible variation in all the factors of the chain it will not be feasible to carry out systematic studies on all the effects. For this reason we propose the development of quantitative predictive models that describes the fate of phytochemicals in the food production chain from 'field to table' and finally into a healthier consumer. Modelling the production chain will consist of a series of sub-models describing the effects of the different steps within the chain. In Figure 5.5 a schematic representation of such a sub-model is given.

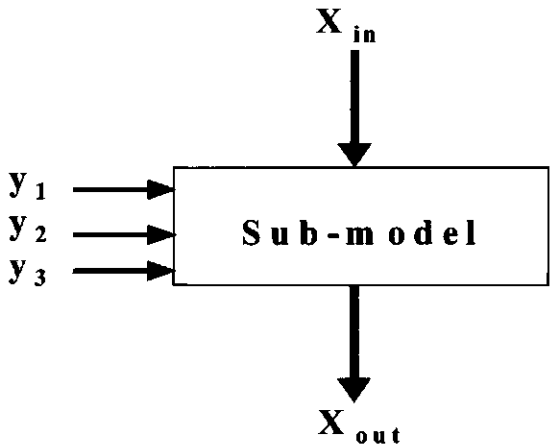

Figure 5.5 Schematic representation of a sub-model describing the effect of a step in the production chain on the level of a bio-active compound (Xin/out) depending on the conditions during that step (Yi).

This sub-model will consists of a set of mathematical equations describing the dynamic changes in the concentration of the phytochemicals in food products in one specific step or process. After linkage of these sub-models the effects of the various processes in (part of) the food production chain on the fate of phytochemicals can be predicted. The development of a predictive model is based on three steps:

• selection of the essential chain elements that determine the final content of the studied compound in the food;
• characterisation of the most critical sub-processes for each factor during the process under study;
• translation of the sub-processes to mathematical equations, usually (partial) differential equations in combination with mass balances, describing what happens during processing.

For the translation it is important to make appropriate assumptions about the system in order to simplify reality as much as possible. With this set of equations the changes in concentration of compounds and other relevant factors can be calculated with appropriate software. The predictive modelling approach must be based upon a sound mechanistic understanding on the most relevant conditions and processes within the entire production chain. A translation of the conceptual (sub)-model to a predictive model is carried out in a case study performed on consumer processing (see chapter 7 for details). The effects of cooking on glucosinolates in cabbage are studied extensively and subsequently translated in a quantitative predictive model. In this case study the important elements for healthiness were selected (content of glucosinolates, breakdown products and active myrosinase) and critical sub processes were identified (cell lysis, leaching, enzyme denaturation and enzyme activity). These processes were translated to mathematical equations and independent analysis on their temperature dependence was performed. By giving the temperature profile during cooking, the amount of cabbage and cooking water to the model, the cooking process can now be simulated and glucosinolate and breakdown product profiles during the cooking process be calculated. In the validation procedure the model was applied to predict the effect of cooking water on the final level of glucosinolates in broccoli and the match between the predicted line and the measurements was almost identical.

Applications of predictive modelling of health aspects

The proposed concept of predictive modelling of health aspects in the production chain is an innovative and challenging approach for the characterisation of the influence of the food production chain on ingestion of phytochemicals by humans. The described method of predictive modelling provides a powerful and efficient tool for handling the variation throughout the chain in a quantitative, scientific way.

A valuable way of using this predictive quantitative model is the use in combination with epidemiological studies. As shown in this chapter these epidemiological studies now have to deal with differences in intake level that can easily vary by a factor 10 to 100 depending on the way of processing (cutting, storage, cooking). By correcting intake data of bioactive compounds derived from e.g. fruit

and vegetables for the way they are processed, either by industry or by consumers themselves, the sensitivity of such studies will be enhanced enormously. Application of predictive models and hence correct intake data for chain effects may possibly separate the anticarcinogenic effect of *Brassica* vegetables from the effect of vegetables in general.

Conclusions

There is a growing interest in the enhancement of levels of desirable phytochemicals in crop plants via classical breeding or biotechnological methods. However, the need for increased levels of specific health promoting compounds in the plants is unsubstantiated when more accurate intake data are lacking. It is demonstrated that many steps in the food production chain of vegetables can have a large impact on the stability and fate of phytochemicals. All these steps contribute to the final intake of specific bio-active compounds and thus, determine the health promoting capacity of the food. This is most clearly illustrated by the influence of processing on the levels of phytochemicals as discussed in this paper. The concept of predictive modelling of the health effects on products throughout the entire production chain can be an extremely valuable tool. An application of this approach as an example of the effect of processing is the predictive model describing the fate of glucosinolates during cooking of cabbage, which is further elaborated in chapter 6 of this thesis. The development of models similar as the one described in this paper for other steps in the production chain and also for other types of compounds (e.g. flavonoids, carotenoids and folates) can increase our knowledge on actual levels in the final product data substantially. A further extension of this modelling concept with the effects on the bio-availability of phytochemicals is a future challenge. Product development, consumer advice and epidemiological research are important areas that can benefit enormously from this approach.

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Chapter 6

Predictive modelling of the glucosinolate-myrosinase system during cooking of cabbage

Ruud Verkerk, Martinus A.J.S. van Boekel, Wim M.F. Jongen and Matthijs Dekker,
submitted for publication.

Abstract

The group of glucosinolates (GS) plays an important role in the health-protective potential of *Brassica* vegetables. Cell damage of the vegetables by processing or mastication releases the endogenous hydrolytic enzyme myrosinase, which converts the glucosinolates to anticarcinogenic breakdown products such as isothiocyanates and indoles.

In this study the glucosinolate-myrosinase system was investigated during processing, that is. cooking of cabbage. By modelling the effects of processing on the system a tool was developed that can be used to assist product and process development. The model can also be used to improve the quality of investigations aimed at understanding the role of dietary glucosinolates and breakdown products in the protection against cancers. Glucosinolates as precursors of health-protective components and the active enzyme myrosinase are selected as most important health related parameters. Cell lysis, leaching of the components and denaturation and activity of myrosinase are identified as critical sub-processes. These sub-processes are translated into mathematical equations describing what happens during the cooking process. Based on the mathematical descriptions of the sub-processes we developed a model that describes the fate of the glucosinolate-myrosinase system during the cooking process of cabbage and predicts the health related parameters in the products consumed by humans. Simulation studies with the model show considerable reduction in GS contents in the cabbage with about 40% to 70% depending on the ratio of vegetables/cooking water, the warm up and cooking time. Also low amounts of breakdown products are formed during cooking (max. 4% of total GS). Ultimately, in different simulation studies it is shown that cabbage prior to consumption contains no active myrosinase anymore. The model predictions show good correlation with experimental data available from literature. The large impact of vegetable processing or food preparation on the variation in levels of health-protective breakdown products can partly explain the weak inverse correlation between consumption of *Brassica* vegetables and cancer incidence.

Introduction

Fruits and vegetables are abundant sources of various, extensively studied, health-protective phytochemicals. One important group of these phytochemicals is that of the glucosinolates. Glucosinolates (GS) comprise a group of thioglucosides naturally occurring in *Brassica* vegetables such as broccoli, cauliflower, radish, Brussels sprouts and cabbage. Glucosinolates co-exist with, but are physically separated from the hydrolytic enzyme myrosinase in the intact *Brassica* plant. Upon mechanical injury of the tissue, the enzyme and substrate come into contact resulting in hydrolysis (Mithen et al., 2000; Verkerk et al., 1998). The products of GS hydrolysis, particularly the isothiocyanates and indoles, have been shown 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 [Sparnins, et al., 1982; Wattenberg, 1978; Zhang et al.,1992). Research is ongoing to establish the biological activities of dietary glucosinolates and breakdown products, their bioavailability and metabolism.

Epidemiological studies indicate that a diet rich in *Brassica* vegetables can reduce the risk from a number of cancers (Steinmetz and Potter, 1991; Verhoeven et al., 1996). However up to now epidemiology cannot reproducibly correlate protection against certain cancers or other diseases with specific vegetables, subgroups or individual components. A plausible explanation for this can be the lack of realistic intake data of specific health protective phytochemicals. Assessment of accurate dietary intake of phytochemicals thus can play a crucial role. In this respect the impact of food processing and storage on the stability and behaviour of phytochemicals such as glucosinolates in the production chain of *Brassica* vegetables has not been investigated intensively (Dekker et al., 2000). With more knowledge on the effects of the complete production chain a more effective choice can be made on how to enhance phytochemical levels in the final consumed product. Moreover, epidemiological studies can possibly be improved by correcting phytochemical intake data for different steps in the food production chain such as processing. The large variability of important dietary phytochemicals within a food production chain of *Brassica* vegetables is illustrated in chapter 5 of this thesis. Furthermore, in chapter 5 it was proposed to develop quantitative predictive models that describes the fate of phytochemicals in the food production chain from 'field to table' and finally into a healthier consumer. This model should consist of a set of mathematical equations describing the dynamic changes in the concentration of the phytochemicals in food products. With this model the effects of the various processes in the food production chain on the fate of phytochemicals can be predicted.

Prior to consumption most of the vegetables are processed, e.g. industrial or domestic. Industrial processing may be minimal or more extensive, involving procedures as washing, cutting, blanching, addition of processing chemicals, drying, fermenting, freezing, canning and sterilising. Domestic processing or preparation of the food is more complex and much less standardised. The most important ones are chopping and cooking of the vegetables. Regarding the *Brassica* vegetables, any process that disrupts cellular integrity may result in some glucosinolate hydrolysis. Besides decline in glucosinolate content also increase of specific glucosinolates occurs in response to cutting and storage of cabbage (Verkerk et al., 2001).

A large number of *Brassica* vegetables are consumed after cooking, and the amounts of glucosinolates are usually reduced considerably in cooked vegetables. Some sub-processes that can take place during cooking are leaching of glucosinolates, breakdown products and myrosinase into the cooking water, inactivation of the enzyme myrosinase, enzymatic breakdown of glucosinolates or heat degradation of glucosinolates and breakdown products. Different studies have shown large effects of cooking *Brassica* vegetables mostly resulting in substantial leaching of glucosinolates into the cooking water (Verkerk et al., 1998; Rosa and Heaney, 1993; Mullin and Sahasrabudhe, 1978; Jiao et al.,1998). These studies emphasise the importance of assessing the intake of glucosinolates after processing prior to consumption. However processing is affecting the glucosinolate levels in a complex manner caused by the variations in process conditions. In the case of cooking of the vegetables, the temperature profile and amount of cooking water are of importance.

Model building

Mathematical description of the cooking process of cabbage

As an example of the modelling of the fate of bio-active compounds in processing and food preparation as part of the food chain we will describe the cooking process of red cabbage. To model the changes in content of glucosinolates in the cabbage we have to look in some detail to the most important processes during cooking. First we have to select the important factors that determine the health-protective compounds of the food product. For these factors the most critical sub-processes during the cooking process have to be selected. When these sub-processes have been identified they have to be translated to mathematical equations, usually (partial) differential equations in combination with mass balances, describing what happens during processing. For the translation it is important to make appropriate assumptions about the system in order to simplify reality as much as possible. With this set of equations, the changes in concentration of compounds and other relevant parameters can be calculated with the appropriate software.

Selection of important parameters for healthiness

The formation of glucosinolate derivatives depends on the enzymatic degradation by the endogenous enzyme myrosinase or by bacterial enzymes in the human gut. For the health protective properties of cabbage the most important parameters are: content of glucosinolates and breakdown products and the amount of active myrosinase. The model to be developed has to be able to predict these parameters after the cooking process.

Identification of critical sub-processes

As described above glucosinolates can be degraded by the endogenous enzyme myrosinase. In intact cells of cabbage the membrane of the vacuole separates the enzyme and its substrate. During cooking cell lysis will occur giving rise to a sudden increase in osmotic pressure difference over the vacuole membrane which will result in collapse of this membrane, resulting in a mixing of the glucosinolates and the myrosinase in the cooking water. Enzymatic degradation can then take place. In Figure 6.1 these sub-processes have been schematically illustrated.

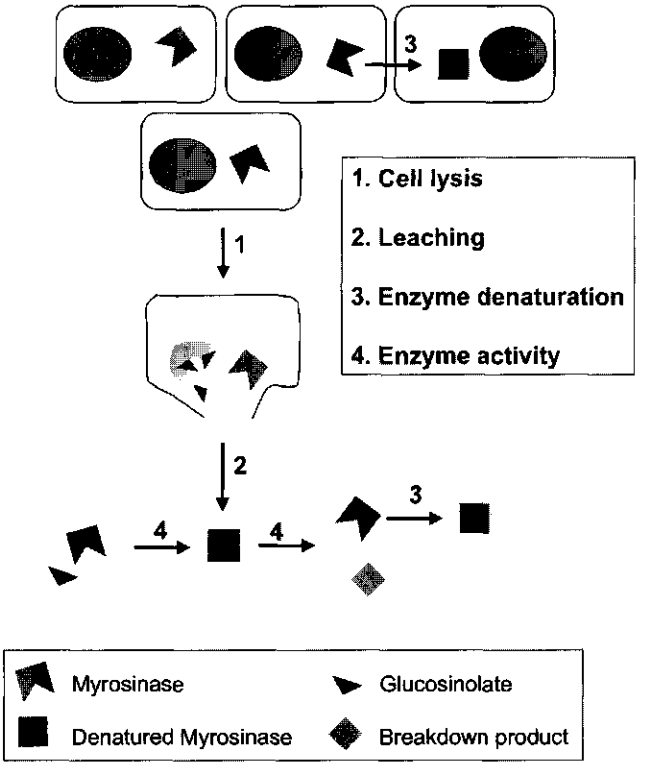

Figure 6.1 Schematic illustration of the most important processes determining the fate of glucosinolates during cooking.

For mathematical treatment of these sub-processes the cell lysis rate has to be known as a function of temperature. Also an assumption has to be made how quickly the enzyme and glucosinolates mix with the cooking water. The kinetics of enzymatic degradation of glucosinolates has to be known as a function of concentrations and of temperature. Although myrosinase is quite a stable enzyme, denaturation will occur at a certain rate depending on the temperature. So also the denaturation rate as a function of temperature has to be determined.

Translation into mathematical equations

Cell lysis is described as a first order process, meaning that the rate of lysis will be proportional to the fraction of cells that is still intact. Experiments were conducted where cell lysis was measured by the increase in conductivity of the cooking water caused by release of cell contents upon lysis after different time/temperature treatments of the cabbage. These experiments could be described quite accurately by the first order equation (1) and mass balance (2):

$$\frac{dCC,i}{dt} = -kl \cdot CC,i \qquad (1)$$

$$C_{C,l} = 1 - C_{C,i} \qquad (2)$$

in which $C_{C,i}$ is the fraction of intact cells and $C_{C,l}$ of lysed cells at time t (min), k_l is the rate constant of cell lysis (min^{-1}).

Because of the high osmotic pressure difference between cooking water and cell contents, free water will dilute the cell content very quickly once the cells are lysed. Also the size of the cut cabbage particles is quite small (~1 cm^2). Therefore the mixing rate of solutes (including enzyme and glucosinolates) from the lysed cells to the cooking water is assumed not to be rate limiting. Based on these assumptions the leaching of compounds from the lysed cells is described by the mass balance (3-5):

$$M_f = M_w + C_{C,l} \cdot M_c \cdot (1 - IM) \qquad (3)$$

M is the mass (g), f refers to not enclosed water (=cooking water + water in lysed cells), w to the initial amount of cooking water and c to the cabbage. IM is the fraction of insoluble matter. After cell lysis, leaching of active myrosinase and glucosinolates is described by the equations (4) and (5).

$$M_c \cdot \frac{dC_{C,i}}{dt} \cdot C_{GS,i} = M_f \cdot \frac{dC_{GS,f}}{dt}\Big|_{i \to f} \quad (4)$$

$$M_c \cdot \frac{dC_{C,i}}{dt} \cdot C_{Myr,i} = M_f \cdot \frac{dC_{Myr,f}}{dt}\Big|_{i \to f} \quad (5)$$

in which *GS* refers to glucosinolates, *Myr* to active myrosinase in intact cells (*i*) or in released water (*f*). The denaturation of myrosinase is described by first order inactivation with the Arrhenius equation describing the temperature dependence of the rate constant (6) and (7).

$$\frac{dC_{Myr,i}}{dt}\Big|_d = -k_d \cdot C_{Myr,i} \quad (6)$$

$$\frac{dC_{Myr,f}}{dt}\Big|_d = -k_d \cdot C_{Myr,f} \quad (7)$$

in which k_d is the rate constant of myrosinase denaturation both in intact cells (*i*) and in released water (*f*). Finally the degradation of glucosinolates by myrosinase is described by the Michaelis-Menten equation (8).

$$\frac{dC_{GS,f}}{dt}\Big|_a = -\frac{k_a \cdot C_{Myr,f}}{\frac{K_m}{C_{GS,f}} + 1} \quad (8)$$

In this equation k_a refers to the rate constant of maximum enzyme activity and K_m to the enzyme M-M constant (μmol.g^{-1}). The temperature dependence was described by the Arrhenius equation (9).

$$k_j = k_{0,j} \cdot e^{\frac{-E_a,j}{R \cdot T}} \quad (9)$$

in which k_j is the experimentally observed rate constant of process *j*, k_o the pre-exponential factor (min^{-1}), E_a the activation energy (kJ/mol), R the gas constant (kJ/mol.K) and T absolute temperature (K). A summary of the different sub-processes, the required experiments and the necessary parameters is presented in Table 6.1.

Table 6.1 Schematic representation of the experimental set-up for parameter estimation.

Experiment	Variable	Analysis	Parameters	Equations
Cell lysis/ leaching	$T_{293\text{-}373K}$ $t_{0\text{-}60\ min}$	Conductivity	k_l $E_{a,l}$; $k_{0,l}$	(1, 2, 3, 4, 5, 9)
Enzyme activity#	T_{313K} $t_{0\text{-}40\ min}$	Hydrolysis of sinigrin	k_a K_m $E_{a,a}$; $k_{0,a}$	(8, 9)
Enzyme denaturation#	$T_{273\text{-}373K}$ $t_{0\text{-}120\ min}$	Enzyme activity	k_d $E_{a,d}$; $k_{0,d}$	(6, 7, 9)

in juiced cabbage; T = Temperature (degrees Kelvin); t = time (min)

Material & Methods

Sample preparation

Red cabbage (*Brassica oleracea* L. var. *Capitata* f. rubra DC.) material was supplied by Novartis Seed BV (Enkhuizen, The Netherlands) and partly purchased from local supermarkets (Wageningen, The Netherlands). The outer leaves of the heads were removed and complete cabbage heads were used for the experiments. The cabbage was chopped into pieces of approximately 1 cm^2 and mixed thoroughly. For glucosinolate analysis of the fresh cabbage the chopped material was directly frozen with liquid nitrogen. The frozen material was ground in a Waring Blender (Model 34BL99, Dynamics Corp. of America, New Hartford, Connecticut, USA) and stored at -30°C until further analysis.

Preparation of cabbage juice for the analysis of the activity and denaturation of myrosinase.

Red cabbage material was chopped and juice was prepared with a commercial juice centrifuge (Braun, type 4290). The obtained juice was sieved to remove the larger parts. Subsequently the juice was incubated for 1 hour at 40°C in an oven to hydrolyse the endogenous glucosinolates present in the cabbage. The obtained batch of glucosinolate-free juice was considered as a crude myrosinase extract in which different cabbage components are present that could affect the myrosinase activity (e.g. ascorbic acid). Part of the juice was incubated for 15 minutes at 100°C in order to inactivate the enzyme myrosinase. This juice was used for dilution purposes in the activity assays.

Microwave cooking

Approximately 2000 g of red cabbage was chopped and divided into portions of 300 g each. Each portion was placed in a 500 ml beaker and cooked in a microwave oven (Daewoo, Model KOC-87-T, Korea) at 2450 MHz for 1, 2, 4, 6, or 8 min with an output power of 540W and 36, 72, 144, 216, or 288 sec with an output power of 900 W. After heating, the vegetables were frozen with liquid nitrogen and stored at -30°C until glucosinolate analysis. Temperature registration during microwave cooking was done using a glassfibre probe (Takaoka, Type FTP3-3003 s/n 31888).

Analysis

Glucosinolate analysis

The glucosinolate 2-propenyl (sinigrin, Aldrich Chemical Co., Milwaukee, WI, USA) present in the fresh cabbage or cabbage juice was analysed using high performance liquid chromatography (HPLC) following on-column desulphation as described by Verkerk et al. (2001).

Conductivity measurement

Cell lysis was determined by measuring the conductivity of the cooking water at 23°C with a Microprocessor Conductivity Meter (Type WTW, LF537). Temperature registration of the cabbage during the conventional cooking experiments was carried out with a Tinytalk-PT Logger (range -50°C - 300°C) that was inserted in the cabbage pieces.

Determination of myrosinase activity

The activity of the enzyme myrosinase present in the juice was measured by analysing the extent of hydrolysis of a known amount of sinigrin added to the juice. To 5.0 g of cabbage juice 1 ml of 6 mM pure sinigrin was added and incubated at 40°C for 0, 5, 10 and 20 minutes. The reaction was stopped by adding 12 ml of 100% hot methanol (for 10 min. at 75°C). The juices were centrifuged (5000 x g, 10 min, RT) and remaining sinigrin was isolated from the collected supernatant and analysed by HPLC.

Experimental set-up

Cell lysis

For the temperature incubations 150 g samples of chopped red cabbage were immersed in 400 ml water at desired temperatures in 800 ml beakers. The beakers were placed in a waterbath and incubated for different preset temperatures. The red cabbage samples were incubated at 40, 60, 80 and 100°C for 10, 20, 40 and 60 minutes. Immediately after incubation the samples were quickly cooled on ice after which the vegetables were separated from the cooking water, weighed and frozen with

liquid nitrogen. After cooling down of the cooking water (23°C) the conductivity was measured (in mSiemens) and samples were collected in tubes and stored at -30°C until further analysis.

Effect of temperature on cabbage myrosinase activity

The cabbage juices (crude myrosinase extract) were equilibrated at various temperatures (20, 40, 60 and 80°C). The hydrolysis was then started by addition of 1.0 ml 6 mM sinigrin standard and stopped after the desired times by adding hot methanol. From the remaining intact sinigrin the rate of hydrolysis was calculated.

Effect of temperature on denaturation of myrosinase

For determining the thermal stability of myrosinase between 25 and 80°C, the remaining activity was measured after different temperature/time incubations of the cabbage juice. For this purpose the 'dilution juice' (heat-treated cabbage juice) was brought at the desired temperature and the incubation was started by addition of the 'active myrosinase' juice. Immediately after cooling of the incubated juices, the myrosinase activity at 40°C was measured as before.

Data analysis

The validity of the Arrhenius equations was evaluated by plotting ln(k) versus the reciprocal temperature and determining the goodness of the fit by means of the correlation coefficient and residual analysis (Microsoft Excel).

Results & Discussion

Kinetics of cell lysis

Cell lysis was determined by measuring conductivity of the cooking water after different time/temperature treatments of the chopped red cabbage. It was found that the electrical conductivity of the cooking water increased with increasing cell damage caused by leaching of the cell content into the water. Being able to measure the extent of cell lysis simply and quickly allows evaluation of what percent of plant cells must be ruptured to achieve optimal conditions for glucosinolate hydrolysis. These experiments could be described quite accurately by the first order equation and the temperature dependence was described by the Arrhenius equation. The rate constant k_l and the apparent activation energy E_a of the cell lysis have been determined for the different temperatures. The temperature dependence according to Arrhenius resulted in $k_{0,l}$ and $E_{a,l}$ values of $2.9 \cdot 10^6$ min^{-1} and 53 kJ/mole·K respectively.

Activity of myrosinase

The activity of the enzyme myrosinase was determined in juices prepared from red cabbage. Previous research (unpublished) showed that juicing of cabbage resulted in high myrosinase activity in the juice and little remained in the cabbage pulp. Since we are interested in the myrosinase activity at cellular conditions, measuring the activity in the juice of cabbage is preferred over the activity of the isolated enzyme, which is usually done (Yen and Wei, 1993; Ludikhuyze et al., 1999, 2000; Wilkinson et al., 1984). The presence of known (e.g. ascorbic acid, $MgCl_2$ and iron) and yet unknown components in the cabbage juice that are important for myrosinase activity gives a valuable advantage to this approach.

The rate of glucosinolate hydrolysis depends on temperature, pH and on the concentrations of enzyme and substrate. The pH dependence is not included in the model. Yen and Wei (1993) reported an optimum pH at 8.0 for myrosinase activity from red cabbage and a relative activity of 90% in a pH range between 5.0 and 8.0. However West et al. (1977) have shown two pH optima at 5.0 and 8.0 for myrosinase in crude extracts from cabbage. For myrosinase from broccoli a pH optimum of 6.5 was found in partly purified extracts (Ludikhuyze et al., 2000). The crude red cabbage juice used in our experiments showed a constant pH of 6.5 (at 23°C), which is according to most literature in the range of high myrosinase activities.

The temperature dependence of myrosinase activity is shown in Figure 6.2. The optimal temperature of myrosinase in the red cabbage juice appeared to be around 40°C. Additional datapoints can possibly result in an optimal temperature just below or above 40°C, however activity was less than 40% at 60°C.

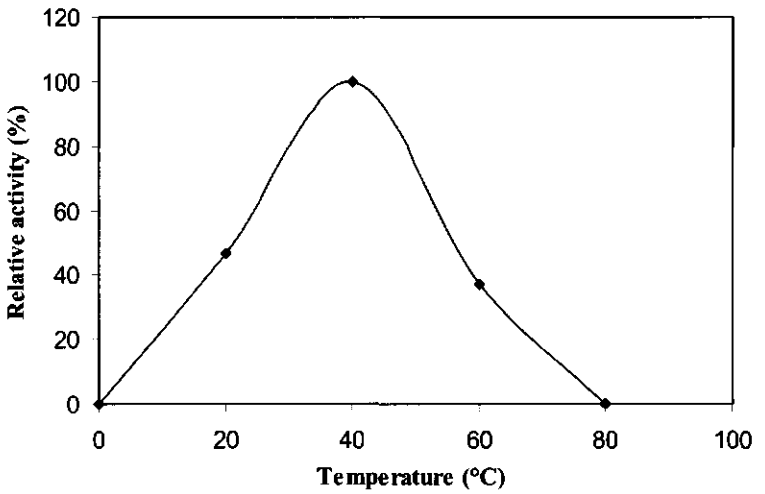

Figure 6.2 Temperature profile of myrosinase activity in red cabbage juice. Experiments carried out under conditions described at Materials &Methods.

The optimal temperature of 30°C found for activity of myrosinase from broccoli (Ludikhuyze et al., 2000) and our own findings are low compared to the reported optimal temperatures of myrosinase from other sources (Bjorkman and Janson, 1972). Yen and Wei (1993) showed optimal temperatures of 60°C for myrosinase from red and white cabbage and still more than 90% activity at 50°C. It is very well possible that different vegetable sources of myrosinase show different physical properties such as temperature dependence. Moreover the difference in procedures of activity measurements, (partly) purified myrosinase versus crude vegetable juice, can also explain differences in behaviour of the enzyme activity. The temperature dependence for myrosinase activity could be described by the Arrhenius equation resulting in $k_{0,a}$ and $E_{a,a}$ values of $1.2 \cdot 10^4 min^{-1}$ and 32 kJ/mole·K, respectively.

Denaturation kinetics of myrosinase

Thermal inactivation of myrosinase was studied at temperatures ranging from 25 to 70°C and was determined by measuring the remaining activity after different temperature-time incubations of the cabbage juice. The rate constant for denaturation was calculated from these experiments. Myrosinase is according to the literature a more thermostable enzyme that seems to survive after cooking of vegetables. Yen and Wei (1993) show that red cabbage myrosinase is more stable than white cabbage myrosinase, however both were destroyed for 90% after heating at 70°C for 30 minutes. Ludikhuyze et al. (1999) studied thermal inactivation of myrosinase in broccoli in the temperature range 30-60°C. They reported a rather thermolabile myrosinase with optimal activity at 30°C, and significant inactivation occurring at 40°C. These latter findings are more in agreement with our results.

Table 6.2 Overview of the parameter estimation of the different cooking sub-processes.

Sub-process	Parameters J/mol.K	
Cell lysis	$k_{0,l}$	$2.9 \cdot 10^6$ min^{-1}
	$E_{a,l}$	53 kJ/mol·K
Myrosinase activity	$k_{0,a}$	$1.2 \cdot 10^4$ min^{-1}
	$E_{a,a}$	32 kJ/mol·K
Myrosinase denaturation	$k_{0,d}$	$5.0 \cdot 10^{23}$ min^{-1}
	$E_{a,d}$	155 kJ/mol·K

Simulation of the cooking process

With the set of equations describing the most relevant sub-processes during cooking of cabbage, cell lysis and myrosinase denaturation and activity, and the parameters that have been estimated it is now possible to simulate the cooking process. For this purpose a temperature profile during cooking had to be chosen as well as the amount of cabbage and cooking water. The applied temperature profile of in total 90 minutes is not realistic to domestic consumer cooking but is chosen here for reasons of clarity to explain the different steps in the cooking process. With an Excel discrete timepoint programme, using Euler's method, these simulations were calculated. In Figure 6.3A and 6.3B the simulation results for the cooking of 150 g red cabbage in 300 g of water is shown.

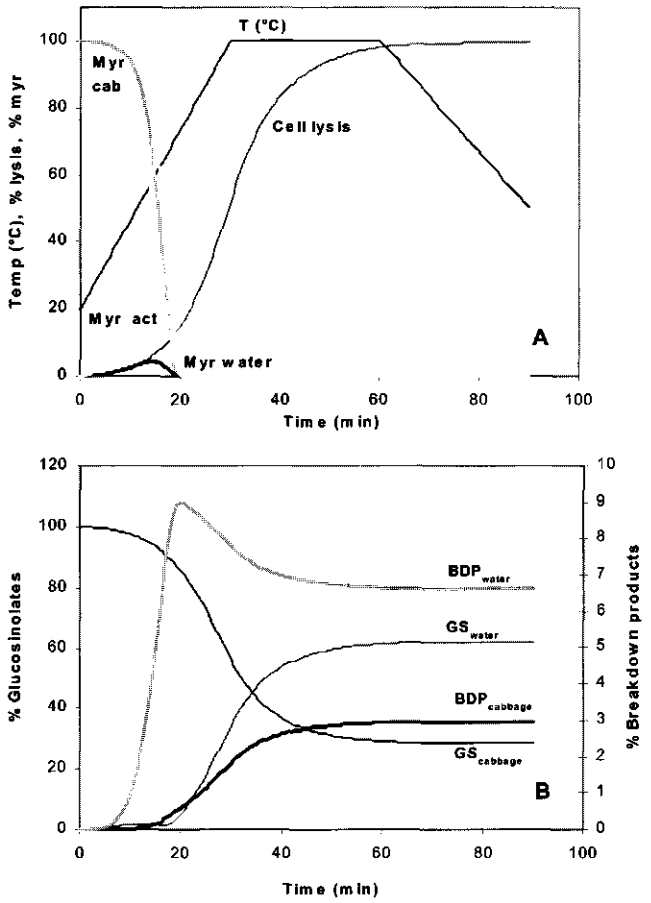

Figure 6.3 Simulation results of a cooking process of 150 g of red cabbage in 300 g of water. A, temperature profile, cell lysis and profile of active myrosinase during the cooking process; B, glucosinolate (GS) and breakdown product (BDP) profiles during the cooking process in cabbage (cab) and water.

The applied temperature profile has been indicated in panel A of Figure 6.3 (heating up from 20 to 100°C, 30 minutes of cooking and finally cooling down to consumption temperature of 50°C). During the heating phase the cells start disrupting which results in the leaching of part of the glucosinolates and myrosinase. However because of the temperature rise also denaturation of the active myrosinase occurs both in the cabbage and in the cooking water after leaching. These two phenomena result in an optimum in the released active myrosinase at around 16 minutes when temperature reaches 60°C. Upon further heating all the myrosinase is quickly denatured and after 20 minutes when temperature reaches 73 °C no active myrosinase is present anymore. As a result of this behaviour of myrosinase the enzymatic breakdown of released glucosinolate is occurring only at a short time period, when the released myrosinase is still active. At this time period the main part of glucosinolate is still present in intact cabbage cells. This part is only released when the myrosinase is already denatured and therefore this glucosinolate fraction will just distribute itself between the cooking water and the cabbage. No degradation is expected anymore. The applied temperature profile, amount of cooking water and cooking time would result for this simulation example in a cabbage for consumption that contains no active myrosinase and 28 % of the initial glucosinolate level. Some 3 % of the initial glucosinolate level is present in the form of breakdown products in the cabbage, 62 % as intact glucosinolate in the cooking water and almost 7 % as breakdown products in the cooking water.

Application and validation of the model

With the derived predictive cooking model it is quite easy to study the effects of the cooking process conditions on the final level of glucosinolate in the cabbage. By doing so it can be shown that one of the most relevant conditions is the amount of cooking water relative to the cabbage. In Figure 6.4 this effect of the amount of cooking water on the final level of glucosinolate in the product is shown. For validating the model cooking experiments that were done with broccoli are shown in the figure as well. It can be observed that the predicted line is in agreement with the experiments (Figure 6.4).

A more challenging validation of the model is the prediction of the dynamic changes in the glucosinolate levels during cooking. This validation was done by cooking red cabbage for different times and analysing the cabbage for glucosinolate levels after different times of treatment. In Figure 6.5 the prediction of the levels of glucosinolate, breakdown products and of active myrosinase are shown together with the measured amount of glucosinolates in the cabbage after different times of the heat treatment.

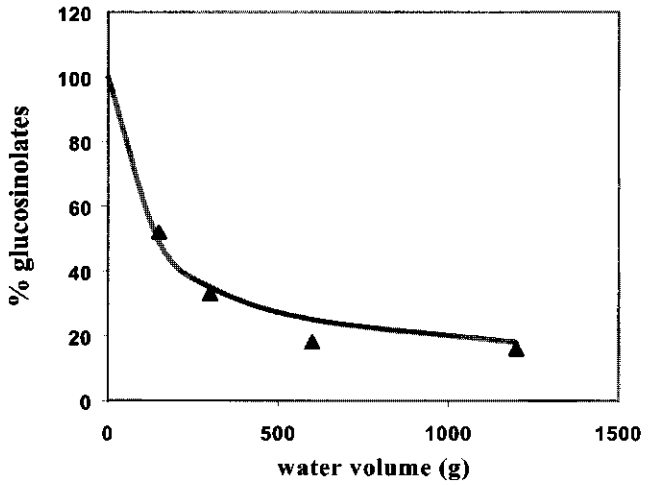

Figure 6.4 Effect of the volume of cooking water on the final level of glucosinolates in 150g of broccoli as predicted by the model, compared with experimental results. The triangles are the experimental data and the line gives the prediction by the model.

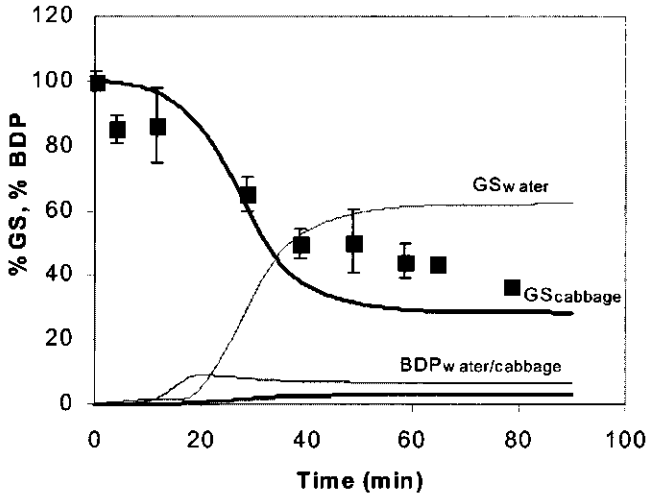

Figure 6.5 Dynamic behaviour of glucosinolate (GS) and breakdown product (BDP) levels during cooking of 150g red cabbage in 300g water as predicted by the model, compared with experimental results of the total glucosinolate levels (■) in the red cabbage.

As can been seen from Figure 6.5 the levels of glucosinolate that are found in the cabbage are somewhat higher than predicted. The final glucosinolate levels in the cabbage are around 50% while the model predicts only 30%. The dynamic behaviour of the glucosinolates in the cabbage is predicted quite well. The predicted decrease in

levels between 15 and 30 minutes is corresponding with the observed decrease. The fact that the predicted levels are lower than the observed ones can possibly be explained by an increase in chemical extractability of the glucosinolates after intense heat treatments (see discussion further on). Another explanation is the fact that glucosinolates do not leach freely out of the lysed cells, but partly bind to parts of the cell contents. This was also observed when juice was made from red cabbage. Although different glucosinolates show different distribution behaviour over the cabbage insoluble parts and the juice, all are preferentially bound to the insoluble parts. To make a more accurate prediction of the glucosinolate behaviour during cooking the model could be extended with a partitioning equation describing this behaviour.

Recently Ciska and Kozlowska (2001) studied the effect of different cooking times on the GS content in white cabbage. Their observation of the largest decrease in GS content during the first minutes of cooking (35%) is in agreement with the prediction carried out with our model when using the described conditions (Figure 6.6).

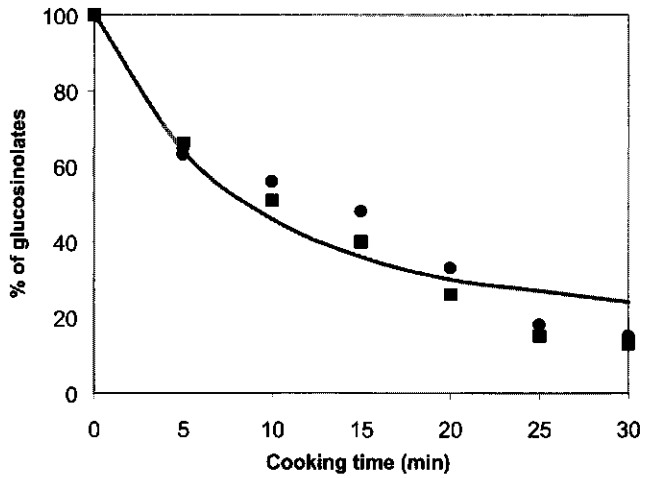

Figure 6.6 Prediction of glucosinolate loss (line) during cooking of red cabbage compared to experimental data of sinigrin (●) and total GS (■) from white cabbage as obtained from literature (Ciska & Kozlowska, 2001). Cooking was carried out with constant cabbage-water ratio (1:3) and started with boiling water.

Also at longer cooking times the model gives a fairly good fit with at maximum 10% differences in GS levels. In another study carried out by Rosa and Heaney (1993) the effect of cooking on glucosinolate content was investigated on different cabbage varieties. They noted a substantial reduction in glucosinolates of more than 50% after

10 minutes of cooking of all tested cabbage varieties. Using the same conditions (10-min cooking, 50g cabbage/250g water) the model predicts a similar reduction of 58% of the glucosinolate content. In addition, because in both studies the vegetables were added to boiling water it is likely that no hydrolytic conversion has taken place and little or no protective components have been formed.

Model predictions of levels of glucosinolates and breakdown products after cooking of cabbage

Conditional on the correctness of the model we are now able, with the use of computer simulations of the cooking model, to make predictions with regard to optimisation of the levels of the protective compounds after cooking, and thus intake levels, by adjusting the cooking conditions.

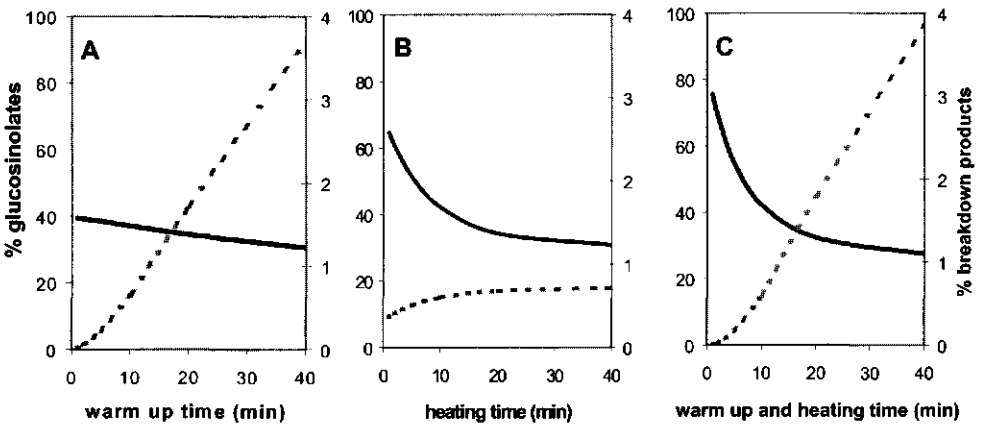

Figure 6.7 Simulations of the cooking model predicting the glucosinolate (solid line) and breakdown product (dotted line) levels in the cabbage for different cooking regimes. A. Variable warm up time from 1 to 40 minutes; heating time of 15 min and cooling down time of 5 min.; B. Variable heating time from 1 to 40 minutes; warm up time of 10 min. and cooling down time of 5 min.; C. Variable warm up and heating time of 1 to 40 minutes and cooling down time of 5 min. 150 g cabbage and 300 g water.

Figure 6.7A shows that the total levels of potentially protective breakdown products are very low varying from 0.6 % at 10-15-5 min of warm up, heating and cooling down, up to a maximum of 3.7 % of the total amount of glucosinolates after the 40-15-5 minutes cooking regime. Besides the breakdown products the simulation shows a low retention of 37% of glucosinolates in the cabbage in the first regime and 30 % in the latter one (Figure 6.7A). When the heating time is varied (1 to 40 min) at constant warm up and cooling down time (10 and 5 min), the level of breakdown products

remain at a constant low level of about 0.7 % of the total amount of glucosinolates (Figure 6.7B). Also after 40 minutes less than 1/3 of the initial amount of glucosinolates is retained in the cabbage. When increasing both warm up and heating time, the levels of breakdown products are only slightly higher (3.9% of total GS) after 40-40-5 minutes (Figure 6.7C) compared to simulation of increasing of warming up time (Figure 6.7A). The above described simulations were carried out assuming a 1:2 ratio of cabbage versus water. Doubling the amount of cooking water (600g instead of 300 g) reduces quantities of breakdown products in the cabbage even more. Reduction of 85, 115 and 86 % in levels of breakdown products for cooking regimes A, B and C respectively, is mostly explained by increased leaching of glucosinolates into the cooking water.

The explanation of these low levels of breakdown products is evident and leads to the glucosinolate/myrosinase system. That is, for production of protective breakdown products enzyme and substrate should be into contact with each other at conditions favoured for hydrolysis. This situation can not be realised during cooking of cabbage. For instance at the 10-15-5 minute regime (Figure 6.7A) the maximum myrosinase enzyme activity is at about 6 minutes while at that time only 4% of the total amount of plant cells are lysed and the cooking water has reached a temperature of about 70°C. In other words glucosinolates and myrosinase are only in limited contact with each other at unfavourable temperatures and therefore little hydrolysis can take place. Increasing the warm up period can result in somewhat, but not much, higher level of breakdown products. However such a temperature profile will lead to undesired aspects such as long preparation time and degeneration of product qualities factors (flavour and texture).

The significance of myrosinase-mediated conversion is emphasised by the bioavailability studies carried out by Conaway et al. (2000). They showed that the bioavailability of isothiocyanates (ITCs) from fresh broccoli was approximately three times higher than that from steamed broccoli, in which myrosinase is inactivated. Based on their results they concluded that, considering the potential chemopreventive activity of ITCs in broccoli, cooking might substantially reduce the health beneficial effects of broccoli in the diet. The assumption that conventional cooking is diminishing the protective effects of vegetables seems to be supported by the epidemiological trend showing most clear protective effects for raw vegetables (Steinmetz and Potter, 1996).

Simulation study of microwave cooking

As discussed above, conventional cooking of cabbage in boiling water causes substantial leaching of glucosinolates and breakdown products from the cooked material. As an alternative we studied a microwave cooking process in which no

additional water is used during the treatment. It is hypothesised that, because no leaching can take place, this way of food preparation will result in higher retention of glucosinolates in the final vegetable product. The experimental data were compared with a simulation of microwave cooking using the developed cooking model without the use of water. Using this model as such, we ignore the possible differences in lysis kinetics between microwave and conventional cooking. Differences in lysis might occur due to a different heat generation system in the cabbage and because of the lack of an osmotic pressure difference between cabbage and cooking water in the case of microwave cooking. The temperature of the cabbage during microwave cooking was described by an experimentally determined relation between cabbage weight and microwave power. The temperature model was based on the energy input, cabbage weight and heat transfer to the surroundings (equations 10 and 11).

$$\frac{dT_c}{dt} = k_1 \cdot \frac{P}{m} - k_2(T_c - T_{sur}), \qquad T_c \le T_{c,max} \qquad (10)$$

$$\frac{dT_{sur}}{dt} = k_3 \cdot (T_c - T_{sur}), \qquad T_{sur} \le T_{sur,max} \qquad (11)$$

in which T_c is the temperature of the cabbage (°C, max. 100°C) and T_{sur} the temperature of the surroundings increasing in time t (s) and P (Watt) refers to the applied microwave power on the mass m (g) of the cabbage. k_1 is an energy conversion coefficient of 10.7 °C·g·J^{-1} and k_2 and k_3 are heat transfer coefficients of 0.26 and 4.0 s^{-1} respectively. The parameters in equation 10 and 11 were estimated from experimental data obtained from microwave treatments of red cabbage as described in chapter 4 (Verkerk et al., 2002). These parameters are specific for the microwave used and the geometry of the container which holds the cabbage. The weight of the cabbage was corrected for evaporation of water from the cabbage by the experimentally determined equation (12).

$$G = G_0 - k_e \cdot t \qquad (G \ge 0) \qquad (12)$$

in which G is the weight of the cabbage (g) at time t (min), G_0 is the starting weight (g) and k_e refers to the evaporation coefficient, which is linearly correlated with the applied microwave power constant (= 0.0168·P (min^{-1})).

Microwave cooking of 300 g cabbage at 540W resulted in a small drop of total glucosinolate content after 2 minutes (from 100% to 88%), after which the amounts

increased up to more than 140% of the untreated cabbage (Figure 6.8). According to the model, which is corrected for weight loss caused by evaporation of the water, there is also a very high amount of glucosinolates in the vegetables predicted after 'cooking without water' for 8 minutes. The cabbage reached a temperature above 60°C after 2.5 minutes and cooking started (100 °C) after 5 minutes. Based on the temperature, glucosinolate hydrolysis could take place within the first two minutes. However hydrolysis will be limited since less than 1% of the cabbage cells are disrupted at that time period.

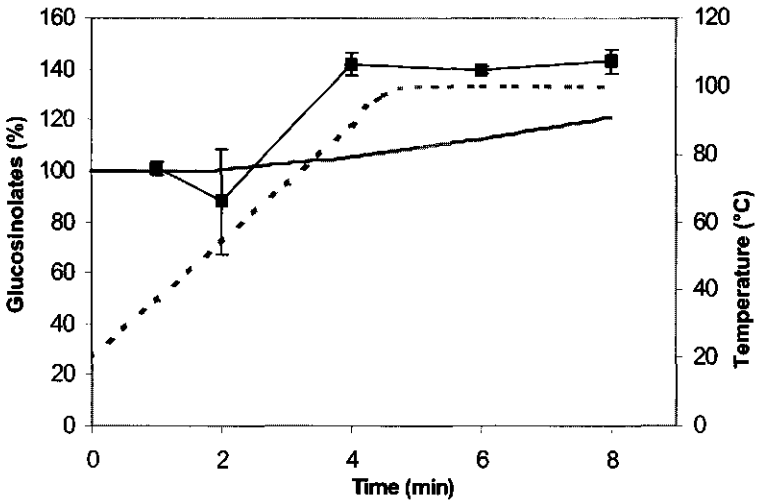

Figure 6.8 Behaviour of glucosinolates in a simulation study on microwave cooking of 300 g red cabbage without water as predicted by the model (solid line), compared with experimental results of the glucosinolate levels in the cabbage (■). Dotted line represents the temperature profile; Microwave cooking is carried out for 8 minutes at 540W; the prediction was corrected for evaporation losses.

Microwave cooking of 300 g cabbage at 900W revealed an increased GS-content with increasing cooking time (Figure 6.9). After almost 5 minutes the GS-content was as high as 178% of the untreated cabbage. Also with this temperature profile, mimicking a 900W treatment, the model predicts very high amounts of glucosinolates (123%) after cooking for 5 minutes. In this cooking profile the temperature of the cabbage reached 100 °C within 3 minutes and continued cooking for another 2 minutes.

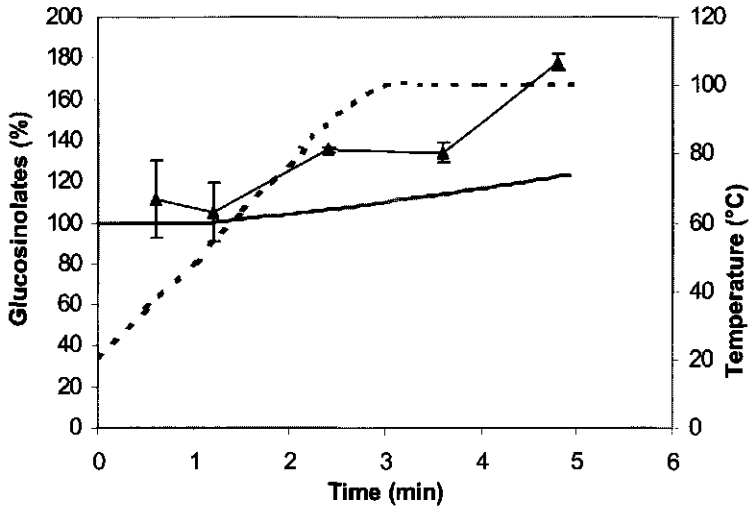

Figure 6.9 Behaviour of glucosinolates in a simulation study on microwave cooking of 300 g red cabbage without water as predicted by the model (solid line), compared with experimental results of the glucosinolate levels in the cabbage (▲). Dotted line represents the temperature profile; Microwave cooking is carried out for 5 minutes at 900W; the prediction was corrected for evaporation losses.

In our study, microwave cooking at intermediate (540W) and high (900W) energy inputs did not result in losses in glucosinolate levels in the cabbage. Unexpectedly, both microwave treatments revealed total GS-contents exceeding the levels present in untreated cabbage. Published data concerning the effects of microwave cooking on glucosinolate levels are limited. In this respect, antioxidant compounds like ascorbic acid (AA) and β-carotene (β-C) were studied in more detail. Howard et al. (1999) reported no effects on the AA or β-C content after microwave cooking of broccoli for 8 min at 700W.

Microwave cooking decreased the moisture content of cabbage by evaporation causing elevated glucosinolate levels in the samples. However a maximum weight loss of 20% after cooking was established experimentally and this could not explain the high increase of glucosinolates. A possible explanation for this phenomenon is an increase in chemical extractability of the glucosinolates after an intense heat treatment. Cooking has been reported to increase extractability of carotenoids. Hart & Scott (1995) showed in different green vegetables, peas and beans an average increase of 24% lutein and 38% β-carotene. These substantial increases of health-protective compounds after cooking can have important consequences with respect to bioavailability of these compounds to humans.

Conclusions

We presented a model that describes the fate of glucosinolates as precursors of health-protective components and an active enzyme myrosinase during the cooking process of red cabbage and predicts the final intake by humans. Conventional cooking resulted in substantial losses of glucosinolates and low levels of protective breakdown products and therefore decreases any health-promoting properties of the cooked vegetables. This study emphasises the importance of assessing the intake of glucosinolates and derivatives after processing or food preparation prior to consumption regarding the health-protective capacity of cooked vegetables.

Further studies characterising changes in the levels of glucosinolates and breakdown products during processing and preparation will be critical to fully understand the role in the diet that vegetables processed in different ways may play in preventing human diseases. Possible other methods of food preparation like microwave cooking of vegetables can offer more controllable and promising alternatives regarding phytochemical intake. To assist these studies the predictive modelling approach presented in this chapter can be a valuable tool. Another way of using the predictive quantitative model is the use in combination with epidemiological studies. The large impact of vegetable processing or food preparation on the variation in levels of health-protective glucosinolate breakdown products can partly explain the weak inverse correlation between consumption of *Brassica* vegetables and cancer incidence. By correcting intake data of bioactive compounds derived from e.g. fruit and vegetables for their way of processing, either by industry or by consumers themselves, the sensitivity of such studies can possibly be enhanced enormously. A third possible application is the simulation of food preparation in different ways for designing human intervention studies.

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Chapter 7

General discussion and conclusions

Introduction

The discovery that a diet rich in fruits and vegetables decreases risk of developing many types of cancer has focused attention on the causal agents and mechanisms underlying this relationship (Steinmetz and Potter, 1991). Evidence that *Brassica* vegetables may have important anticarcinogenic effects associated with the biological activity of glucosinolate breakdown products raised questions about how to investigate these compounds from a food technology point of view.

The research described in this thesis was done to evaluate how levels of glucosinolates and their derivatives are affected by various factors within the production chain of *Brassica* vegetables towards a better understanding of the alleged health effects of glucosinolates in *Brassica* vegetables. The research focused mostly on the effects of processing, namely chopping and cooking, on the content of glucosinolates. A novel predictive modelling approach is proposed (and elaborated in a case study) to handle these variations in the production chain and to provide a tool that can be used to assist product and process development. This model provides us with more insight in the behaviour and fate of glucosinolates and protective derivatives and may lead to options for improvement of investigations aimed at understanding the role of dietary glucosinolates and breakdown products in the protection against various cancers. The implications of the effects for human intake are discussed in this chapter in relation to the health-protective potential of *Brassica* vegetables and vegetable products in the human diet.

Health effects of *Brassica* vegetables

The health value of food products is determined by the level of both nutrients and biologically active non-nutrients in the final product and by their bioavailability and bioactivity after digestion. The protective effect of *Brassica* vegetables against cancer has been suggested to be partly due to their relatively high content of glucosinolates, which distinguishes them from other vegetables. Vegetables of the *Brassica* genus, including cabbage, Brussels sprouts, broccoli, cauliflower and kohlrabi contribute most to the daily intake of glucosinolates. Epidemiological studies strongly suggest that high intakes of *Brassica* vegetables are associated with a reduced risk of cancer at many sites (Verhoeven et al., 1996). This epidemiological evidence is consistent with many experimental studies, which from the 1960s onwards have indicated that glucosinolate breakdown products exert anticarcinogenic activity in experimental animal models (Verhoeven et al., 1997; Hecht, 1999).

However, from the epidemiological literature it is not clear whether the protective effects were attributable to *Brassica* vegetables *per se* or specific groups of *Brassica* vegetables or to vegetables in general (Voorrips et al., 2000). Furthermore, because of

the existence of many other bioactive compounds in vegetables, the question remained which (combination of) compounds might be responsible for the protective effect.

Variation within the production chain of *Brassica* vegetables

General

Because of the nature of glucosinolates, their vulnerability to tissue damage and the highly reactive nature of their breakdown products, it is essential to consider their behaviour and biological effects in the context of the whole food chain from farm to fork. There is only scattered information about the effects of the conditions prevailing at each step in the production chain on the level of specific bio-active components. Even less information is available on the effects of steps in the production chain on the bioavailability, although one important exception is the effect of processing on the bioavailability of carotenoids (Castenmiller et al., 1999; Thane and Reddy, 1997).

Processing is a prerequisite for several foodstuffs in order to improve palatability, shelf life, digestibility and food safety. The methods involved in processing and preparation of foods vary widely and several studies have shown that nutritive values may be improved or diminished. Less information is available about the effect of processing on non-nutritive, bioactive compounds with alleged health protective properties.

Fresh vegetables

Obviously, raw and fresh *Brassica* vegetables contain highest levels of glucosinolates. However, there is a substantial variation in glucosinolates content between groups of vegetables and between and within different varieties due to genetic and environment factors (Kushad et al., 1999). Also, it is worthwhile to mention the analytical variation and inaccuracies in the quantitative determination of glucosinolates. A number of factors contribute to both, within and between laboratory variation. Most important are the discrepancies of analytical data obtained from raw versus cooked vegetables. In our research, microwave cooking of red cabbage led to an apparent increase in the levels of glucosinolates in the cooked vegetables (Chapter 4). This was probably due to an increased chemical extractability of glucosinolates from the vegetable matrix. Cooking has been reported to increase extractability of carotenoids in various green vegetables, peas and beans (Hart & Scott 1995; Granado et al., 1992), by which the increment varied according to the type of vegetable and carotenoid. It is therefore important to further investigate this underestimation of the glucosinolate content of raw *Brassica* vegetables. Moreover, increases of glucosinolates, as observed after microwave cooking, can have important

consequences with respect to (bio)availability of these compounds and thus, the health-protective potential of (microwave) cooked vegetables to humans.

The existing variation in individual and total glucosinolates levels between and within different varieties can be ascribed to environmental factors as cultivation (variety, cultivation practices, nutrition, climate, harvest time) and storage, packaging and transportation (time, temperature, humidity, physical damage, atmosphere) (Chapter 5). It is of importance to recognise the large differences in glucosinolate profiles between various *Brassica* vegetables. Vegetables like white cabbage and savoy cabbage do not contain the glucosinolate glucoraphanin (4-methylsulphinylbutyl), while glucoraphanin is the predominant glucosinolate in broccoli (more than 50% of the total amount of glucosinolates). Sinigrin (2-propenyl glucosinolate) is present in all *Brassica* vegetables except for broccoli (or in very low amounts). In cauliflower the most abundant glucosinolates are sinigrine and glucobrassicin (3-indolymethyl glucosinolate)(Kushad et al., 1999; Rosa et al., 1997).

There is a scientific debate about which glucosinolate breakdown products play a role in the protection against cancers. Most attention has been paid to specific breakdown products, mainly isothiocyanates, which are able to inhibit phase I enzymes responsible for the bio-activation of carcinogens (Guo et al., 1992) and to induce phase II detoxification enzymes (Sparnins et al., 1982; Zhang et al., 1992). In this respect, the indolic compounds (released from indolyl glucosinolates) reveal a dual role, inducing both phase I and phase II enzymes (McDanell et al., 1988).

The large variation in glucosinolate levels between and within each group of *Brassica* vegetables suggests differences in their health-promoting potentials (Kushad et al., 1999). Since not all *Brassica* vegetables contain the "healthy" glucosinolates, or at least in appreciable amounts, this should be taken into account in epidemiological studies.

Clearly, ingestion of raw vegetables will result in hydrolysis by an unaffected, active enzyme myrosinase during consumption of the vegetables. However, it is unknown to what extent mastication of the vegetables contributes to the release of the protective breakdown products. Since consumption is the final step in which glucosinolates can be released from the food matrix and hydrolysed into protective derivatives, mastication and possibly fermentation in the body needs to be thoroughly investigated.

Cooked vegetables

Brassica vegetables are mostly not consumed raw but are often industrially or domestically subjected to thermal processing. Industrial heating of the vegetables is usual blanching or sterilising. Domestic heating or preparation of the food is more complex and much less standardised. The glucosinolate content in vegetables after

cooking depends on the method and duration of cooking, degree of raw material disintegration and on the raw material itself. Some sub-processes that take place during cooking are leaching of glucosinolates, breakdown products and myrosinase into the cooking water, inactivation of the enzyme myrosinase, enzymatic breakdown of glucosinolates or heat degradation of glucosinolates and breakdown products (Chapter 5). Our research showed that cooking is affecting the glucosinolate levels in a complex manner caused by the variations in process conditions of which the temperature profile and amount of cooking water are the most important ones (Chapter 6). Conventional cooking resulted in substantial losses of glucosinolates and low levels of protective breakdown products and therefore decreases any health-promoting properties of the cooked vegetables. These reductions in glucosinolate levels are in agreement with earlier reports (Rosa and Heaney, 1993; Mullin and Sahasrabudhe, 1978; Ciska & Kozlowska, 2001).

As an alternative we studied microwave cooking of vegetables in which no additional water is used during the treatment (Chapter 4). It was hypothesised that, because no leaching can take place, this way of food preparation resulted in higher retention of glucosinolates in the prepared vegetable product. In this study, microwave cooking showed a high retention of the glucosinolates, in some instances even exceeding the levels in untreated cabbage material. High intensity microwave cooking of red cabbage led to an apparent increase in the levels of glucosinolates in the cooked vegetables (Chapter 4). Moreover the increase in levels seems to be associated with the energy input applied. This was probably due to reduced glucosinolate breakdown in combination with more efficient release of glucosinolates during extraction for analysis. Microwave-cooked cabbage would result in a relatively higher intake of glucosinolates as compared to conventional cooked cabbage in water. Above all, a reasonable retention of myrosinase activity was shown at less intense microwave conditions, which is essential for the release of protective glucosinolate breakdown products during consumption.

Ready-to-eat vegetables

The ready-to-eat products and meals form an important market growing every year. The application of new technologies has resulted in products with extended shelf life, better taste and higher nutritional value. *Brassica* vegetables as cabbage, Brussels sprouts, cauliflower and broccoli are often used in ready-to-eat meals and can therefore contribute to an important part of our glucosinolate intake. However, preliminary analysis of data of an extensive survey of glucosinolates and breakdown products conducted on a broad range of ready-to-eat products and meals showed negligible amount of both groups of compounds in most of the meals (Verkerk, 2001, unpublished results).

In this respect, food companies producing ready-to-eat meals containing *Brassica* vegetables are facing a challenge to develop products and/or meals with high levels of health-protective glucosinolates. A ready-to-eat product can probably be transformed into a functional food if the food industry succeeds in increasing the concentration of glucosinolates in the product by retaining the original levels in vegetables throughout the entire process. Nevertheless, a health beneficial effect of the product still needs to be demonstrated and for this further research is required.

Glucosinolates and epidemiology

Food composition tables are important tools for epidemiological studies on nutrients and non-nutrient phytochemicals in relation with diseases. However, accurate knowledge of the nutrient and non-nutrient intake of individuals and groups of people requires information on the contents of prepared foods, in other words prior to consumption. Unfortunately, dietary calculations are frequently made on the basis of foods as brought into the kitchen. It is shown in this thesis (Chapter 3, 4, and 6) that vegetable processing and food preparation have large impact on glucosinolate content.

Epidemiological studies indicate that cancer incidence may be lowered due to the intake of vegetables. For the group of *Brassica* vegetables the presence of glucosinolates is assumed to play a role in the protection against cancer. This would imply a direct relation between glucosinolate intake and glucosinolates measured in the fresh, unprocessed vegetables. However, this epidemiological observation does not take into account the effects of food processing and preparation on glucosinolate content and myrosinase activity, and thus of the consequences for potential health-protection. Therefore, we emphasise that food composition tables should be used with some restraint or rather, should be adapted to accommodate these new insights.

The possible consequences of these highly variable levels of glucosinolates in epidemiological studies can be illustrated in a hypothetical example in which the intake of a bioactive compound (e.g. a glucosinolate) is associated with the decrease of the relative risk of developing a certain type of cancer. In Figure 7.1 it is illustrated that high intake of a protective compound would be inversely associated with cancer incidence: the relative risk (RR) is 0.6 for a relative intake of the compound (RI) of 0.5. In epidemiological studies (cohort or case control) usually only the intake of products is estimated and a fixed content of bioactive compounds is assumed. The consequence of this is that variation in the content of bioactive compounds, such as glucosinolates, is not taken into account. If we assume a situation in which there is a 2-fold deviation in content and thus intake of a protective glucosinolate (RI 0.25-1.0), caused by variation in e.g. vegetable processing and food preparation, it will alter the

bandwidth of the relative risk considerably (RR 0.2-0.8). In other words, the study population consists of people varying not only in the intake of the food products, but also in the content of protective compounds in those products. These confounding effects lowers the sensitivity of epidemiological studies considerably.

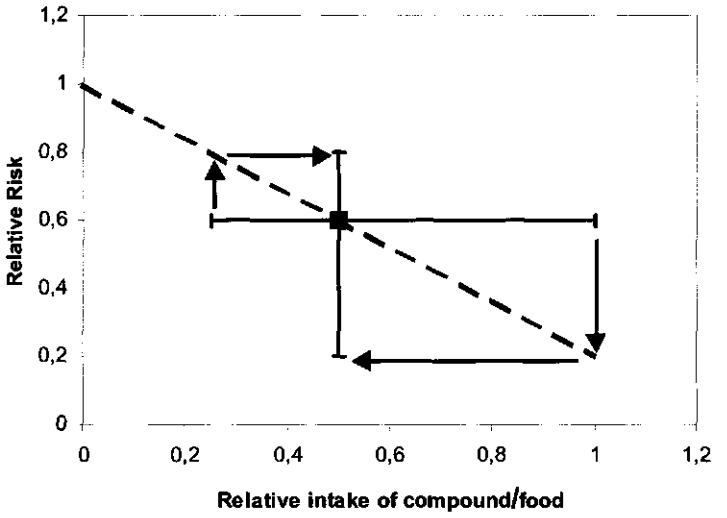

Figure 7.1 Hypothetical example of the deviation in relative risk of cancer incidence according to relative compound/food intake.

This example is a simplified representation of a much more complex situation. If individual compounds, as glucosinolates and breakdown products in *Brassica* vegetables are responsible for alleged health-protection it is likely that their activity will be positively or negatively affected by other dietary factors (e.g. ascorbic acid, fibers). In this example we used a reasonably low variation in content (2-fold) of protective compound, while it is more likely that this variation will be several orders of magnitudes higher as described in this thesis. So the actual confounding effect of this variation can be much higher. Therefore, we emphasise that confounding of epidemiological results, caused by this glucosinolate intake paradigm, will very likely contribute to the uncertainties in the association between *Brassica* vegetable intake and cancer incidence.

The complexity of the glucosinolate-myrosinase system hampers the use of food composition tables in epidemiological studies even more. Glucosinolates themselves do not possess any health-protective properties but rather some of their breakdown products. Cell damage of the vegetables by processing or mastication releases the

endogenous hydrolytic enzyme myrosinase, which converts the glucosinolates to anticarcinogenic breakdown products such as isothiocyanates and indoles. Thus, protection is only possible if an active plant myrosinase or microbial enzymes in the gut with similar activity are present and able to convert the glucosinolates.

Predictive modelling

The effects of all steps in the production chain on health-promoting components have not been systematically studied and because of the almost infinite amount of possible variation in all the factors they never will be. Due to this lack of information we advocate a predictive modelling approach as a very effective tool to estimate the effects of variation in conditions and processes on the levels of glucosinolates and protective derivatives and thus the health-protective potential of vegetable products. This modelling approach should then be based upon mechanistic understanding of the most relevant conditions and processes within the entire production chain of *Brassica* vegetables.

Research on the protective effects of dietary glucosinolates from *Brassica* vegetables in human cancer development requires an accurate assessment of the dietary intake of these components.

Glucosinolate-myrosinase system

The conversion of anticarcinogenic breakdown products from glucosinolates can in theory take place at three different stages; i) during processing, e.g. cooking by plant myrosinase; ii) during mastication of the cooked vegetables and iii) by intestinal microbial enzymatic activity in the human body. For the first stage our model simulations (Chapter 6) predict that during cooking very little hydrolysis takes place resulting in about 3 to 4 % of the total original amount of glucosinolates. For the second stage, hydrolysis during mastication of the vegetables in the mouth, an active myrosinase enzyme is needed. Our predictive model indicates that myrosinase is completely inactivated after different cooking simulations (not shown) and therefore can not contribute to hydrolysis of glucosinolates in the mouth. Hydrolysis by intestinal microflora of the remaining intact glucosinolates present in the cabbage can most likely contribute to the release of breakdown products and subsequently being absorbed from the intestinal tract (Getahun and Chung, 1999; Elfoul et al., 2001 and Rabot et al., 1995). However, it is demonstrated that this third stage of conversion of glucosinolates, depending on the used bacterial strain, is taking place in only limited quantities in the human body (Krul et al., 2001).

The significance of myrosinase-mediated conversion is emphasised by bioavailability studies carried out by Conaway et al. (2000). They showed that the

bioavailability of isothiocyanates (ITCs) from fresh broccoli is approximately three times higher than that from steamed broccoli, in which myrosinase is inactivated. Based on their results they concluded, considering the potential chemopreventive activity of ITCs in broccoli, that cooking might substantially reduce the health beneficial effects of broccoli in the diet. The assumption that conventional cooking is diminishing the protective effects of *Brassica* vegetables seems to be supported by the epidemiological trend showing most clear protective effects for raw vegetables (Steinmetz and Potter, 1996).

Future prospects

It is demonstrated that many steps in the food production chain of *Brassica* vegetables can have a large impact on the behaviour and fate of glucosinolates, breakdown products and the hydrolytic enzyme myrosinase. All these steps contribute to the final intake of glucosinolates and their protective breakdown products and thus, determine the health promoting potential of the food. In an attempt to deal with the variability in the food production chain it will be useful to develop a health protection index of *Brassica* vegetables or vegetable products that contains a correction factor for effects of the production chain. In this respect, an index for effects of processing, affecting intake mostly, is especially of interest. In this health protection index the presence or absence of "healthy" glucosinolates in defined *Brassica* vegetables can be included as well as the levels of the desired glucosinolate and breakdown product (high, medium, low) after post-harvest processes or treatments and the amount of retained active myrosinase enzyme. In future epidemiological studies evaluating possible associations between intake of glucosinolates and cancer incidence the food intake data that is converted to glucosinolate data can be adjusted by using this health protection index. This can lead to an enhancement of the sensitivity of these studies. The concept of predictive modelling within the entire production chain as developed and discussed in this thesis could be a valuable tool in developing this health protection index. In summary, the approach of predictive modelling based on mechanistic insight in the underlying (bio)chemical processes and the development of a health protection index for *Brassica* vegetables can be helpful in enhancing the sensitivity of epidemiological studies.

There is a growing interest in the enhancement of levels of desirable phytochemicals in crop plants via classical breeding or biotechnological methods. However, the need for increased levels of specific health-promoting glucosinolates in the crops is unsubstantiated as long as more accurate intake data are lacking and no strong associations between *Brassica* vegetable consumption and cancer incidence can be postulated.

When products are developed with substantial higher levels of glucosinolates, possible harmful effects of these compounds should be taken into account. It has been shown by *in vitro* experiments that glucosinolate breakdown products are capable of inducing genotoxic effects. However this only occurs at very high doses, which exceed by more than 100 fold the exposure level in humans (Kassie and Knasmüller, 2000). Also there is no epidemiological evidence that this is an important cause of human disease.

In conclusion, the approach of predictive modelling and the development of a health protection index for processed *Brassica* vegetables can be helpful in enhancing the sensitivity of epidemiological studies and eventually provide solid evidence for assessment of the risks and benefits of glucosinolate consumption. This approach can also be applied to other phytochemicals, where similar variability can be expected. Furthermore, predictive modelling of health aspects in the production chain of vegetable products can be used for the development of tools to facilitate both product and process development for health products.

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Summary

Phytochemicals are a group of "non-nutrient" plant secondary metabolites, found in many plant foods, which can have important implications for human health. Their added value can be ascribed to the important role in the prevention of various diseases, most importantly ageing diseases like cancer and coronary heart diseases. Consequently, vegetable quality has to be defined not only by the presence of essential nutritive compounds, favourable sensory attributes and the absence of undesirable compounds but also by presence of health-protective or health-promoting phytochemicals.

There is substantial epidemiological evidence for positive health effects from the consumption of fruits and vegetables on various cancers and cardiovascular diseases. In this respect, protective effects of *Brassica* vegetables against cancer have been suggested to be mainly due to their relatively high content of glucosinolates, which distinguish them from other vegetables. Vegetables of the *Brassica* genus, including cabbage, Brussels sprouts, broccoli, cauliflower and kohlrabi contribute almost exclusively to our intake of glucosinolates. There is considerable scientific evidence for the anticarcinogenic action of certain glucosinolate breakdown products, especially sulphoraphane and other isothiocyanates, which induce detoxifying enzymes in a number of different organs *in vivo*. In general, this induction leads to enhanced excretion of carcinogens and is associated with a reduction in the formation of DNA adducts. Several epidemiological studies show that high intakes of *Brassica* vegetables are associated with a reduced risk of cancer at different sites. However, it is not yet clear whether these associations are to be attributed to *Brassica* vegetables *per se* or to vegetables in general.

In this thesis an extensive overview of the relative unknown group of glucosinolates is presented, describing the current state of knowledge regarding the genetics and biosynthesis of glucosinolates, their chemical analysis and their bioavailability to humans (*Chapter 2*). The myrosinase-mediated hydrolysis of glucosinolates plays an important role in the release of protective breakdown products. In principle, disruption of the food matrix and subsequent release of glucosinolates and myrosinase triggers this hydrolytic reaction. In *Chapter 3* it is demonstrated that chopping of raw *Brassica* vegetables results only in limited breakdown of aliphatic glucosinolates whereas, unexpectedly, increased levels of indolyl glucosinolates are observed after chopping and storage of cabbage and broccoli under ambient conditions. In white cabbage a 15-fold increase of 4-methoxy- and 1-methoxy-3-indolylmethyl glucosinolates was noted after 48 h of storage of chopped cabbage. Chopping and storage of broccoli vegetables resulted in a strong

reduction of most glucosinolates, except for 4-hydroxy- and 4-methoxy-3-indolylmethyl glucosinolates, which increased 3.5- and 2-fold respectively. In this study we showed that the well-known and accepted breakdown mechanism of glucosinolates (hydrolysis by the endogenous enzyme myrosinase) appeared to be counteracted by a yet unknown mechanism causing an increase of some indolyl glucosinolates. It is postulated that chopping, by mimicking pest damage, triggers a defence mechanism in harvested *Brassica* vegetables.

As most vegetables are processed in some way before consumption, the effects of industrial processing and household preparation should be taken into account in order to know what the intake of these protective compounds will be. A large number of *Brassica* vegetables are consumed after cooking. Various studies on glucosinolates have shown that conventional cooking can lower their contents in foods. Leaching of the glucosinolates into the cooking water causes considerable reduction of glucosinolates levels. A study conducted on microwave cooking of red cabbage showed it to be an interesting alternative for conventional cooking (*Chapter 4*). In general, high total glucosinolate levels were observed for various microwave treatments probably reflecting the absence of leaching of glucosinolates into cooking water that takes place in conventional cooked vegetables. Therefore, consumption of microwave-cooked cabbage would result in a higher intake of glucosinolates compared to conventional cooked cabbage. It is striking that many of the various time/energy input combinations resulted in levels exceeding the total glucosinolate content of the untreated cabbage. Moreover the increase in levels seemed to be associated with the energy input applied. It is hypothesised that this is due to an increased chemical extractability of glucosinolates from the vegetable matrix after the microwave treatment. The various applied microwave powers affected the myrosinase enzyme differently. Substantial myrosinase activity was retained in cabbage at low (24 min 180 Watt) and intermediate microwave powers (8 min 540 Watt) while microwave cooking for 4.8 minutes at 900 W (259.2 kJ energy input) resulted in complete loss of hydrolytic activity. In this respect, differences in observed temperature profiles of the various microwave treatments play an important role. Microwave prepared *Brassica* vegetables can offer a higher retention of glucosinolates and controllable amounts of active myrosinase, thereby increasing the health-promoting potential.

In *Chapter 5* it is demonstrated that many steps in the food production chain of *Brassica* vegetables or vegetable products can have a large impact on the glucosinolate content and thus, affect the final intake of health-protective glucosinolates and breakdown products for humans. Summarising it is concluded that the glucosinolate content of processed vegetables depends on:

- genetic and environmental factors (determining the glucosinolate content in the original raw foods);
- the extent and nature of processing (industrial and domestic);
- packaging, storage and distribution conditions.

The large amount of variables for each step in the chain makes an experimental quantification of dietary intake of glucosinolates and consequently their contribution to health-protection very difficult. For this reason a concept of predictive modelling of glucosinolates in the production chain of *Brassica* vegetables and derived products was developed. This model can be used as a tool to facilitate both product and process development for health products as well as to obtain more realistic epidemiological input data for bioactive substances in the diet.

The proposed predictive modelling concept was elaborated in a study on the glucosinolate-myrosinase system during cooking of cabbage (*Chapter 6*). The level of glucosinolates as precursors of health-protective components and the activity of the enzyme myrosinase were selected as most important parameters. Subsequently cell lysis, leaching of the components and denaturation and activity of myrosinase are identified as critical sub-processes. These sub-processes were translated into mathematical equations describing the changes during the cooking process. Based on the mathematical descriptions of the sub-processes a model was developed that describes the fate of the glucosinolate-myrosinase system during the cooking process of red cabbage and predicts their level prior to consumption of the vegetables. Validation of the model was done by comparing the results of cooking experiments of broccoli and red cabbage with the model prediction.

Simulation studies with the predictive model show considerable reduction in glucosinolate contents in the cabbage of about 40% to 70% depending on the ratio of vegetables/cooking water, the warm up and cooking time. Also very low amounts of breakdown products are formed during cooking (max. 4% of total glucosinolate content). Ultimately, in different simulation studies it is shown that cabbage prior to consumption contains no or very little active myrosinase. In conclusion, conventional cooking of cabbage resulted in substantial losses of glucosinolates and low levels of protective breakdown products and therefore diminishes the health-protective potential of the cooked vegetables.

It is demonstrated that many steps in the food production chain of *Brassica* vegetables can have a large impact on the fate of glucosinolates, breakdown products and the hydrolytic enzyme myrosinase. All these steps contribute to the final intake of glucosinolates, their protective breakdown products and myrosinase and thus, determine the health-promoting potential of the food. This variation is a strong confounding factor in epidemiological studies and will very likely contribute to the

uncertainties in the association between *Brassica* vegetable intake and cancer incidence.

In an attempt to deal with the variability in the food production chain the introduction of a "health protection index" of *Brassica* vegetables or vegetable products is proposed that can be used as a correction factor for effects of the production chain. In the future, epidemiological studies evaluating possible associations between intake of *Brassica* vegetables and cancer incidence can be adjusted by using this health protection index. The concept of predictive modelling within the entire production chain as developed and discussed in this thesis could be a valuable tool in developing this health protection index. Refining epidemiological studies in this way may result in separating the anticarcinogenic effect of the glucosinolates from *Brassica* vegetables from the effect of vegetables in general. In conclusion, the approach of predictive modelling based on mechanistic insight in the underlying (bio)chemical processes and the development of a health protection index for processed *Brassica* vegetables can be helpful in enhancing the sensitivity of epidemiological studies and eventually provide solid evidence for assessment of the risks and benefits of glucosinolate consumption. This approach can also be applied to other phytochemicals, where similar variability can be expected. Furthermore, predictive modelling of health aspects in the production chain of vegetable products can be used for the development of tools to facilitate both product and process development for health products.

Samenvatting

Fytochemicaliën zijn een groep van bioactieve secundaire plantmetabolieten die voorkomen in veel plantaardig voedsel. Ze kunnen een belangrijke rol vervullen voor de gezondheid en het welzijn van de mens. Hun belang in de voeding kan toegeschreven worden aan de mogelijke rol die zij spelen bij het voorkomen van enkele ziekten, voornamelijk verouderingsziekten zoals kanker en hart- en vaatziekten. Dit betekent dat de aanwezigheid van gezondheidsbeschermende en -bevorderende fytochemicaliën als een additioneel kwaliteitskenmerk van groenten kan worden aangemerkt, naast de gewenste sensorische eigenschappen, de aanwezigheid van essentiële voedingscomponenten en de afwezigheid van ongewenste verbindingen.

De laatste jaren hebben diverse epidemiologische studies (bevolkingsonderzoeken) aangetoond dat een hoge consumptie van groente en fruit samenhangt met een lager risico op het krijgen van bepaalde soorten kanker, hart- en vaatziekten en andere chronische ziekten. De groep van *Brassica* groenten lijkt daarbij een expliciete rol te spelen bij het voorkomen van kanker. Groenten behorend tot het *Brassica* geslacht, zoals alle koolsoorten, spruiten, broccoli, bloemkool en koolrabi, bevatten een relatief grote hoeveelheid aan bio-actieve stoffen, glucosinolaten genaamd. Verondersteld wordt dat de beschermende effecten van *Brassica* groenten tegen kanker toe te schrijven zijn aan de aanwezigheid van glucosinolaten die deze groep onderscheidt van andere groenten. Glucosinolaten zelf vertonen nauwelijks anti-kanker activiteit, echter bij beschadiging van het plantenweefsel komt een hydrolytisch enzym (myrosinase) vrij dat in staat is om de glucosinolaten om te zetten in een reeks biologisch actieve componenten. Enkele van deze afbraakproducten, zogenaamde isothiocyanaten en indolen, hebben het vermogen om in het lichaam ontgiftigingsenzymen te induceren. In het algemeen leidt deze enzyminductie tot een toename in uitscheiding van carcinogene en andere lichaamsvreemde stoffen en gaat samen met een afname van DNA schade. Hoewel diverse epidemiologische studies aangeven dat een hoge inname aan *Brassica* groenten samen gaat met een verlaging van het risico van diverse soorten kanker kunnen deze studies vooralsnog niet reproduceerbaar aangeven of dit verband toe te schrijven is aan *Brassica* groenten op zich of aan groenten in het algemeen. Met andere woorden, het is moeilijk om, op basis van deze epidemiologische studies, de groep van glucosinolaten aan te wijzen als een van de beschermende factoren. Bij het onderzoek naar de gezondheidsbeschermende eigenschappen van glucosinolaten in *Brassica* groenten is het van belang om te weten wat de gehalten zijn na verwerking en/of bereiding van groenten, om uiteindelijk de inname van deze stoffen bij de mens te kunnen schatten.

De uitgangsgedachte is dat in de gehele productieketen van *Brassica* groenten er factoren zijn die de gehalten kunnen beïnvloeden. Het doel van het onderzoek zoals beschreven in dit proefschrift was daarom om de effecten van activiteiten in de productieketen van *Brassica* groenten op de glucosinolaat gehalten te bestuderen. Daarbij is met name gekeken naar de bereiding van groenten waarvan de grootste effecten werden verwacht. Een onderzoeksaanpak gebaseerd op het voorspellend modelleren van processen binnen de productieketen is ontwikkeld om de glucosinolaat gehalten en beschermende afbraakproducten na een proces te kunnen kwantificeren en zodoende een uitspraak te kunnen doen over hun inname.

In dit proefschrift wordt als eerste een uitgebreid overzicht gegeven van de betrekkelijk onbekende groep van glucosinolaten. Hierin wordt de huidige stand van zaken weergegeven aangaande de genetica en biosynthese van glucosinolaten, hun chemische analyse en hun biologische beschikbaarheid voor de mens (Hoofdstuk 2). De myrosinase-gereguleerde hydrolyse van glucosinolaten speelt een belangrijke rol bij het vrijmaken van de beschermende afbraakproducten. In principe zal beschadiging van het plantenweefsel en het vervolgens vrijkomen van glucosinolaten en het enzym myrosinase leiden tot deze hydrolyse. Echter, in Hoofdstuk 3 is beschreven dat het snijden van rauwe *Brassica* groenten slechts resulteerde in een beperkte afbraak van alifatische-glucosinolaten terwijl een onverwachte toename van indolyl-glucosinolaten werd waargenomen na snijden en opslag van kool en broccoli onder omgevingscondities. In witte kool werd zelfs een 15-voudige toename van het 4-methoxy-3-indolylmethyl-glucosinolaat en 1-methoxy-3-indolylmethyl-glucosinolaat waargenomen na 48 uur opslag van gesneden kool. Snijden en opslag van broccoli resulteerde in een sterke afname van de meeste glucosinolaten, behalve het 4-hydroxy-3-indolylmethyl-glucosinolaat en 4-methoxy-3-indolylmethyl-glucosinolaat die respectievelijk een 3,5- en 2-voudige toename lieten zien. In deze studie is aangetoond dat tegenover het bekende afbraakmechanisme van glucosinolaten (hydrolyse door het endogene enzym myrosinase) een nog onbekend mechanisme staat dat een toename van met name indolyl-glucosinolaten kan veroorzaken. Verondersteld wordt dat het snijden, overeenkomend met insectenvraat, een verdedigingsmechanisme in geoogste *Brassica* groenten activeert.

De meeste groenten worden voor consumptie op de een of andere wijze bewerkt. De effecten van bewerking dienen daarom in ogenschouw te worden genomen teneinde te bepalen wat de gevolgen zijn voor de inname van de beschermende componenten. Veelal worden *Brassica* groenten gekookt. Voorgaande studies hebben aangetoond dat koken het glucosinolaat gehalte in voedsel aanzienlijk kan verlagen. Het uitlekken van glucosinolaten naar het kookvocht is de belangrijkste oorzaak voor deze afname van glucosinolaten. In een studie beschreven in Hoofdstuk 4 zijn de effecten van magnetronbereiding van rode kool, als alternatief voor conventioneel

koken, onderzocht op de glucosinolaat gehalten en de myrosinase activiteit. Bij magnetron bereiding van de groenten was geen water toegevoegd en verondersteld werd dat uitlek van de glucosinolaten dan ook beperkt zou zijn. In het algemeen werden zeer hoge gehalten aan glucosinolaten waargenomen bij verscheidene magnetronbehandelingen variërend in tijd en vermogen. Aangenomen kan dan ook worden dat consumptie van magnetron-bereide kool zal leiden tot een hogere inname aan glucosinolaten dan kool gekookt in water. Het is opvallend dat veel van de verschillende tijd-energie input combinaties leidden tot glucosinolaatgehaltes die zelfs hoger waren dan in onbehandelde kool. Bovendien nam het gehalte aan glucosinolaten toe met de hoeveelheid toegevoerde energie door de magnetron. Verondersteld wordt dat de magnetronbehandeling de matrix van de kool verandert hetgeen de chemische extractie van de glucosinolaten vergroot. Verder is gebleken dat de resterende myrosinase enzymactiviteit afhankelijk is van de tijdsduur en het vermogen van de magnetronbehandeling. Enzymactiviteit werd nog waargenomen bij een laag (24 minuten 180Watt) en een tussenliggend magnetronvermogen (8 minuten 540 Watt) waardoor er tijdens consumptie van de bereide groenten nog hydrolyse van glucosinolaten kan plaatsvinden. Een intensieve behandeling van 4,8 minuten bij 900 Watt (259,2 kJ energie) resulteerde in een complete inactivatie van het enzym. De waargenomen verschillen in temperatuurprofielen van de diverse behandelingen spelen waarschijnlijk hierbij een rol. Geconcludeerd kan worden dat magnetron bereiding van kool kan resulteren in een hoog gehalte aan glucosinolaten en een beheersbare hoeveelheid actief myrosinase, hetgeen een toename in het gezondheidsbeschermend vermogen van de bereide groenten kan opleveren.

In Hoofdstuk 5 staat beschreven dat een groot aantal stappen in de productieketen van *Brassica* groenten of afgeleide producten een grote invloed kan hebben op het glucosinolaatgehalte en dus op de uiteindelijke inname van de beschermende glucosinolaten en hun afbraakproducten. Samenvattend kan worden vastgesteld dat het glucosinolaatgehalte in verwerkte groenten afhankelijk is van:

- de genetica en omgevingfactoren (bepalen het glucosinolaatgehalte in het ruwe uitgangsmateriaal);
- de mate van verwerking (industrieel en huishoudelijk)
- verpakking, opslag en distributie omstandigheden.

Het grote aantal variabelen van de diverse schakels in de keten maakt een experimentele kwantificering van de glucosinolaatgehalten en vervolgens hun gezondheidsbeschermende bijdrage voor de mens zeer moeilijk uitvoerbaar. Daarom is een concept ontwikkeld voor het voorspellend modelleren van de glucosinolaat gehalten in de productieketen van *Brassica* groenten en afgeleide producten. Dit model zou gebruikt kunnen worden bij bevolkingsstudies voor het verkrijgen van reële inname gegevens van bioactieve componenten via onze voeding. Anderzijds

biedt een modelmatige aanpak mogelijkheden voor product- en procesontwikkeling voor gezonde producten. Het voorgestelde concept van voorspellend modelleren is in Hoofdstuk 6 verder uitgewerkt in een studie naar het glucosinolaat-myrosinase systeem tijdens het koken van kool. Het gehalte aan glucosinolaten, als precursors van de beschermende afbraakproducten, en de activiteit van het enzym myrosinase zijn geselecteerd als meest belangrijke parameters. Vervolgens zijn lysis van cellen, uitlek van de componenten en de activiteit en het denatureren van myrosinase geïdentificeerd als kritieke sub-processen. Deze sub-processen zijn bestudeerd en vertaald in wiskundige vergelijkingen die de veranderingen tijdens het kookproces beschrijven. Gebaseerd op de wiskundige beschrijvingen van de sub-processen is een model ontwikkeld dat het glucosinolaat-myrosinase systeem beschrijft tijdens het kookproces van rode kool en de gehalten glucosinolaten en afbraakproducten voorspelt in de groente vlak vóór consumptie. De werking van het model werd getoetst door de resultaten van kookexperimenten van broccoli en rode kool en experimenten beschreven in de literatuur te vergelijken met model voorspellingen.

Simulatiestudies met het model laten een aanzienlijke afname zien van glucosinolaat gehalten in de kool van ongeveer 40% tot 70% afhankelijk van de verhouding groente/kookwater, de opwarmtijd en de kooktijd. Ook voorspelt het model dat er lage hoeveelheden afbraakproducten gevormd worden tijdens het koken (maximaal 4% van het totale glucosinolaat gehalte). Bovendien laten verschillende simulatiestudies zien dat de kool na behandeling zeer weinig of geen actief myrosinase meer bevat. Dus, conventioneel koken (in water) van kool leidt tot aanzienlijke verliezen van glucosinolaten en lage hoeveelheden van beschermende afbraakproducten hetgeen het gezondheidsbeschermend vermogen van de gekookte groenten substantieel kan verminderen.

Samenvattend kan gesteld worden dat een groot aantal factoren in de productieketen van *Brassica* groenten een grote invloed hebben op de glucosinolaat gehalten, beschermende afbraakproducten en de myrosinase activiteit. Al deze factoren dragen bij aan de uiteindelijke inname van glucosinolaten en daarvan afgeleide beschermende stoffen en bepalen dus voor een groot gedeelte het gezondheidsbeschermend vermogen van dit voedsel. Het is zeer goed denkbaar dat de genoemde variatie in de keten, met name de verwerking en bereiding van groenten, een sterk complicerende factor is in epidemiologische studies en interventiestudies en dus mogelijk een van de verklaringen is voor de vaak uiteenlopende bevindingen.

Om met deze variatie in de productieketen om te gaan is voorgesteld een "gezondheidsbeschermingsindex" te introduceren voor *Brassica* groenten of afgeleide producten die als correctiefactor gebruikt kan worden. In epidemiologisch onderzoek naar inname van *Brassica* groenten en verbanden met kanker incidentie zou correctie met behulp van deze beschermingsindex kunnen plaatsvinden. De aanpak van het

voorspellend modelleren in de productieketen, zoals besproken in dit proefschrift, kan gebruikt worden bij de verdere ontwikkeling van deze index. Verondersteld wordt dat het op deze manier verfijnen van epidemiologische studies zou kunnen leiden tot het scheiden van aanwezige beschermende effecten van de bioactieve componenten in *Brassica* groenten van de effecten van groenten in het algemeen. Verder onderzoek is nodig om dit te onderbouwen.

Concluderend kan gesteld worden dat de aanpak van voorspellend modelleren, gebaseerd op mechanistische inzichten van de onderliggende (bio)chemische processen en de ontwikkeling van een "beschermingsindex" voor verwerkte *Brassica* groenten helpt bij het vergroten van de gevoeligheid van epidemiologisch onderzoek. Deze aanpak opent de weg om een sterker bewijs te kunnen leveren voor mogelijke beschermende van effecten van glucosinolaten. Voor andere fytochemicaliën, waarbij een vergelijkbare variatie is te verwachten in de productieketen, zijn de concepten zoals beschreven in dit proefschrift voor glucosinolaten in *Brassica* groenten, ook goed toepasbaar.

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Ruud

Over de auteur

Ruud Verkerk werd op 16 juli 1964 geboren te Utrecht. Na het behalen van zijn diploma MAVO-4 in 1978 is hij in hetzelfde jaar begonnen met een laboratorium opleiding MBO-botanie. Aansluitend heeft hij de opleiding HAS-microbiologie te Wageningen gevolgd. Na het afronden van zijn HBO studie in 1987 werd hij aangesteld als analist bij de werkgroep 'genoomorganisatie van de tomaat' aan de vakgroep Moleculaire Biologie van de Landbouwuniversiteit te Wageningen. In april 1995 trad hij in dienst bij de toenmalige sectie Zuivel en Levensmiddelennatuurkunde, later Geïntegreerde levensmiddelentechnologie genaamd, aan de vakgroep Levensmiddelentechnologie van de Landbouwuniversiteit, waar hij tot oktober 1997 werkzaam is geweest als analist. In oktober 1997 begon hij als toegevoegd onderzoeker aan het promotieonderzoek dat in dit proefschrift staat beschreven en werd uitgevoerd in het kader van een Europees project getiteld "Effects of food-borne glucosinolates on human health". Sinds oktober 2000 is hij werkzaam als postdoc bij dezelfde leerstoelgroep met de huidige naam Productontwerpen en Kwaliteitskunde, departement Agrotechnologie en Voedingswetenschappen aan Wageningen Universiteit.

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