Isothiocyanates from Cruciferous Vegetables: Kinetics, Biomarkers and Effects

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ABSTRACT

Cruciferous vegetables like cabbages, broccoli, mustard and cress, have been reported to be beneficial for human health. They contain glucosinolates, which are hydrolysed into isothiocyanates that have shown anticarcinogenic properties in animal experiments. To study the bioavailability, kinetics and effects of isothiocyanates from cruciferous vegetables, biomarkers of exposure and for selected beneficial effects were developed and validated.

As a biomarker for intake and bioavailability, isothiocyanate mercapturic acids were chemically synthesised as reference compounds and a method for their quantification in urine was developed. The validity of this biomarker was proven in a study in which three volunteers consumed 19 different raw and cooked vegetables and condiments. Urinary excretion levels of isothiocyanate mercapturic acids were higher after the consumption of raw vegetables and condiments (bioavailability 60%, range 8.2-113%) compared to cooked vegetables (10%, range 1.8-43%). In a second study, in which eight smoking men consumed cooked and raw broccoli, higher levels of sulforaphane were found when broccoli was eaten raw (bioavailability 37%) versus cooked (3.4%).

Also as a biomarker for kinetics, sulforaphane conjugates were determined in blood samples also collected in the second study. The area under the blood-concentration curve was higher when broccoli was eaten raw (0.50 μM·h, dose of 9.9 μmol sulforaphane) versus cooked (0.29 μM·h, dose of 61 μmol glucoraphanin). It is concluded that the bioavailability of isothiocyanates from glucosinolates is higher from raw than from cooked vegetables.

The induction of phase 2 metabolism enzymes via an electrophile responsive element (EpRE), that is present in the promotor region of the genes coding for these enzymes, was used as a biomarker for a beneficial health effect. Isothiocyanates containing a methyl-sulfur side chain (like sulforaphane, EC_{50} value 1.2 μM) are more potent inducers, in this *in vitro* assay, than aliphatic and aromatic isothiocyanates (e.g. allyl isothiocyanate, EC_{50} value 6.5 μM). The estimated concentrations of individual isothiocyanates in the body after consumption of cruciferous vegetables may amount to 0.04-4 μM. Since cruciferous vegetables contain several isothiocyanates which act synergistically, concentrations might thus reach these EC_{50} values for EpRE induction, and represent biologically active concentrations.

In conclusion, the biomarkers developed and validated in the present thesis show that higher and physiologically relevant amounts of isothiocyanates are absorbed after the consumption of raw cruciferous vegetables, compared to cooked vegetables. Broccoli (cress) is favorite since it contains high amounts of sulforaphane, which is, based on the results of the present studies, the most promising isothiocyanate because it shows the lowest EC_{50} for EpRE induction.

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Chapter 1

General Introduction

Based on:

Potential power:

What is the best way to exploit the health effects of glucosinolates?

Martijn Vermeulen, Robin van den Berg, Wouter H. J. Vaes

Ingredients, Health and Nutrition. **2004**, 7(2), 16-20.

Nutrition, Brassica and Health

People with a high consumption of fruits and vegetables are at lower risk of getting cardiovascular diseases. Overall, there is a consistent inverse association between the consumption of a wide variety of vegetables and fruits and the risk of cancer at most sites (*1*). The World Cancer Research Fund states: 'Nutrition and exercise are crucial to our general health and well-being'. They estimated the overall extent to which dietary modification may be expected to reduce cancer risk at 30-40% (*2*). Data from seven prospective cohort studies and 87 case-control studies showed that high consumption of one or more Brassica or cruciferous vegetables (all kales and cabbages, as well as broccoli, cauliflower, turnip and radishes are part of this family) is associated with a reduced risk of cancer of the lung, stomach, colon and rectum, with a possible reduction in endometrial and ovarian cancer, and a decrease in total cancer incidence (*3*).

Cruciferous vegetables are a unique source of glucosinolates, sulfur containing phytochemicals with a variable side chain ranging from alkyl, alkenyl, aromatic, to indolyl (see **table 1**). These glucosinolates are only present in cruciferous vegetables and several factors in culturing and preparation have an influence on the final content. Glucosinolates are converted upon chopping or chewing into isothiocyanates which are readily absorbed in the gastro-intestinal tract (*4*). When eating crucifers the intake consists of glucosinolates, somewhat bitter components, as well as isothiocyanates, causing the pungent taste of mustard. Since isothiocyanates are electrophiles they are eliminated via the mercapturic acid pathway and excreted in urine. Isothiocyanates circulate through the body as different conjugates and can exert a healthy effect when deconjugated at the site of action (see **figure 1**). Valid methods to determine food intake in a population are required since estimations of daily intake by questionnaires are subjective. As a selective and objective biomarker of cruciferous vegetable intake, isothiocyanate mercapturic acids measured in urine can be used. These mercapturic acids additionally reflect the total active dose of isothiocyanates taken up.

Culturing and preparation

Glucosinolates are present in a wide range of plants (see **table 2**); crop plants (seed oils), condiments and relishes (mustard, horseradish, wasabi), salad crops (radish, water cress, garden cress, rocket), leaf vegetables (cabbage, Brussels sprouts, cauliflower, broccoli, mustard spinach and greens) and root vegetables (kohlrabi, rutabaga or swede,

turnip) (*5*). Content and relative abundance of the glucosinolates in cabbage differ within the pith, cambial cortex and leaves. In the cortex, flavor precursors are abundant but these are absent in the pith. Sinigrin, 2-propenyl-glucosinolate present in Brussels sprouts and mustard, is a bitter compound where its isothiocyanate is pungent. Progoitrin, a glucosinolate present in Brussels sprouts, however is without bitterness but its breakdown product, 5-vinyl-oxazolidine-2-thione is intensely bitter. In nature, glucosinolates play a major role in plant defence against fungal disease and pest infestation. They are derived from amino acids and the majority of side chains require structural modification, e.g., homologation, oxidation, elimination, or hydroxylation. Factors affecting glucosinolate content are genetic origin, nature, age of the growing plant, cultural and environmental factors, period of harvest, further processing and storage. Amounts of the major glucosinolates present in e.g. rapeseed are controlled by different gene systems which are not under identical genetic control. It is therefore possible to selectively reduce those glucosinolates producing undesirable aglucones and enhance those with appreciated flavour and positive health effect. The soil composition, season, climate and time of growing, water regime and spacing all have effect on the amounts of glucosinolates finally present in the vegetable.

Glucosinolates are physically separated in the plant from an enzyme called myrosinase. Upon cutting or chewing, this enzyme is liberated and converts glucosinolates into isothiocyanates, indoles and other metabolites. The conversion of glucosinolates by myrosinase is pH dependent. Although mainly isothiocyanates are formed during hydrolysis in a neutral environment (pH 7), at low pH (pH 3) and in the presence of compounds that modify the action of myrosinase, nitriles, epithionitriles, and thiocyanates may also be produced (*5*). In the mouth the pH (6.2-7.2) is optimal for conversion into isothiocyanates. During processing of cabbage, e.g. washing, cutting, packaging, and transport, glucosinolates are lost due to leakage and due to conversion into volatile metabolites. Upon cutting and subsequent storage, amounts of indole glucosinolates rise three- to four-fold (*6*). This is an interesting post-harvest response of the vegetable. In the kitchen, chopping, cooking, and especially boiling of cabbages reduces the content of glucosinolates considerably with losses ranging from 50 to 85% (*7*). Upon microwave cooking, 25 to 50% is lost. Thorough chewing of raw cruciferous vegetables releases and converts most of the glucosinolates into isothiocyanates. Cooking inactivates the enzyme myrosinase which is responsible for this conversion.

Since isothiocyanates are the active ingredients and not glucosinolates, care must be taken to retain glucosinolates for subsequent breakdown in the body or vegetables should be consumed raw.

Absorption of glucosinolates and isothiocyanates

Depending on food preparation and extent of chewing, either glucosinolates, isothiocyanates or both reach the stomach after consumption of cruciferous vegetables. Glucosinolates seem to be hydrolysed in the stomach to some extent and bioavailability in the small intestine is limited. Acidic hydrolysis can take place in the stomach because of the low pH (1.0-3.0), whereas in the small intestine (pH 4.8-8.2) no hydrolysis or enzymatic conversion takes place. Glucosinolates are to some degree taken up intact but the fate of these compounds is unknown. Intact glucosinolates are, to a lesser degree, converted by the large intestinal microflora into isothiocyanates (pH in the colorectum is 7.8-8.0). Subsequently, if they are not absorbed or converted, they will be excreted unchanged in the faeces (*8, 9*). Isothiocyanates are readily absorbed in the small as well as in the large intestine.

Table 1. Glucosinolates are present, in vegetables and condiments, with a variable side chain

R—C(=N—OSO_3^-)—S—ß-D-glucose

nr	MW[a]	R =	name	main dietary source
1	371.5	H_3C-	glucocapparin	capers
2	397.5	$H_2C{=}CHCH_2$-	sinigrin	cabbage, mustard
3	411.5	$H_2C{=}CH(CH_2)_2$-	gluconapin	Chinese cabbage
4	425.5	$H_2C{=}CH(CH_2)_3$-	glucobrassicanapin	Chinese cabbage
5	427	$H_2C{=}CHC(OH)HCH_2$-	progoitrin	Brussels sprouts, kale
6	461.6	$H_3CS({=}O)(CH_2)_3$-	glucoiberin	broccoli
7	459.6	$H_3CS(CH_2)_4$-	glucoerucin	rutabaga, turnip
8	475.6	$H_3CS({=}O)(CH_2)_4$-	glucoraphanin	cabbage, cauliflower
9	589.6	$H_3CS({=}O)(CH_2)_5$-	glucoalyssin	paksoi
10	463.5	$pHOC_6H_4CH_2$-	glucosinalbin	mustard
11	447.5	$C_6H_5CH_2$-	glucotropaeolin	garden cress
12	461.5	$C_6H_5(CH_2)_2$-	gluconasturtiin	water cress
13	486	(indol-3-yl)-CH_2— (N-H)	glucobrassicin /	all vegetables
14	516	(indol-3-yl)-CH_2— (N-OCH_3)	neoglucobrassicin[b]	

[a] The Molecular Weight of each glucosinolate is calculated as its potassium salt in g/mol. [b] Brassicins are 3-indolylmethyl compounds.

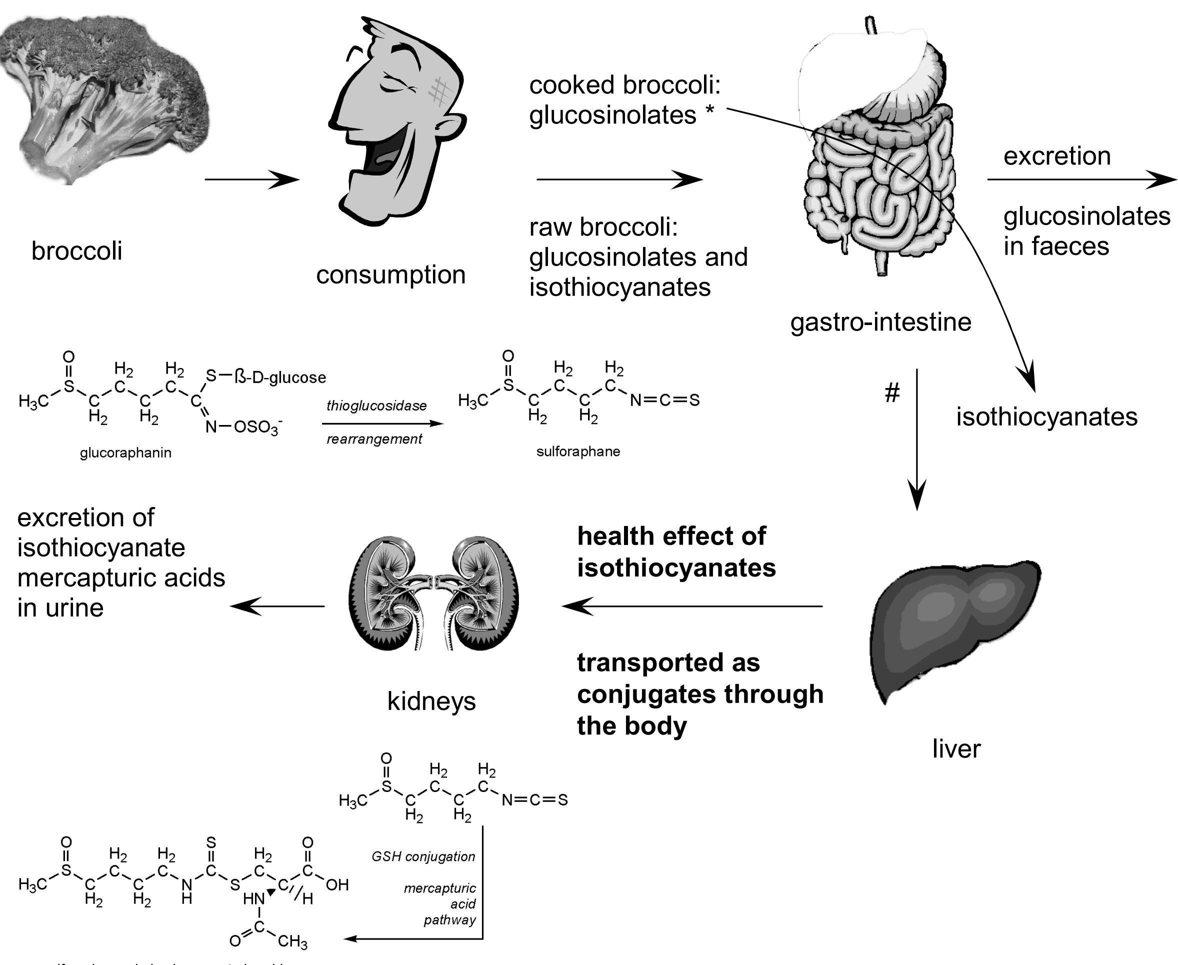

Figure 1. Broccoli contains glucoraphanin which, upon chewing, is enzymatically hydrolysed to sulforaphane. The enzyme responsible, a thioglucosidase called myrosinase, is present in all cruciferous vegetables. By cooking, this enzyme is deactivated and only glucosinolates are ingested. *10-15% of these intact glucosinolates is converted into isothiocyanates in the colon. With raw broccoli, glucosinolates and isothiocyanates are ingested. #100% of ingested isothiocyanates is absorbed in the intestine. Intact glucosinolates which are not converted are excreted with the faeces. Absorbed isothiocyanates are conjugated to glutathione (GSH) and further metabolised to mercapturic acids, which are excreted in the urine. Glucoraphanin is also present in high amounts in red cabbage and cauliflower and less in Brussels sprouts.

Metabolism of isothiocyanates

The body disposes of toxic compounds, exogenous and endogenous, by several routes. Isothiocyanates are exogenous and are therefore excreted the same way. In phase I metabolism compounds are oxidised or reduced to create chemical sites for conjugation to highly water soluble substrates in phase II metabolism. An example of a phase II enzyme reaction is conjugation to glutathione (GSH) which can be catalysed by the enzyme glutathione S-transferase (GST). Another example of a phase II enzyme is NADPH quinone oxidoreductase (NQO1) which catalyses the reduction of quinones (strictly spoken a phase I reaction). It also acts as a catalyst in other, phase II, detoxification pathways. Isothiocyanates are conjugated to glutathione in the liver, these conjugates are converted through the mercapturic acid pathway and excreted in the urine. As a consequence, several conjugates of isothiocyanates are present in the body. All conjugations are reversible, prolonging the residence time of the isothiocyanate in the body (*10*). The consecutive steps in the mercapturic acid pathway are: conjugation to the tripeptide GSH (glutaminyl-cysteinyl-glycine) catalysed by GST, separation of glutamine catalysed by glutamyl-transpeptidase, separation of glycine catalysed by cysteinyl-glycinase, and finally N-acetylation catalysed by N-acetylase.

Excretion of isothiocyante mercapturic acids in humans

Isothiocyanates are rapidly absorbed but are quickly excreted as well. After conjugation and further metabolism mercapturic acids are excreted in urine but not 100% of the dose is recovered. The fate of this remaining amount of isothiocyanate might be in exhaled air, deposit in (fat) tissue, excretion in faeces or metabolism to other compounds.

There are several studies describing the excretion in urine. The mercapturic acid of benzyl isothiocyanate (BITC) was the only metabolite detected in urine (46-62 %) after administration of BITC (0.1 mmol) in a capsule to male volunteers (*11*). BITC is usually consumed as glucotropaeolin from garden cress. Also phenethyl isothiocyanate from water cress, a rich source of gluconasturtiin, was solely excreted as its mercapturic acid in urine of human volunteers (30-67 % of the dose) (*12*). About 44-63 % of the initial dose of allyl isothiocyanate from brown mustard was excreted as its mercapturic acid by four volunteers (*13*). On average 44 % (range 34-50 %) of several doses of allyl isothiocyanate from horseradish (12-74 µmol) was excreted by one male volunteer within 24 h, with a half-life of urinary excretion of 2 hours (*14*). After ingestion of 200 g of raw and cooked broccoli (± 210

µmol of glucosinolates) by 12 volunteers, the average urinary excretion of total isothiocyanate conjugates was 68 µmol and 21 µmol respectively (*15*). Cumulative excretion of isothiocyanate conjugates following 111-µmol doses of isothiocyanates (fresh broccoli sprouts) was greater than that after glucosinolates (boiled broccoli sprouts), 89 ± 6 and 13 ± 2 µmol, corresponding to 80 % and 12 % of the ingested dose, respectively (*16*).

A 'quick and dirty' assay to quantify urinary isothiocyanates and their conjugates was developed earlier. The method is based on a quantitative cyclocondensation reaction with 1,2-benzedithiol but background levels of cyclocondensation product often occur in urine and plasma and underline the low specificity of the assay (*14,17-23*). A specific method to measure mercapturic acid excretion by HPLC coupled to mass spectrometry has been developed recently (*24*). Very low levels of excretion can be determined and from the analysis in urine a distinction can be made between, e.g. white cabbage or Chinese cabbage consumption. Glucobrassicines or indole glucosinolates do not yield isothiocyanates upon hydrolysis but are converted to indoles and thiocyanate ion (SCN^-). This ion is excreted in urine and can also be used as a biomarker of cruciferous vegetable intake. Baseline excretion levels of thiocyanate-ion are elevated in smokers and other individuals prone to inhalation of toxic nitriles, which complicates the use of this biomarker for cruciferous vegetable intake.

Excretion of isothiocyanate mercapturic acids in animals

Guinea pigs and rabbits do not readily form mercapturic acids but excrete isothiocyanates as mercaptopyruvic acid conjugates instead (*25*). Hippuric acid was the major urinary metabolite in dogs dosed benzyl isothiocyanate (*26*). Mice excreted phenethyl isothiocyanate mainly as its cyclic mercaptopyruvic acid conjugate and less as its mercapturic acid (*27*). By 48 h, 89 % of ^{14}C was collected in the urine after administration of radiolabeled ^{14}C phenethyl isothiocyanate to rats. Greater than 90 % of urinary ^{14}C was mercapturic acid (*28*).

Table 2. Principal glucosinolates occurring in the main cruciferous vegetables (*5,6,44*)

mmol/kg fresh weight (average (range))	glucosinolate sinigrin	gluconapin	glucobrassica-napin	progoitrin	glucoiberin
broccoli		0.03 (0-0.06)		0.25 (0.20-0.31)	0.74 (0.01-3.3)
Brussels sprouts	0.86 (0.04-3.9)	0.34 (0.01-2.2)		0.63 (0.01-3.0)	0.44 (0-1.5)
cauliflower	0.28 (0.01-1.6)				0.34 (0.01-3.3)
Chinese cabbage		0.12 (0-0.59)	0.20 (0.03-0.62)	0.08 (0-0.44)	
collards	0.21 (0.13-0.29)				0.39 (0.08-0.69)
garden cress					
horseradish	65-70				
kale	0.97 (0.63-2.0)	0.21 (0-0.38)		0.70 (0.17-1.3)	0.12 (0-0.50)
kohlrabi		0.11			0.19
mustard seed, black	39-175				
mustard seed, brown	1-43	23-163	0-0.7		
red cabbage	0.12 (0.02-0.26)	0.20 (0.05-0.30)		0.10 (0.04-0.14)	0.16 (0.05-0.31)
rutabage / swede				0.63 (0.09-1.5)	
savoy cabbage	0.53 (0.01-1.6)			0.07 (0-0.30)	0.11 (0.15-2.8)
turnip tops		0.61 (0.01-2.9)	0.43 (0.01-1.5)	0.52 (0-1.0)	
water cress					
white cabbage	0.44 (0.04-1.6)	0.13		0.10	0.58 (0.05-2.8)

Glucosinolates in vegetables can be analysed by either ELISA or HPLC. The advantage of an Enzyme-Linked Immunosorbent Assay (ELISA) is its speed and convenient use. Disadvantages are that an ELISA has to be developed for each single component, that sensitivity is low and background noise is high. For sinigrin and progoitrin, two major glucosinolates in Brussels sprouts, ELISAs have been developed (*45*). Using High Performance Liquid Chromatography (HPLC), all glucosinolates can be determined in one single run. Specificity and reliability are high and it is a more discriminating technique since chromatography separates the compounds from other related materials that may cause assay interference. One vegetable generally contains two to five different glucosinolates in high content (table 2) but in all cruciferous plants together some hundred and twenty different glucosinolates exist.

Table 2. Continued

glucosinolate					
glucoerucin	glucoraphanin	glucoalyssin	gluconasturtiin	glucobrassicin	mmol/kg fresh weight (average (range))
	0.81 (0.29-1.9)			0.58 (0.23-1.0)	broccoli
	0.08 (0-0.23)			2.1 (0.45-4.7)	Brussels sprouts
	0.64 (0.02-1.9)			0.50 (0.14-1.6)	cauliflower
		0.13 (0.01-0.51)	0.15 (0.05-0.67)	0.41 (0.19-1.1)[a]	Chinese cabbage
				0.50 (0.44-0.70)	collards
			present (+ glucotropaeolin)		garden cress
			8.8-15		horseradish
				1.1 (0.67-1.7)	kale
				0.26[a]	kohlrabi
					mustard seed, black
					mustard seed, brown
0.02 (0.01-0.07)	0.55 (0.32-0.82)			0.73 (0.32-1.0)[a]	red cabbage
0.20 (0.01-0.89)		0.08 (0-0.22)	0.14 (0.01-1.0)	0.42 (0.14-1.1)[a]	rutabage / swede
				1.2 (0.7-2.0)	savoy cabbage
0.10 (0.01-0.29)			0.39 (0.02-1.2)	0.50 (0.12-1.1)[a]	turnip tops
			present		water cress
	0.1 (0-0.29)			0.5 (0.09-2.0)	white cabbage

[a] This figure is the total of all indole glucosinolates.

In bile collected from rats administered (ip) sulforaphane, five metabolites were identified. Besides GSH and NAC conjugates of sulforaphane, erucin conjugates were found pointing to S-oxide reduction. Dehydrogenation probably is the reaction responsible for the fifth, desaturated, derivative. Urinary metabolites collected during 24 h following administration of sulforaphane were the mercapturic acid of sulforaphane and of erucin, 60% and 12% of the dose respectively. When erucin was administered, the 24 h urinary metabolites consisted of 67% sulforaphane and 29% erucin mercapturic acid (*29*).

Since the metabolism in animals differs from humans, not all laboratory animals are suitable as experimental models for the biotransformation of isothiocyanates. From the mentioned experimental animals, the rat seems to be the only appropriate animal model.

Biomarkers of exposure to isothiocyanates

Valid methods to determine food intake in a population are being developed. Food frequency questionaires (FFQs) are not useful for measuring vegetable consumption since correlation with true intake is poor. On the other hand, FFQs turn out to be a reliable dietary assessment method to estimate alcohol intake. Where FFQs retrospectively cover a month of food intake, seven day diet recalls and 24-hour recalls cover a shorter period and are repeated to give adequate values for food intake. Biomarkers are more and more used to measure (vegetable) intake since they give an objective reflection of the intake of specific food ingredients. Excretion of isothiocyanate derived mercapturic acids reflects the amount of isothiocyanate absorbed and correspondingly the amount of cruciferous vegetables consumed by humans.

Effects of isothiocyanates

Isothiocyanates are inhibitors of some phase I enzymes and inducers of phase II enzymes and are therefore thought to be cancer chemopreventors (*30*). Indoles on the other hand are inducers of phase I enzymes but are present at much lower concentrations than isothiocyanates. A metabolite formed in the stomach especialy after consumption of raw vegetables is 3,3'-diindolylmethane (DIM). DIM is formed from indoles and has multiple cell suppressive effects on human breast cancer cell growth (*31*). Human intervention trials with large quantities of cruciferous vegetables gave similar effects on phase I and phase II enzymes (*32, 33*). Isothiocyanates are electrophilic compounds but are not direct antioxidants. In recent studies, isothiocyanates and their conjugates have been reported to

inhibit the cell cycle and cause apoptotic cell death. Sulforaphane, 4-methylsulfinylbutyl isothiocyanate, which occurs as glucoraphanin in broccoli and in other vegetables (see **figure 1**), was reported to be an extremely potent inducer of phase II enzymes (*34, 35*). Phenethyl isothiocyanate is derived from gluconasturtiin, the major glucosinolate in water cress. Consumption of water cress for three days increased the urinary excretion of metabolites of the tobacco specific nitrosamine NNK in smokers (*33*). The phase II enzyme inducing potencies of two other compounds of water cress, 7-methylsulfinylheptyl and 8-methylsulfinyloctyl isothiocyanate, were similar to that reported for sulforaphane (*36*) and greater than that for phenethyl isothiocyanate (*37*). Besides positive effects, isothiocyanates could cause negative effects as well. Like Paracelsus stated: 'Sola dosis facit venonum', only the dose makes a toxic, it is therefore essential to also determine the maximal safe intake of isothiocyanates.

Biomarkers of effect

A promising approach to strengthen epidemiological studies is the use of biomarkers. A biomarker can be defined as an indicator on a biochemical, genetic or cellular level, reflecting exposure to a compound, susceptibility for a disease or the health status of a subject. In the physiological evaluation of a biomarker, it is important that there is a well-established relationship between the response of the biomarker and the effect monitored. In the causal pathway of disease occurrence one might distinguish biomarkers of exposure (dietary intake), biomarkers of biological response and of (subclinical) disease and biomarkers of susceptibility. For example, in relation to the consumption of cruciferous vegetables that contain glucosinolates, isothiocyanates are ingested and subsequently excreted as the corresponding mercapturic acids. The isothiocyanate mercapturic acids then are a biomarker of exposure to cruciferous vegetables. A biomarker is always an objective measure as opposed to questionnaires which are subjective. The major advantage of a biomarker is its relative quick response since it may take many years for a disease to become apparent. Another advantage of a marker is its accessibility in clinical samples.

Glutathione acts as a transporter of isothiocyanates since the conjugation is reversible and isothiocyanates can be transfered from glutathione to another endogenous nucleophile (*38*).

The enzyme GST consists of multiple isoenzymes that have been catalogued under four classes of cytosolic enzymes, referred to as alpha, mu, pi, and theta, and a class of microsomal enzymes. GST-isoenzymes might be expressed to a very different extent in different tissues. In human liver, alpha class isoenzymes represent more than 80% of total GSTs (*39*). GST-α can be measured in plasma and is an accurate index for induction of GST in the liver when there is no hepatocellular damage. Unlike GST-α, which is found in all human livers, the major class mu isoenzyme is found only in about half of the adult caucasian population. This enzyme is encoded at the GSTM1 gene locus. Individuals who are homozygous GSTM1-0, who lack the GSTM1-enzyme, may be more sensitive to certain genotoxic and carcinogenic compounds. A second polymorphism of GST-activity is found at the GSTT1 gene where in 25 to 40% of the human population the GSTT1-enzyme is absent.

In a study of Lin et al. (*40*), the protective effect of broccoli on the prevalence of colorectal adenomas was observed only among subjects with the GSTM1-null phenotype. It was proposed that GSTM1-null individuals would excrete isothiocyanates more slowly because of insufficient activities of GSTs, but in this study there was no significant difference in urinary excretion of isothiocyanates between GSTM1-null and GSTM1-positive subjects (*22*). Among GSTT1-positive individuals, however, urinary excretion of isothiocyanates was significantly higher than among GSTT1-null subjects. In a recent study (*41*), the protective effect of dietary isothiocyanates was more pronounced in persons with the homozygous GSTM1-null genotype and was particularly strong in subjects with deletion of both GSTM1 and GSTT1. It seams that the reduced rate of excretion of isothiocyanates in persons lacking the specific genotypes for GSTs that conjugate isothiocyanates may result in higher levels of isothiocyanates in the body, thus reducing the incidence of lung cancer.

The supposed protective effect of broccoli might act by up-regulation of transcription of certain specific genes, specifically the earlier mentioned GST-isoenzymes. For example, glutathione can react directly with reactive oxygen species via a sensitive SH-group. Glutathione can also be inactivated by disulphide formation, which can be reversed by GST-α. Thus, an up-regulation of GST-α may lead to a reduced damage of reactive oxygen species and increased levels of active glutathione. As a consequence thereof, biomarkers related to oxidative damage and measured in urine, e.g. 8-oxodG (marker of oxidative DNA damage), and F2-isoprostanes (marker of oxidative lipid damage) may be affected by

consumption of cruciferous vegetables. Verhagen et al. (*42*) showed in a clinical trial that a simple vegetable like Brussels sprouts increases the detoxification of xenobiotics by GST induction. The same study showed that oxidative damage of DNA was reduced.

Biotransformation enzymes appear to be selectively induced by the activation of an electrophile responsive element (EpRE or ARE) located in the 5'-upstream region of many phase II enzymes (*43*). A transcription factor involved in this activation is Nrf2, which together with other transcription factors binds to the EpRE and activates phase II gene transcription. Nrf2 gene knock-out mice exposed to a carcinogen developed more tumors than did wild-type controls. Tumor multiplicity was reduced in wild-type mice but not in knock-out mice when a phase II inducer was administered. This proves the critical role of phase II enzymes in the susceptibility to carcinogens and the importance of the EpRE.

Recommendations for food preparation

Boiling vegetables, in large amounts of water, results in the loss of almost all glucosinolates. In contrast, microwave cooking, and frying improve not only the taste but also the preservation of glucosinolates in the vegetables. Cruciferous vegetables should, from a health point of view, preferable be eaten raw. This greatly enhances the conversion of glucosinolates into bioactive isothiocyanates, which protect in vivo by GST induction against electrophiles and free radicals.

Objective of this thesis

A healthy diet contains all necessary macro and micro nutrients; carbohydrates, proteins, fats, vitamins and minerals. Additionally, each foodstuff contains its own characteristic non-nutrients profile as well, i.e. compounds which are not essential but are nonetheless bioactive. Several classes of non-nutrients exist but clear dose-response relations have not been established. Two examples are flavonoids, antioxidants present in vegetables, fruits, tea and red wine, and glucosinolates, precursors for isothiocyanates present in cabbages. The major difference between these two is that isothiocyanates are not direct antioxidants like flavonoids. Isothiocyanates induce biotransformation enzymes and thereby boost the bodies' natural antioxidant response. There are many structurally different glucosinolates present in the diet, and therefore the question arises which of these glucosinolates yield the most active isothiocyanate, in the highest amount per gram vegetable.

Also, how much of this vegetable should be consumed for a safe but health promoting effect, and is it possible to discriminate between different glucosinolate containing vegetables by measuring metabolites in urine. The goal of this thesis is to obtain a biomarker to determine cruciferous vegetable intake and to establish the association between cruciferous vegetable intake, and isothiocyanate uptake, excretion and effect.

To obtain a biomarker for isothiocyanate uptake, mercapturic acids of the most important isothiocyanates were chemically synthesised as reference compounds. In up to four synthesis steps commercially not available isothiocyanates were prepared and in a fifth step conjugated to N-acetyl-*L*-cysteine (NAC) yielding the mercapturic acid, methods of synthesis are described in **chapter 2**. These compounds were used to achieve separation on high-performance liquid chromatography. At first, ultraviolet detection was used but this turned out to lack enough sensitivity and specificity for the analysis of low levels of isothiocyanate mercapturic acids in urine. Ion-trap mass spectrometry was therefore used as described in **chapter 3**. Using the TNO *in vitro* gastro-intestinal model (TIM 2) the bioavailability and bioconversion of sinigrin and allyl isothiocyanate (from Brussels sprouts) in the large intestine was determined (not described in this thesis, *8*). To determine bioavailability and excretion kinetics of glucosinolates, and its derived isothiocyanates in humans, 3 volunteers consumed 19 different raw and cooked vegetables and condiments. Results of this study are described in **chapter 4**.

Isothiocyanates are indirect antioxidants and induce phase II metabolism enzymes via an electrophile responsive element (EpRE) which is present in the promotor region of the genes coding for these enzymes. The induction factor differs between the various isothiocyanates, induction of gene expression via an EpRE is described in **chapter 5**. Absorption and excretion kinetics and the effect on biomarkers of glucoraphanin derived sulforaphane, from raw and cooked broccoli, was studied in a clinical trial with smoking men. The kinetic data of isothiocyanates in blood and urine and the effect on GST-α, iPF2-α, and 8-oxodG are described in **chapter 6**. Finally, the results are summarised and discussed in **chapter 7**.

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Chapter 2

Synthesis of Isothiocyanate-derived Mercapturic Acids

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ABSTRACT

Twelve mercapturic acids derived from saturated and unsaturated aliphatic and aromatic isothiocyanates were synthesised, by adding isothiocyanate to a solution of *N*-acetyl-*L*-cysteine and sodium bicarbonate, in a typical yield of 77%. Isothiocyanates were synthesised first by adding the corresponding alkyl bromide to phthalimide potassium salt. The obtained *N*-alkyl-phthalimide was hydrazinolysed yielding the alkyl amine, which subsequently was reacted with thiophosgene yielding the isothiocyanate with an overal yield of 16%. Mercapturic acids in urine can serve as a biomarker of intake to determine the health promoting potential of isothiocyanates present in cruciferous vegetables.

INTRODUCTION

Consumption of fruits and vegetables is associated with a reduced risk on degenerative diseases such as cancer and cardiovascular diseases, as indicated by epidemiological studies (*1, 2*). A reasonable estimate of the overall extent to which dietary modification may be expected to reduce cancer risk is 30-40% (*3*). In particular cruciferous vegetables appear to have beneficial health potential (*4, 5*). Since cruciferous vegetables differ from other vegetables by the presence of glucosinolates, these health promoting effects seem to be attributable to these phytochemicals or breakdown products thereof (*6*). Glucosinolates are broken down into indoles, nitriles and isothiocyanates (**Figure 1**) by a thioglucosidase (myrosinase, EC 3.2.3.1) present in cruciferous vegetables (*7, 8*) and to a lesser degree by microbes present in the human gut (*9, 10*). Isothiocyanates are strong inhibitors of phase I enzymes and inducers of phase II enzymes, and therefore are thought to be strong cancer chemopreventors (*11-13*). Many different isothiocyanates (more than 25) block the carcinogenic effects of more than 12 chemically different types of carcinogens in at least 10 different target sites in three species of rodents (*14*). Phenethyl isothiocyanate is a particularly effective inhibitor of lung tumor induction by the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and, therefore, is currently being developed as a chemopreventive agent against lung cancer (*14*). Human intervention trials with large quantities of cruciferous vegetables gave similar effects on phase I and phase II enzymes (*15, 16*).

Figure 1. Isothiocyanates are enzymatically hydrolysed from glucosinolates, in the body conjugated to glutathione and excreted as mercapturic acids in the urine.

Isothiocyanates are conjugated to glutathione in the body and excreted into the urine as their corresponding mercapturic acids (**Figure 1**) as was demonstrated in rats (*17*), guinea pigs and rabbits (*18*) and in humans (*19, 20*). Mercapturic acids reflect the intake of glucosinolates present in cruciferous vegetables (*21-23*) and could be used as a selective biomarker for cruciferous vegetable intake. Different cruciferous vegetables can botanically be differentiated by the variety and amount of glucosinolates (*24*). Broccoli, for instance, is rich in 4-methylthiobutyl glucosinolate, whereas Brussels sprouts are rich in 2-propenyl and 2-hydroxy-3-butenyl glucosinolate (progoitrin), and cress is rich in aromatic glucosinolates (*7*).

Several reports describing the synthesis of isothiocyanates and the corresponding mercapturic acids are available (**Table 1**, comments in the discussion section). We here describe the synthesis of 12 mercapturic acids derived from saturated and unsaturated aliphatic and aromatic isothiocyanates, namely methyl, butyl, isopropyl, 1-methylpropyl, 2-propenyl, 3-butenyl, 4-pentenyl, phenyl, benzyl, phenylethyl, 4-methylthiobutyl and 4-methylsulfinylbutyl isothiocyanate (**Figure 2**). The HPLC-MS/MS analysis of isothiocyanate derived mercapturic acids in urine is described in Ref. (*41*).

CHEMISTRY

In cruciferous plants glucosinolates are formed from amino acids (*25*). Tissue disruption by, e.g. chewing starts the breakdown of glucosinolates into isothiocyanates and this provides protection from plants towards insects as isothiocyanates are pungent metabolites. In humans, chewing of Brussels sprouts releases 39% of the glucosinolates as isothiocyantes, measured in the urine, whereas no chewing results in the excretion of 26% of isothiocyanates (*26*). Ingestion of pure benzyl isothiocyanate, introduced as a drug for the treatment of infections of the respiratory and urinary tract under the trade-mark Tromacaps®, resulted in 43-60% excretion of the compound in the urine as its mercapturic acid (*20*). Sulforaphane is the bioactive compound found in broccoli (*27*). This synthesis is described in **Scheme 1**.

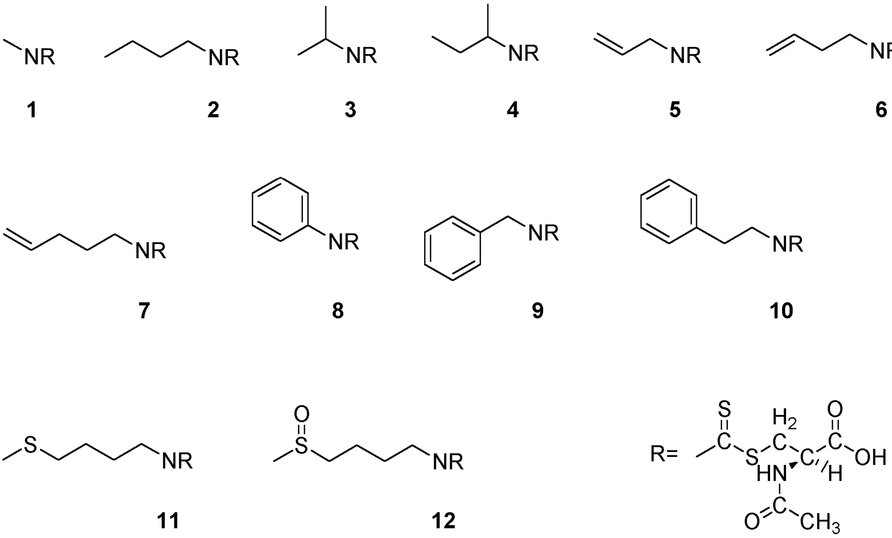

Figure 2. Structures of twelve synthesised mercapturic acids derived from isothiocyanate.

DMF 0 °C
± 72%
$NaSCH_3$ DMF 0 °C
± 85%
$N_2H_4.H_2O$ / HCl
ethanol reflux
± 45%
$CSCl_2$ chloroform / NaOH
0 °C to rt
± 80%
MCPBA
DCM 0 °C
±90%
N-acetyl-L-cysteine
$NaHCO_3$
50% aqeous ethanol rt
± 77%

Scheme 1. Synthesis of N-acetyl-S-(N-4-methylsulfinylbutylthiocarbamoyl)-L-cysteine (sulforaphane mercapturic acid).

RESULTS AND DISCUSSION

Twelve isothiocyanate mercapturic acids have been synthesised (**Figure 2, Table 1**). The synthesis of isothiocyanates from phthalimide is a multistep reaction with an overal yield of 16%. The synthesis from *N*-acetyl-*L*-cysteine (NAC) and isothiocyanate was a convenient one step reaction which proceeds in a typical yield of 77%. Before adding isothiocyanate to NAC, we converted NAC to its sodium salt using sodium bicarbonate, thus enhancing the reaction rate. After the reaction was complete, the mercapturic salt was converted into the poorly water-soluble mercapturic acid using hydrochloric acid. Several solvents were tested for recrystallisation. The products dissolved best in boiling ethyl acetate which allowed crystallisation on cooling. The mercapturic acids derived from isopropyl and of 1-methylpropyl isothiocyanate were obtained in a yield of 50%. The solubility of these products in ethyl acetate is perhaps higher than, for instance, the mercapturic acid derived from benzyl isothiocyanate. A better solvent for recrystallisation could not be found.

The synthesis of 3-butenyl isothiocyanate has previously been described by Kjær et al. (*28*) starting from allyl cyanide. The boiling point observed by us and that reported (*28*) are the same (60 °C at 12 mmHg). Ettlinger and Hodgkins (*29*) started from allylcarbinol and reported the boiling point to be 77.5 °C at 28 mmHg. Leoni (*30*) obtained 3-butenyl isothiocyanate by enzymatic hydrolysis of gluconapin. Our NMR data are identical with that reported. 4-Pentenyl isothiocyanate has been synthesised previously by Kjær and Jensen (*31*) and in the same way by Gilbert and Nursten (*32*). Only the boiling point, density and elemental analysis were mentioned. 4-Methylthiobutyl isothiocyanate (trivial name erucin) has first been synthesised by Schmid and Karrer (*33*) and later by Kjær and Gmelin (*34*). Our boiling point and NMR data are identical with those reported. 4-Methylsulfinylbutyl isothiocyanate (trivial name sulforaphane) has first been synthesised by Schmid and Karrer (*33*) who also described the synthesis of optically pure *L*- and *D*-sulforaphane. Sulforaphane was later synthesised by Zhang et al. (*27*) and Kuhnert et al. (*35)* (conversion of the amine into the isothiocyanate unit preceeds the oxidation of sulfur). The physical and spectral data are identical. All authors who published their results between 1948 and 1972, used pyridine and benzene. These toxic compounds can be replaced by aqueous ethanol and heptane, respectively. The presence in nature and isolation from plants of isothiocyanates was summarised by Kjær (*7*).

Table 1. Trivial names of isothiocyanate yielding glucosinolates, structures of the corresponding isothiocyanates, (main) dietary source and literature references to isothiocyanate and mercapturic acid synthesis

glucosinolate	isothiocyanate	(main) dietary source	[a]	previous isothiocyanate synthesis by others[b]	previous isothiocyanate mercapturic acid synthesis by others[b]
glucocapparin	H_3CNCS	cauliflower, capers	1	commercially available	(*36*)
unknown	$H_3C(CH_2)_3NCS$	cabbage, horse radish	2	commercially available	(*36*)
glucoputranjivin	$H_3CCH(CH_3)NCS$	Brussels sprouts, turnip	3	commercially available	not yet reported
glucocochlearin	$H_3CCH_2CH(CH_3)NCS$	black mustard	4	commercially available[c]	not yet reported
sinigrin	$H_2C=CHCH_2NCS$	cabbage, brown mustard	5	commercially available	(*36, 37*)
gluconapin	$H_2C=CH(CH_2)_2NCS$	Chinese cabbage	6	(28, 29)	not yet reported
glucobrassicanapin	$H_2C=CH(CH_2)_3NCS$	Chinese cabbage	7	(31, 32)	not yet reported
unknown	C_6H_5NCS	ambiguous[d]	8	commercially available	not yet reported
glucotropaeolin	$C_6H_5CH_2NCS$	garden cress	9	commercially available	(*17*)
gluconasturtiin	$C_6H_5(CH_2)_2NCS$	water cress	10	commercially available	(*38, 39*)
glucoerucin	$H_3CS(CH_2)_4NCS$	broccoli, rocket	11	(27, 33, 34)	(*40*)
glucoraphanin	$H_3CS(=O)(CH_2)_4NCS$	cabbage	12	(27, 33, 35)	(*40*)

[a] Numbers refer to structures in figure 2. [b] For comments, see Discussion section. [c] Not optically pure. [d] Presence in nature is unlikely.

Mennicke et al. (*36*) described the synthesis of **1**, **2** and **5**. Physical data were only given for the dicyclohexylamine salt. Ioannou et al. (*37*) (only brief description of the synthesis) described the isolation of **5** from rat urine and the synthesis of ^{14}C radio-labeled **5** using column chromatography to obtain the pure compound. All NMR data are comparable with ours. Brüsewitz et al. (*17*) described the synthesis of benzyl isothiocyanate conjugates. They reported an m.p. of 58-62 °C for **9**, which is very different from ours (136.12 °C). Since optical rotation, elemental analysis and NMR data are in agreement with ours, the product obtained by Brüsewitz is probably contaminated by some solvent which lowers the m.p. Toxic solvents can be replaced by non-toxic. Eklind et al. (*38*) synthesised **10**, by adding the isothiocyanate to a solution of *N*-acetyl-*L*-cysteine in methanol in poor yield (39%). The NMR data are the same as ours. Jiao et al. (*39*) used the method of synthesis described by Brüsewitz, however, their NMR data for the obtained product (**10**) differ from those reported by Eklind and found by us. Kassahun et al. (*40*) prepared **11** and **12** by adding the isothiocyanate to an aqueous ethanol solution of *N*-acetyl-*L*-cysteine at basic pH. These products were purified by column chromatography, the NMR data (in $D_2O/CDCl_3$) are in agreement with ours.

In our synthesis of **11** and **12**, phthalimide potassium salt was added to three equivalents of 1,4-dibromobutane to minimise the formation of 1,4-diphthalimidylbutane. A top piece containing potassium hydroxide was used to avoid moisture and to prevent the reaction of sodium methylmercaptide with carbon dioxide to give methylmercaptan. We compared triethylamine, sodium hydroxide and sodium bicarbonate as the basic reagent in the formation of isothiocyanate from the amine and thiophosgene. Three equivalents of the base were used, after which chloroform and excess of thiophosgene were evaporated and the residue was poored in water and diethyl ether. This resulted in black solids when triethylamine was used. Because of gas forming after adding sodium bicarbonate, we preferred the use of sodium hydroxide as a base. Distillation of the isothiocyanate formed was performed immediately after evaporating chloroform and thiophosgene in order to prevent residues of thiophosgene to react with the isothiocyanate. 4-Methylsulfinylbutyl isothiocyanate was obtained by oxidation of 4-methylthiobutyl isothiocyanate. 4-Methylsulfinylbutylamine could be obtained by oxidising *N*-(4-methylthiobutyl)-phthalimide with MCPBA, followed by hydrazinolysis. Subsequent reaction of the amine with thiophosgene led to complete reduction of the sulfinyl group yielding 4-methylthiobutylamine rendering this route of synthesis impossible. This deoxygenation in the absence of a reducing agent has been observed also by Kuhnert et al. (*35*) and cannot be explained.

Thiophosgene used to convert amines to isothiocyanates can be replaced by di-2-pyridyl thionocarbonate which is not toxic and easier to handle. We were unable to derive optimal conditions for this conversion using 1,1'-thiocarbonyl diimidazole.

Product **7** has an brownish-orange color, while all other mercapturic acids are colored yellow to white. The reaction mixture containing 4-pentenyl isothiocyanate and NAC also contained some black residue which may be due to side products resulting from thiophosgene.

Isothiocyanate-derived mercapturic acids and its precursors can be separated using TLC. Isothiocyanates and their mercapturic acids are UV 254 nm active, whereas NAC is not UV active. Potassium dichromate followed by heat colors all products and precursors and has the advantage that it colors NAC orange before heat treatment. In ethyl acetate-water-formic acid (60:35:18, v/v/v) all components showed only minor retention, NAC R_F=0.78, phenyl mercapturic acid R_F=0.90 and phenyl isothiocyanate R_F=0.99. Adding just enough acetic acid (8 mL) to mix 80 mL of ethyl acetate with 10 mL of water gave a satisfactory retention and separation of the components.

The melting of the mercapturic acid derived from phenyl isothiocyanate resulted in the explosion of a DSC sample cell. This is probably due to an exothermal reaction with formation of gas following the melt. Melting of NAC also resulted in the formation of gas but the reaction products could not be analysed.

CONCLUSIONS

In conclusion, we have synthesised 12 mercapturic acids derived from isothiocyanates in good yield. These 12 compounds were prepared because they probably represent the most important urinary excretion products after eating a cruciferous vegetable meal. Phenyl glucosinolate is not a natural compound so the mercapturic acid derived from phenyl isothiocyanate can be used as internal standard for the analysis of mercapturic acids obtained from isothiocyanates. Future research will include the development of a liquid chromatographic method to analyse mercapturic acids in urine and validation of mercapturic acids as biomarkers to measure the effective dose of isothiocyanates absorbed.

Table 2. Chemical and physical data of the synthesised isothiocyanate derived mercapturic acids

No. [a]	m.p. (°C) [b]	optical rotation	elemental analysis calculated / found	mass	UV absorbance (nm)
1	144-146	-35.0°	$C_7H_{12}N_2O_3S_2$ C 35.58, H 5.12, N 11.85, S 27.14 C 35.82, H 5.01, N 11.70, S 27.29	FW 236.316 exact 236.0 MS 237.0	267 (1st max) 249 (2nd max)
2	rt	-19.2°	$C_{10}H_{18}N_2O_3S_2$ C 43.14, H 6.52, N 10.06, S 23.04 C 43.61, H 6.81, N 9.67, S 21.64	FW 278.397 exact 278.1 MS 279.0	251 (1st max) 270 (2nd max)
3	165-169	-17.6°	$C_9H_{16}N_2O_3S_2$ C 40.89, H 6.10, N 10.60, S 24.26 C 40.85, H 6.12, N 10.40, S 24.01	FW 264.370 exact 264.1 MS 265.0	253 (1st max) 270 (2nd max)
4	140-144	-14.2°	$C_{10}H_{18}N_2O_3S_2$ C 43.14, H 6.52, N 10.06, S 23.04 C 43.13, H 6.63, N 10.09, S 22.66	FW 278.397 exact 278.1 MS 279.0	253 (1st max) 270 (2nd max)
5	rt	-21.7°	$C_9H_{14}N_2O_3S_2$ C 41.20, H 5.38, N 10.68, S 24.44 C 40.53, H 5.47, N 10.26, S 23.40	FW 262.354 exact 262.1 MS 263.0	251 (1st max) 269 (2nd max)
6	rt	-21.8°	$C_{10}H_{16}N_2O_3S_2$ C 43.46, H 5.84, N 10.14, S 23.20 C 42.95, H 5.89, N 9.94, S 22.19	FW 276.381 exact 276.1 MS 277.0	251 (1st max) 269 (2nd max)
7	rt	missing	$C_{11}H_{18}N_2O_3S_2$ missing	FW 290.41 exact 290.1 MS 291.0	251 (1st max) 269 (2nd max)
8	176-178 (176.50)	-16.7°	$C_{12}H_{14}N_2O_3S_2$ C 48.30, H 4.73, N 9.39, S 21.49 C 48.36, H 4.72, N 9.29, S 20.77	FW 298.387 exact 298.1 MS 299.0	277
9	133-135 (136.12)	-15.8°	$C_{13}H_{16}N_2O_3S_2$ C 49.98, H 5.16, N 8.97, S 20.53 C 50.10, H 5.37, N 9.06, S 20.50	FW 312.414 exact 312.1 MS 313.0	251 (1st max) 270 (2nd max)
10	50-58	-14.9°	$C_{14}H_{18}N_2O_3S_2$ C 51.51, H 5.56, N 8.58, S 19.64 C 50.92, H 5.67, N 8.46, S 18.82	FW 326.441 exact 326.1 MS 327.0	253 (1st max) 270 (2nd max)
11	rt	-17.6°	$C_{11}H_{20}N_2O_3S_3$ C 40.72, H 6.21, N 8.63, S 29.65 C 40.04, H 6.07, N 8.34, S 28.34	FW 324.490 exact 324.1 MS 325.0	251 (1st max) 269 (2nd max)
12	60-62	missing	$C_{11}H_{20}N_2O_4S_3$ missing	FW 340.486 exact 340.1 MS 341.0	252 (1st max) 269 (2nd max)

[a] Numbers refer to compounds as depicted in **figure 2**, methods of analysis are described in the chemistry section. [b] melting point , rt = room temperature, indicating a non-crystalline product.

Table 2. Continued.

No.	^{1}H-NMR (δ in ppm, J in Hz)
1	(300 MHz) 1.82 (s, 3H, $C(O)CH_3$), 3.00 (d, 3H, J=4.4, NCH_3), 3.30 (dd, 1H, J_{AB}=13.6, J_{BX}=9.2, CH_B), 3.75 (dd, 1H, J_{AX}=5.0, CH_A), 4.38 (m, 1H, CH_X), 8.30 (d, 1H, J=8.1, OCNH), 9.96 (t, 1H, SCNH), 11.87 (s, 1H, OH)
2	(100 MHz) 0.86 (t, 3H, $NCCCCH_3$), 1.23 (m, 2H, $NCCCH_2$), 1.45 (m, 2H, $NCCH_2$), 1.82 (s, 3H, $C(O)CH_3$), 3.27 (dd, 1H, J_{AB}=13.6, J_{BX}=9.2, CH_B), 3.54 (t, 2H, NCH_2), 3.76 (dd, 1H, J_{AX}=5.1, CH_A), 4.37 (m, 1H, CH_X), 8.32 (d, 1H, J=8.0, OCNH), 10.00 (t, 1H, SCNH)
3	(100 MHz) 1.13 (d, 6H, J=6.5, $C(CH_3)CH_3$), 1.81 (s, 3H, $C(O)CH_3$), 3.26 (m, 1H, CH_B), 3.75 (dd, 1H, J_{AB}=13.6, J_{AX}=5.0, CH_A), 4.41 (m, 2H, CH_X and CH(C)C), 8.31 (d, 1H, J=7.9, OCNH), 9.88 (d, 1H, J=7.3, SCNH), 12.83 (s, 1H, OH)
4	(100 MHz) 0.86 (t, 3H, $C(C)CCH_3$), 1.15 (d, 3H, J=6.6, $C(CH_3)CC$), 1.52 (dd, 2H, $C(C)CH_2C$), 1.87 (s, 3H, $C(O)CH_3$), 3.34 (dd, 1H, J_{AB}=13.3, J_{BX}=9.1 CH_B), 3.80 (dd, 1H, J_{AX}=5.1, CH_A), 4.41 (m, 2H, CH_X and CH(C)CC), 8.36 (d, 1H, J=8.0, OCNH), 9.89 (d, 1H, J=7.7, SCNH), 12.87 (s, 1H, OH)
5	(100 MHz) 1.82 (s, 3H, $C(O)CH_3$), 3.29 (dd, 1H, J_{AB}=13.6, J_{BX}=9.2, CH_B), 3.77 (dd, 1H, J_{AX}=5.0, CH_A), 4.20 (dd, 2H, NCH_2), 4.39 (m, 1H, CH_X), 5.14 (dd, 2H, CH_2=C), 5.84 (m, 1H, C=CH), 8.32 (d, 1H, J=8.1, OCNH), 10.17 (t, 1H, SCNH), 12.85 (s, 1H, OH)
6	(100 MHz) 1.82 (s, 3H, $C(O)CH_3$), 2.31 (m, 2H, $NCCH_2$), 3.28 (dd, 1H, J_{AB}=13.6, J_{BX}=9.2, CH_B), 3.61 (m, 2H, NCH_2), 3.75 (dd, 1H, J_{AX}=5.1, CH_A), 4.38 (m, 1H, CH_X), 5.05 (dd, 2H, CH_2=C), 5.78 (m, 1H, C=CH), 8.31 (d, 1H, J=8.0, OCNH), 10.03 (t, 1H, SCNH), 12.84 (s, 1H, OH)
7	(100 MHz) 1.66(m,2H,$NCCH_2$), 1.82(s,3H,$C(O)CH_3$), 2.03(m,2H,$NCCCH_2$), 3.28(dd,1H,J_{AB}=13.6, J_{BX}=9.2,CH_B), 3.54 (m, 2H, NCH_2), 3.76 (dd, 1H, J_{AX}=5.0, CH_A), 4.37 (m, 1H, CH_X), 5.01(dd,2H, CH_2=C), 5.77(m,1H,C=CH), 8.32(d,1H,J=8.1,OCNH), 10.02 (t, 1H, SCNH), 12.85 (s, 1H, OH)
8	(400 MHz) 1.83 (s, 3H, CH_3), 3.28 (dd, 1H, J_{AB}=13.7, J_{BX}=10.0, CH_B), 3.81 (dd, 1H, J_{AX}=4.8, CH_A), 4.46 (m, 1H, CH_X), 7.22 (m, 1H, aromatic), 7.40 (m, 2H, aromatic), 7.70 (m, 2H, aromatic), 8.39 (d, 1H, J=8.0, OCNH), 11.70 (s, 1H, SCNH), 12.90 (s, 1H, OH)
9	(100 MHz) 1.82 (s, 3H, CH_3), 3.32 (dd, 1H, J_{AB}=13.7, J_{BX}=10.0, CH_B), 3.79 (dd, 1H, J_{AX}=4.8, CH_A), 4.41 (m, 1H, CH_X), 4.82 (d, 2H, J=5.3, NCH_2), 7.29 (m, 5H, aromatic), 8.33 (d, 1H, J=8.0, OCNH), 10.50 (t, 1H, SCNH), 12.85 (s, 1H, OH)
10	(300 MHz) 1.83 (s, 3H, CH_3), 2.88 (t, 2H, J=7.6, ØCH_2), 3.30 (dd, 1H, J_{AB}=13.6, J_{BX}=9.0, CH_B), 3.75 (m, 1H, J_{AX}=5.0 CH_A), 3.75 (m, 2H, NCH_2), 4.39 (m, 1H, CH_X), 7.25 (m, 5H, aromatic), 8.28 (d, 1H, J=8.0, OCNH), 10.17 (t, 1H, SCNH), 12.86 (s, 1H, OH)
11	(100 MHz) 1.57 (m, 4H, $NCCH_2$ and $NCCCH_2$), 1.82 (s, 3H, $C(O)CH_3$), 2.01 (s, 3H, SCH_3), 2.46 (t, 2H, $NCCCCH_2S$), 3.28 (dd, 1H, J_{AB}=13.6, J_{BX}=9.2, CH_B), 3.56 (t, 2H, NCH_2), 3.76 (dd, 1H, J_{AX}=5.1, CH_A), 4.37 (m, 1H, CH_X), 8.31 (d, 1H, J=8.1, OCNH), 10.02 (t, 1H, SCNH),12.8 (s, 1H, OH)
12	(600 MHz) 1.66 (m, 4H, $NCCH_2CH_2$), 1.82 (s, 3H, CH_3CO), 2.50 (s, 3H, CH_3SO), 2.70 (m, 2H, CCH_2SO), 3.28 (dd, 1H, J_{AB}=13.2, J_{BX}=8.1, CH_B), 3.58 (wide m, 2H, NCH_2), 3.68 (dd, 1H, J_{AX}=4.8, CH_A), 4.30 (m, 1H, CH_X), 8.15 (d,1H,J=7.2,OCNH), 10.34(wide s,1H,SCNH), 12.86(wide s,1H,OH)

EXPERIMENTAL PROTOCOLS

Methyl, 2-propenyl, phenyl, benzyl and phenylethyl isothiocyanate as well as *N*-acetyl-*L*-cysteine (NAC), 1,4-dibromobutane, 4-bromo-1-butene and 5-bromo-1-pentene were purchased from Acros Organics. Butyl isothiocyanate, iso-propyl isothiocyanate and sodium methylmercaptide were purchased from Sigma-Aldrich Co., 1-methylpropyl isothiocyanate was obtained from Maybridge Chemical Company Ltd. and phthalimide potassium salt was purchased from Merck. Solvents were dried using the following methods: Dichloromethane was distilled from P_2O_5. Diethyl ether was distilled from NaH. Hexane and heptane were distilled from CaH_2. All other chemicals were of analytical grade. All chemical and physical data are mentioned in **Table 2**.

^{1}H-NMR (100 MHz) spectra were recorded on a Bruker AC 100 spectrometer, 300 MHz ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AC 300 spectrometer, 400 MHz ^{1}H-NMR spectra were recorded on a Varian Unity-400 spectrometer, and 600 MHz ^{1}H-NMR spectra were recorded on a Bruker Avance spectrometer with DMSO-d_6 as internal standard unless stated otherwise (Aldrich, δ = 2.49, and 39.7 ppm respectively, *J* in Hz). Optical rotation (OR) was measured on a Perkin-Elmer 241 Polarimeter at 589.3 nm, products were dissolved in methanol (10 mg mL^{-1}), measured three times and the average was taken. Melting points (m.p.) were determined with a Reichert Thermopan microscope and figures between brackets on a differential scanning calorimeter (DSC-2920, TA Instruments), both are uncorrected. Elemental analyses (EA) were performed on a Carlo Erba instruments CHNS-O 1108 elemental analyser at the Department of Microanalysis of the University of Nijmegen. The formula weight (FW) is the average molecular weight. The exact molecular mass was calculated using the atomic masses of the most abundant isotopes. Mass spectra (MS) were collected on a Finigan MAT LCQ mass spectrometer coupled to a Waters 2690 liquid chromatograph (HPLC). All isothiocyanate-derived mercapturic acids were separated on a C18 column using a gradient of acetonitrile in water (both with 0.1% formic acid) and detected using an LCQ ion-trap MS with electrospray ionisation (positive mode). Mercapturic acids were detected as their $[M + H]^+$ ion which is the MS data mentioned in the syntheses (*41*). Ultra violet (UV) spectra were collected on a Waters 996 Diode-array detector (DAD) after chromatographic separation as described for MS. Thin-layer chromatography (TLC) was carried out on Merck precoated silica gel 60 F254 plates (0.25 mm) using the eluents indicated. Spots were visualised with UV and using a potassium dichromate spray. Potassium dichromate colors NAC orange, but not its isothiocyanate mercapturic acid, the latter is made visible by heating the TLC plate with hot air.

General procedure for commercially available isothiocyanates. Compounds **1**, **2**, **3**, **4**, **5**, **8**, **9**, and **10** were synthesised by gradually adding the diluted isothyocianate to a solution of *N*-acetyl-*L*-cysteine and sodium bicarbonate. The reaction was monitored by TLC with ethyl acetate-water-acetic acid (80:10:8, v/v/v) as eluent.

*N-acetyl-S-(N-methylthiocarbamoyl)-L-cysteine (**1**), mercapturic acid derived from methyl isothiocyanate*. Methyl isothiocyanate (16.5 mmol) was dissolved in 20 mL of ethanol and gradually added to a solution of N-acetyl-L-cysteine (NAC, 15.0 mmol) and sodium bicarbonate (15.8 mmol) in 20

20 mL of water. TLC: NAC R_F=0.48, **1** R_F=0.43. All NAC had reacted immediately after methyl isothiocyanate was added. Using a Dowex 50X8-200 cation exchange column the sodium salt of the mercapturic acid was converted to the free carboxylic acid. The product was crystallised from ethyl acetate yielding 11.0 mmol (73.3%) of **1** as bright white crystals.

*N-acetyl-S-(N-butylthiocarbamoyl)-L-cysteine (**2**), mercapturic acid derived from butyl isothiocyanate*. Butyl isothiocyanate (14.0 mmol) was dissolved in 15 mL of ethanol and gradually added to a solution of NAC (12.8 mmol) and sodium bicarbonate (13.4 mmol) in 15 mL of water. TLC: NAC R_F=0.47, **2** R_F=0.57. All NAC has reacted within a few hours after butyl isothiocyanate was added. The aqueous ethanol was evaporated and the residue was dissolved in 15 mL of brine. The excess of butyl isothiocyanate was extracted with heptane-diethyl ether (10:5, v/v) and the mixture was acidified. The mercapturic acid was extracted with 15 mL of ethyl acetate, concentrated until dryness and traces of ethyl acetate were removed by subsequent washing and evaporating with dichloromethane yielding 9.3 mmol (72.7%) of **2** as a yellow sticky product.

*N-acetyl-S-(N-isopropylthiocarbamoyl)-L-cysteine (**3**), mercapturic acid derived from isopropyl isothiocyanate*. Isopropyl isothiocyanate (27.2 mmol) was dissolved in 20 mL of ethanol and gradually added to a solution of NAC (24.8 mmol) and sodium bicarbonate (26.0 mmol) in 20 mL of water. TLC: NAC R_F=0.45, **3** R_F=0.53. All NAC has reacted immediately after isopropyl isothiocyanate was added. Ethanol was evaporated, the mixture was acidified with HCl and the product was crystallised from boiling ethyl acetate yielding 12.3 mmol (49.6%) of **3** as bright white crystals.

*N-acetyl-S-(N-1-methylpropylthiocarbamoyl)-L-cysteine (**4**), mercapturic acid derived from 1-methylpropyl isothiocyanate*. 1-Methylpropyl isothiocyanate (105.1 mmol) was dissolved in 50 mL of ethanol and gradually added to a solution of NAC (100.0 mmol) and sodium bicarbonate (110.0 mmol) in 50 mL of water. TLC: NAC R_F=0.44, **4** R_F=0.54. All NAC has reacted within a few hours after 1-methylpropyl isothiocyanate was added. Ethanol was evaporated and the excess of 1-methylpropyl isothiocyanate was extracted with heptane. The mixture was acidified with HCl and the product was crystallised from boiling ethyl acetate, yielding 49.7 mmol (49.7%) of **4** as bright white crystals.

*N-acetyl-S-(N-2-propenylthiocarbamoyl)-L-cysteine (**5**), mercapturic acid derived from 2-propenyl isothiocyanate*. 2-Propenyl isothiocyanate (57.3 mmol) was dissolved in 50 mL of ethanol and gradually added to a solution of NAC (51.2 mmol) and sodium bicarbonate (56.3 mmol) in 50 mL of water. TLC: NAC R_F=0.46, **5** R_F=0.57. All NAC has reacted within a few hours after 2-propenyl isothiocyanate was added. Ethanol was evaporated and the mixture was acidified with HCl which gave a yellow oily precipitate. The mercapturic acid was extracted with ethyl acetate and traces of organic solutes were evaporated using high vacuum, yielding 42.5 mmol (83.0%) of **5** as a yellow sticky product.

*N-acetyl-S-(N-phenylthiocarbamoyl)-L-cysteine (**8**), mercapturic acid derived from phenyl isothiocyanate*. Phenyl isothiocyanate (13.3 mmol) was dissolved in 15 mL of ethanol-water (8:2, v/v) and gradually added to a solution of NAC (12.3 mmol) in 70 mL of tetrahydrofuran (THF). TLC: NAC R_F=0.39, **8** R_F=0.46.

Sodium bicarbonate (14.0 mmol) and 30 mL of water were added to the mixture which started the reaction. All NAC had reacted within twenty-four hours. THF was evaporated, the mixture was acidified with HCl and the product was crystallised from boiling ethyl acetate-methanol, yielding 8.9 mmol (72.4%) of **8** as white crystals.

*N-acetyl-S-(N-benzylthiocarbamoyl)-L-cysteine (**9**), mercapturic acid derived from benzyl isothiocyanate*. Benzyl isothiocyanate (14.9 mmol) was gradually added to a solution of NAC (13.6 mmol) and sodium bicarbonate (13.6 mmol) in 33 mL of aqueous 82% (v/v) ethanol. TLC: NAC R_F=0.42, **9** R_F=0.55. All NAC had reacted within a few hours after benzyl isothiocyanate was added. The ethanol was evaporated and the mixture was acidified with HCl. After crystallisation the product was washed on filter with cold water and dried on filter with gentle suction, yielding 11.5 mmol (84.6%) of **9** as yellow-white crystals.

*N-acetyl-S-(N-phenylethylthiocarbamoyl)-L-cysteine (**10**), mercapturic acid derived from phenylethyl isothiocyanate*. Phenylethyl isothiocyanate (13.8 mmol) was gradually added to a solution of NAC (12.5 mmol) and sodium bicarbonate (13.1 mmol) in 25 mL of aqueous 70% (v/v) ethanol. TLC: NAC R_F=0.41, **10** R_F=0.53. All NAC had reacted within a few hours after phenylethyl isothiocyanate was added. The ethanol was evaporated, the excess of phenylethyl isothiocyanate was extracted with hexane, the remaining mixture was acidified with HCl and the mercapturic acid was extracted with ethyl acetate. Traces of organic solutes were finally evaporated using high vacuum, yielding 9.1 mmol (72.8%) of **10** as white crystals. ^{13}C-NMR: 195.6 (C=S), 172.2 (COOH), 169.5 (C=O), 139.0 (C-1 Ø), 128.8 (C-3 Ø), 128.6 (C-2 Ø), 126.5 (C-4 Ø), 51.9 (CCOOH), 48.3 (ØCCH_2), 35.9 (CH_2S), 33.5 (ØCH_2), 22.6 (CH_3).

General procedure for the complete synthesis. Compounds **6**, **7**, **11**, and **12** were synthesised by first adding the corresponding alkyl bromide to phthalimide potassium salt. The obtained N-alkyl-phthalimid was hydrazinolysed, yielding the alkylamine, which was subsequently reacted with thiophosgene to produce the isothiocyanate. The diluted isothiocyanate was gradually added to a solution of *N*-acetyl-*L*-cysteine (NAC) and sodium bicarbonate which was monitored by TLC with ethyl acetate-water-acetic acid (80:10:8, v/v/v) as eluent.

*N-acetyl-S-(N-3-butenylthiocarbamoyl)-L-cysteine (**6**), mercapturic acid derived from 3-butenyl isothiocyanate*. Phthalimide potassium salt (224.0 mmol) was slowly added to a solution of 25 mL of 4-bromo-1-butene (246.3 mmol) in 75 mL of dimethylformamide (DMF). All phthalimide potassium salt had reacted after overnight stirring. DMF and the excess of 4-bromo-1-butene were evaporated. With heat, the product and by-product (potassium bromide) were dissolved in 400 mL of ethyl acetate-water (1:1, v/v). The ethyl acetate layer was evaporated until dryness and the product was crystallised from boiling diisopropyl ether, yielding 183.0 mmol of *N*-(3-butenyl)-phthalimide as white, needle-shaped crystals. ^{1}H-NMR (in $CDCl_3$, 100 MHz, δ in ppm relative to TMS): 7.78 (m, 4H), 5.82 (m, 1H), 5.07 (dd, 2H), 3.78 (t, 2H), 2.46 (m, 2H). Hydrazine monohydrate (233 mmol) was added to a solution of 179.0 mmol of *N*-(3-butenyl)-phthalimide in 200 mL of ethanol under nitrogen. After 2 hours of reflux at 75 °C, HCl was added and the mixture was further heated for 1 h at 100 °C. The mixture was left to cool

overnight, filtered and the residue was washed with water. The filtrate containing 3-butenylamine was extracted twice with an equal volume of diethyl ether, distilled, and dissolved in 100 mL of chloroform. Slowly 1.1 molar equivalent of thiophosgene was added to the mixture with stirring, followed by 3.0 molar equivalents of sodium hydroxide. The reaction was monitored by TLC with chloroform as eluent, a spot was formed at R_F=0.60. The R_F of butyl isothiocyanate was 0.61. The mixture was left overnight at room temperature. The chloroform and thiophosgene were evaporated and 3-butenyl isothiocyanate was obtained by distillation, b.p. 60 °C 12 mmHg (60 °C 12mmHg (*28*); 77.5 °C 28mmHg (*29*)), yielding 28.9 mMol. ^{1}H-NMR (in $CDCl_3$, 100 MHz): 5.80 (m, 1H), 5.18 (dd, 2H), 3.57 (t, 2H), 2.45 (m, 2H). 3-Butenyl isothiocyanate (28.2 mmol) was dissolved in 20 mL of ethanol and gradually added to a solution of NAC (27.8 mmol) and sodium bicarbonate (29.2 mmol) in 20 mL of water. TLC: NAC R_F=0.49, **6** R_F=0.59. All NAC had reacted within 1 h after 3-butenyl isothiocyanate was added. Ethanol was evaporated and the excess of 3-butenyl isothiocyanate was extracted with heptane. The mixture was acidified with HCl, the mercapturic acid was extracted with ethyl acetate and ethyl acetate was evaporated yielding 22.9 mmol (10.9%) of **6** as a white sticky product.

*N-acetyl-S-(N-4-pentenylthiocarbamoyl)-L-cysteine (**7**), mercapturic acid derived from 4-pentenyl isothiocyanate*. 5-Bromo-1-pentene was used. N-(4-pentenyl-phthalimide was dissolved in boiling ethanol, ^{1}H-NMR (in $CDCl_3$, 100 MHz, δ in ppm relative to TMS): 7.78 (m, 4H), 5.80 (m, 1H), 4.98 (dd, 2H), 3.70 (t, 2H), 2.00 (m, 4H). The yield of 4-pentenyl isothiocyanate was 24.4 mMol. ^{1}H-NMR (in $CDCl_3$, 100 MHz, δ in ppm) 5.78 (m, 1H), 5.08 (dd, 2H), 3.53 (t, 2H), 2.21 (m, 2H), 1.80 (m, 2H). TLC: NAC R_F=0.48, **7** R_F=0.64. The yield of **7** was 14.6 mmol (12.0%) as an brownish-orange sticky product.

*N-acetyl-S-(N-4-methylthiobutylthiocarbamoyl)-L-cysteine (**11**), mercapturic acid derived from 4-methylthiobutyl isothiocyanate*. 1,4-Dibromobutane was used (**Scheme 1**). *N*-(4-bromobutyl)-phthalimide (103.0 mmol) was yielded as white, needle-shaped crystals, ^{1}H-NMR (in $CDCl_3$, 100 MHz, δ in ppm): 7.79 (m, 4H), 3.73 (m, 2H), 3.45 (m, 2H), 1.88 (m, 4H). *N*-(4-bromobutyl)-phthalimide (14.1 mmol) was slowly added to a solution of sodium methylmercaptide (14.8 mmol) in 10 mL of ice-cooled DMF. The reaction was complete within a few hours. The mixture was poored into 100 mL of ice cold water while stirring, resulting in crystallisation of the product. Crystallisation from diisopropyl ether yielded 9.9 mmol of *N*-(4-methylthiobutyl)-phthalimide as white, needle-shaped crystals. ^{1}H-NMR (in $CDCl_3$, 300 MHz, δ in ppm relative to TMS): 7.83 (m, 2H), 7.70 (m, 2H), 3.71 (t, 2H), 2.54 (t, 2H), 2.09 (s, 3H), 1.80 (m, 2H), 1.66 (m, 2H). Hydrazinolysis yielded 4-methylthiobutylamine which was distilled at reduced pressure, b.p. 72.9 °C (12.0 mmHg), yielding 21.1 mmol, ^{1}H-NMR (in $CDCl_3$, 100 MHz, δ in ppm): 2.72 (t, 2H), 2.52 (t, 2H), 2.11 (s, 3H), 1.59 (m, 4H), 1.16 (s, 2H). After reaction with thiophosgene, 4-methylthiobutyl isothiocyanate was obtained by distillation at 14.5 mmHg, b.p. 135 °C (136 °C at 12mmHg (*34*); 130-140 °C at 9mmHg (*33*)), yielding 12.5 mmol of a yellow oil, ^{1}H-NMR (in $CDCl_3$, 100 MHz, δ in ppm): 3.56 (t, 2H), 2.54 (t, 2H), 2.11 (s, 3H), 1.78 (m, 4H). A singlet peak at 2.58 ppm belonging to a methyl adjacent to a sulfinyl group and a singlet at 2.91 belonging to a methyl adjacent to a sulfonyl group were also observed.

4-Methylthiobutyl isothiocyanate (10.5 mmol) was dissolved in 10 mL of ethanol and gradually added to a solution of NAC (9.5 mmol) and sodium bicarbonate (10.0 mmol) in 20 mL of water. TLC: NAC R_F=0.45, **11** R_F=0.59. After acidification and extraction, 7.1 mmol (13.0%) of **11** was yielded as a yellow sticky product.

*N-acetyl-S-(N-4-methylsulfinylbutylthiocarbamoyl)-L-cysteine (**12**), mercapturic acid derived from 4-methylsulfinylbutyl isothiocyanate*. To a solution of 4-methylthiobutyl isothiocyanate (10.3 mmol, prepared as described above) in 15 mL of DCM, a solution of *m*-chloroperbenzoic acid (MCPBA, 12.6 mmol) in 15 mL of DCM was gradually added (**Scheme 1**). The reaction was monitored by TLC with ethyl acetate as eluent (4-methylthiobutyl isothiocyanate R_F=0.64, 4-methylsulfinylbutyl isothiocyanate R_F=0.06). All isothiocyanate was oxidised after 30 min. The DCM layer was separated and evaporated, yielding 10.4 mmol (100%) of 4-methylsulfinylbutyl isothiocyanate as a yellow oil. ^{1}H-NMR (in $CDCl_3$, 100 MHz, δ in ppm relative to TMS): 1.92 (m, 4H), 2.61 (s, 3H), 2.74 (m, 2H), 3.61 (t, 2H). 4-Methylsulfinylbutyl isothiocyanate reacted completely after overnight stirring with NAC. TLC: NAC R_F=0.36, **12** R_F=0.09. The yield of mercapturic acid **12** was 6.3 mmol (69.5%) as white crystals.

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Chapter 3

Analysis of Isothiocyanate Mercapturic Acids in Urine: A Biomarker for Cruciferous Vegetable Intake

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ABSTRACT

Cruciferous vegetables contain glucosinolates, which are degraded to isothiocyanates. These are easily absorbed, conjugated to glutathione, and excreted into the urine as their corresponding mercapturic acids. We have developed and validated a solid phase extraction high-performance liquid chromatography electrospray ionization mass spectrometry/mass spectrometry method for the specific analysis of individual isothiocyanate mercapturic acids in urine. The range of reliable analysis was 1.0-310 μM in urine. Urine samples fortified with three different levels of isothiocyanate mercapturic acids were measured on six different days by three independent technicians. The relative standard deviation (RSD) of repeatability was 12, 6, and 3%; the RSD of reproducibility was 19, 14, and 8%, and spike recoveries were 103, 104, and 103%, respectively, for 1.04, 10.5, and 313 μM levels. In 24h urine collected from two volunteers after they consumed broccoli and cauliflower, clearly sulforaphane mercapturic acid (133 μmol) and allyl isothiocyanate mercapturic acid (4.7 μmol) were found. This procedure demonstrates a reliable and efficient method to study the intake and mode of action of isothiocyanates in animal studies and clinical trials.

INTRODUCTION

Consumption of fruits and vegetables is associated with a reduced risk on degenerative diseases such as cancer and cardiovascular diseases, as indicated by epidemiological studies (*1*, *2*). In particular, cruciferous vegetables, i.e. cabbages, broccoli, Brussels sprouts, radish, mustard, and cress, appear to have beneficial health potentials (*3*-*5*). In 1978, the daily per capita consumption of standard cruciferous vegetables, in the United States and Canada was reported to be 18 g and in the U.K. 45 g (*15*). In The Netherlands, the average daily consumption of cruciferous vegetables in 1992 ranged from 29 g (*19*) to 32 g (*5*). Because cruciferous vegetables differ from other vegetables by the presence of glucosinolates, health-promoting effects seem to be attributable to these phytochemicals or their breakdown products, e.g., isothiocyanates (*6*). Isothiocyanates are strong inhibitors of phase I enzymes and inducers of phase II enzymes and are therefore thought to be strong cancer chemopreventors (*7*-*9*). Human intervention trials with large quantities of cruciferous vegetables gave similar effects on phase I and phase II enzymes (*10*-*13*).

Glucosinolates remain intact within the plant cytoplasm until tissue disruption by chewing or cutting which releases the hydrolysis enzyme myrosinase (see **Figure 1**) (*14*, *15*). Unmetabolised glucosinolates can be degraded, to a lesser degree, by microbes present in the human gut (*16*-*18*). Major breakdown products are isothiocyanates, which are responsible for the pungent taste of mustard. Other breakdown products are indoles, and nitriles. Additionally, acid hydrolysis inside the stomach can degrade glucosinolates into isothiocyanates. Glucosinolates may be absorbed without change, to some degree, but the subsequent fate of these compounds in the metabolic scheme remains unknown. Thus, when eating crucifers, both glucosinolates and isothiocyanates are important components to be considered in the metabolic and excretory pathways. Verhoeven et al. (*6*) estimated that an average daily intake in The Netherlands of 21 mg of glucosinolates, corresponding to 50 µmol, can be calculated.

Figure 1. Glucosinolates are enzymatically hydrolysed into isothiocyanates and are subsequently conjugated in the body to glutathione followed by excretion as mercapturic acids in the urine.

Figure 2. These 12 isothiocyanate mercapturic acids were previously synthesised (*30*) and were used here as reference standards for the analysis of mercapturic acids in urine. Compound **1**, methyl; **2,** 4-methylsulfinylbutyl; **3**, allyl; **4**, isopropyl; **5**, 3-butenyl; **6**, 1-methylpropyl; **7**, phenyl; **8**, butyl; **9**, 4-methylthiobutyl; **10**, 4-pentenyl; **11**, benzyl; **12**, 2-phenylethyl.

Isothiocyanates are conjugated to glutathione in the body and excreted into the urine as their corresponding mercapturic acids (**Figure 1**) as demonstrated in rats (*20*), guinea pigs, rabbits (*21*), and humans (*22*, *23*). Mercapturic acids reflect the uptake of isothiocyanates, and thus the intake of glucosinolates present in cruciferous vegetables (*24-26*). Therefore, mercapturic acids can be used as a selective biomarker for cruciferous vegetable intake. Different cruciferous vegetables can be differentiated by the variety and amount of glucosinolates (*27*). For example, broccoli is rich in 4-methylthiobutyl glucosinolate, whereas Brussels sprouts are rich in allyl and 2-hydroxy-3-butenyl glucosinolate (progoitrin). On the other hand, garden and water cress are rich in arylaliphatic glucosinolates (*15*).

Isothiocyanates and their corresponding mercapturic acids can be measured in physiological fluids by a sum parameter assay. The assay utilises 1,2-benzenedithiol as a vicinal dithiol reagent and measures the reaction product, 1,3-benzodithiole-2-thione, spectrophotometrically (*28, 29*). Chemical specificity of the cyclocondensation reaction is not restricted to isothiocyanates, but includes dithiocarbamates and related thiocarbonyl compounds such as carbon disulfide, certain substituted thiourea derivatives, and xanthates (*28*). Besides, the sum parameter does not give any information about which kind of crucifer has been consumed.

We therefore developed an analytical method by which the isothiocyanate mercapturic acids not only can be determined quantitatively, but also individually characterised. Mercapturic acids were extracted from urine using solid phase extraction (SPE) followed by separation using reversed phase liquid chromatography. Because specificity by UV detection (absorption maxima in acetonitrile-water at 250 nm and at 270 nm) is too low, mass spectrometric (MS/MS) detection was used. Linearity, repeatability, reproducibility, accuracy, and detection limits of the method were determined. The strength of the application of the current method is demonstrated by the analysis of urine samples collected for 24 h after consumption of cooked broccoli and cauliflower and a salad of mustard, garden cress, water cress, rocket, winter radish, and radish.

MATERIAL AND METHODS

Materials. Urine was collected in clear polyethylene flasks. Solid Phase Extraction using Bakerbond octadecyl 100 mg SPE columns (Mallinckrodt Baker B.V., Deventer, The Netherlands) was performed on a vacuum manifold with Teflon fittings.

Chromatographic separation was performed using a Waters Alliance 2690 quaternary high-performance liquid chromatograph (HPLC, Waters Chromatography B.V., Etten-Leur, The Netherlands) equipped with a Waters SymmetryShield RP18 3.5 µm guard (2.1 mm x 10 mm cartridge) and analytical (2.1 mm x 150 mm cartridge) column thermostated at 35 °C. This system was equipped with a Finnigan MAT LCQ ion trap mass spectrometer (Thermo Quest B.V., Breda, The Netherlands) and operated by XCalibur software.

Chemicals. Water was demineralised using an ELGA Option 7 Plus water purifier (Salm en Kipp, Breukelen, The Netherlands). All chemicals were from Merck (Merck KGaA, Darmstadt, Germany) and of analytical grade. Phenyl isothiocyanate mercapturic acid (internal standard), and 11 reference compounds (methyl, 4-methylsulfinylbutyl, allyl, isopropyl, 3-butenyl, 1-methylpropyl, butyl, 4-methylthiobutyl, 4-pentenyl, benzyl, and 2-phenylethyl isothiocyanate mercapturic acids) were prepared as described elsewhere (*30*, **Figure 2**). In short, isothiocyanate was dissolved in alcohol and added to a solution of *N*-acetyl-*L*-cysteine and sodium bicarbonate in water. The corresponding mercapturic acid was obtained with a typical yield of 77%. Because few isothiocyanates are commercially available, others were first synthesised by adding the corresponding alkyl bromide to phthalimide potassium salt. The obtained *N*-alkyl-phthalimide was hydrazinolysed yielding the alkylamine, which was subsequently substituted with thiophosgene yielding the isothiocyanate with an overall yield of 16%. The internal standard was dissolved in tetrahydrofuran and diluted with 0.25 M hydrochloric acid. Tetrahydrofuran, methanol and acetonitrile for solid phase extraction and chromatography were HPLC grade.

Sample Preparation. Twenty microliters of internal standard solution was added to 200 µL of urine in a 0.5 mL Eppendorf tube followed by vortexing. Next, SPE columns were washed with methanol and hydrochloric acid buffer (pH 2), and subsequently, the samples were applied. SPE columns were washed with water and aspirated until dryness. Mercapturic acids were eluted from the column with phosphate buffer (pH 8):acetonitrile (v:v, 1:1) into a vial containing hydrochloric acid buffer (pH 2). From the mixed eluate, 5 µL was analysed on an HPLC-MS/MS system.

HPLC-MS/MS Analysis. Chromatography was performed using a SymmetryShield column and an acetonitrile gradient. The flow rate of mobile phase was 0.25 mL/min using a linear gradient of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The amount of B was raised from 0 to 30% in 55 min. Electrospray ionization (ESI) in positive

positive mode was found optimal with the following ESI source settings: sheath gas flow, 80%; auxiliary gas flow, 20%; capillary temperature, 225 °C; source voltage, 4.5 kV. Parent masses $[M + H]^+$ were isolated with a *m/z* window of 3.0 and fragmented using 30% collision energy, which resulted in 12 different scan filters, one for each mercapturic acid. The spectra of fragments in the range *m/z* 100-350 were collected, and peak areas were obtained using the appropriate scan filter and the response of the fragment with *m/z* 164.0 (*N*-acetyl-*L*-cysteine) with a *m/z* window of 1.0. For 4-methylsulfinylbutyl isothiocyanate mercapturic acid, the fragment 178.0 (4-methylsulfinylbutyl isothiocyanate) instead of 164.0 was used. Urinary concentrations of mercapturic acids were quantitated using an internal standard and external calibration (**Figure 3**). Because phenyl glucosinolate is not a naturally occurring compound, the mercapturic acid of phenyl isothiocyanate was used as the internal standard.

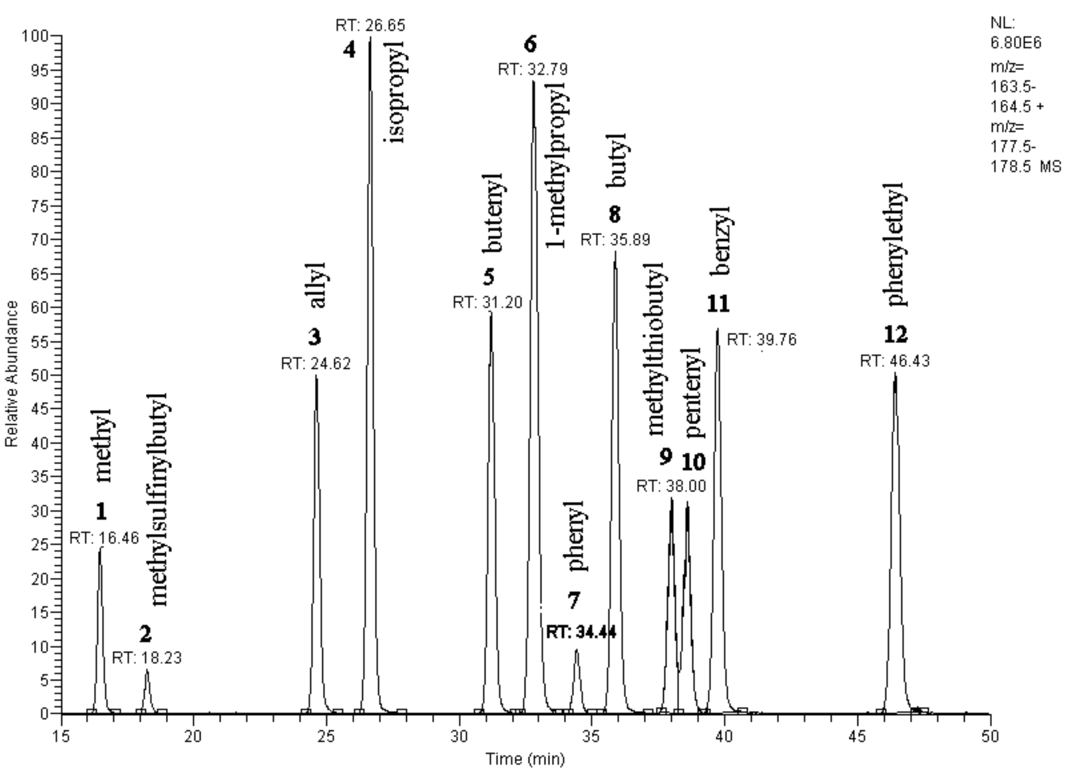

Figure 3. HPLC-MS/MS chromatogram of twelve available isothiocyanate mercapturic acids. Each peak is labeled with the side chain (R) mentioned in **Figure 2**. Concentrations are (µM) as follows: **1** (77), **2** (48), **3** (66), **4** (70), **5** (65), **6** (65), **7** (14), **8** (61), **9** (56), **10** (69), **11** (57), **12** (55).

Method Validation. For the purpose of validation and quality control, calibration standards were added to blank urine at three levels: low (on average 1.04 μM), medium (on average 10.5 μM), and high level (on average 313 μM) in order to determine repeatability, recovery, and reproducibility of analysis. Linearity of analysis was checked by preparing two stock solutions containing all mercapturic acids with subsequent dilutions.

RESULTS AND DISCUSSION

Solid Phase Extraction (SPE) using only C18 columns resulted in acceptable reproducibilities (**Table 1**) and a rapid cleanup method. Urine samples and C18 columns were acidified to pH 2.0 to protonate the mercapturic acids. The measured pK_a of allyl isothiocyanate mercapturic acid was 3.3. Sufficient separation of isothiocyanate mercapturic acids was obtained on a SymmetryShield RP18 HPLC column, which has improved water wettability. A linear gradient of acetonitrile and water is used to separate all compounds. Acetonitrile was used as an HPLC modifier because this resulted in better peak shape as compared to a gradient of methanol and water (data not shown). Electrospray ionization (ESI) proved more suitable than atmospheric pressure chemical ionization (APCI), because of its better performance at low HPLC eluent flow rates (0.25 mL/min) and negligible in-source fragmentation. In time segments of approximately 1 min surrounding the retention times of all 12 isothiocyanate mercapturic acids, the masses $[M + H]^+$ of each individual component were isolated and fragmented (**Figure 3**).

Mass Spectra. Fragmentation spectra generally contain fragments with relative abundances as shown in **Figure 4B** for allyl isothiocyanate mercapturic acid where *m/z* 122 is cysteine, 130 is 2-*N*-acetyl-propanoic acid, 164 is *N*-acetyl-*L*-cysteine, and 134 is the variable side chain allyl-N(H)-C(=S)-S. The most abundant fragment ion has a *m/z* of 164 except for compounds **2** and **7**. Phenyl isothiocyanate mercapturic acid (**7**) fragmented into ions with a base peak at *m/z* 170 (phenyl-N(H)-C(=S)-S), fragment *m/z* 164 (70%), and some minor fragments. 4-Methylsulfinylbutyl isothiocyanate mercapturic acid (**2**) fragmented into ions with a base peak at *m/z* 178 (H_3C-S(=O)-$(CH_2)_4$-N-C=S), and fragments at *m/z* 212 (H_3C-S(=O)-$(CH_2)_4$-N(H)-C(=S)-S, 45%), and 164 (1%). Quantitation was performed using *m/z* 164, except for compound **2** where *m/z* 178 was used. Clearly, this MS/MS detection method provides high specificity.

Table 1. Validation Results of SPE-HPLC-MS/MS Analysis of Isothiocyanate Mercapturic Acids in urine [a]

compd nr.[b]	linearity[c] range (μM)	R^2	slope	intercept (95% CI)	repeatability RSD (%)			recovery (%)			reproducibility RSD (%)			LOQ (μM)[d]
					l	m	h	l	m	h	l	m	h	
1	0.113-113	0.996	29.0	-0.02 (-0.14, 0.09)	15	6	4.8	93	98	92	23	17	11	0.5
2	0.127-125.5	0.998	58.5	-0.07 (-0.13, 0.28)	18	4	2.4	87	100	96	16	9	6	0.1
3	0.098-98.1	0.997	78.3	-0.04 (-0.27, 0.18)	5	7	3.0	103	106	107	25	18	7	0.6
4	0.104-104	0.997	149	-0.1 (-0.6, 0.4)	11	5	2.0	112	104	104	17	12	8	0.6
5	0.096-95.6	0.999	99.5	-0.01 (-0.14, 0.11)	7	8	4.1	92	108	105	15	13	8	0.3
6	0.096-96.5	0.998	186	0 (-0.5, 0.4)	20	6	2.1	108	107	104	14	12	8	0.4
8	0.091-90.7	0.990	133	0 (-0.7, 0.7)	10	4	3.3	103	106	102	19	13	9	0.8
9	0.083-83.5	0.993	149	-0.1 (-0.7, 0.5)	10	11	3.2	103	96	108	19	17	7	0.2
10	0.102-102	0.999	107	-0.03 (-0.26, 0.19)	13	9	7	103	104	100	19	16	9	0.6
11	0.085-85	0.996	136	-0.1 (-0.5, 0.4)	12	5	3.1	115	106	105	18	14	10	0.4
12	0.081-80.9	0.995	147	-0.1 (-0.5, 0.4)	15	6	2.3	111	105	105	21	11	8	0.5

[a] Urine samples fortified at three different levels of 1.04 μM (l), 10.5 μM (m), and 313 μM (h) were measured six times to determine the relative standard deviation (RSD) of repeatability, and the recovery. These samples were measured on six different days by three different technicians to determine the RSD of reproducibility. Blank urine was measured six times to determine the limit of quantification (LOQ). [b] Compound nr refers to isothiocyanate mercapturic acids depicted in **Figure 2**. Compound nr **7** is not included because this compound is used as the internal standard. [c] Linearity is defined as $Y = aX + b$ where Y = area ratio, and X = concentration in mM. [d] The LOQ is quantified as 10/3 x (the average value of the noise + (3 x SD)).

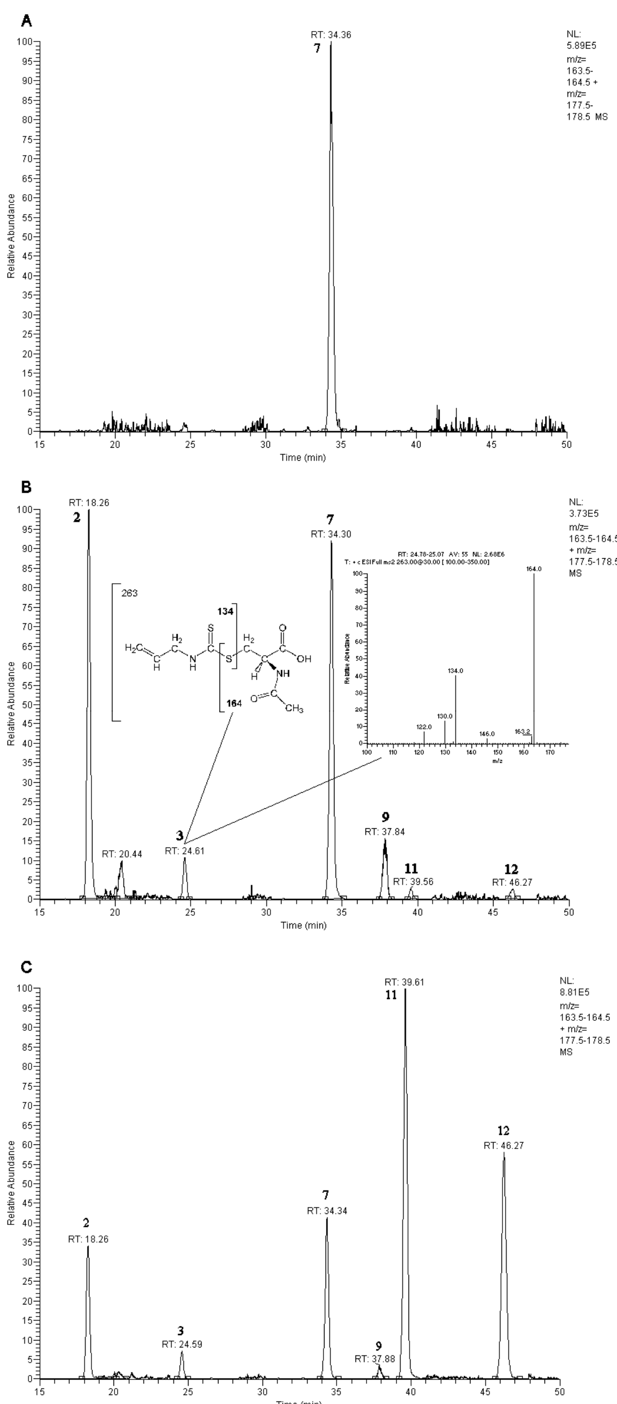

Figure 4. HPLC-MS/MS chromatograms of isothiocyanate mercapturic acids in urine. Each peak is labeled with the side chain (R) mentioned in **Figure 2**. The absence (<0.5 μM) of isothiocyanate mercapturic acids in blank urine is shown in chromatogram **A**. Peak 7 is the internal standard, 13.7 μM phenyl isothiocyanate mercapturic acid. After cauliflower and broccoli were consumed, chromatogram **B** was obtained. The fragmentation structures and spectrum of allyl isothiocyanate mercapturic acid are inserted. After mustard, garden cress, water cress, rocket, winter radish, and radish were consumed, chromatogram **C** was obtained. Method settings are as described in the Material and Methods.

Method Validation. The ruggedness of the method was tested by changing six parameters slightly and analyzing urine spiked at the medium level. The tested parameters were the sample volume of urine applied to the SPE column, the volume of water for washing, the pH of the hydrochloric acid buffer, the composition of buffer for elution of the components from the SPE column, the injection volume for HPLC analysis, and the collision energy used for fragmentation of the compounds in the ion trap. Using the saturated design of Plackett and Burman (*31*), the method appears to be rugged for all but two parameters. These two parameters, applied sample volume and collision energy, were only found not to be rugged for one or two of the 11 isothiocyanate mercapturic acids. Concluding, no more than 200 µL of urine should be extracted and the collision energy used, to fragment trapped ions, should be no less than 30%.

From 11 solutions of mercapturic acids two calibration standards were prepared. These standards were subsequently diluted to give the mentioned (**Table 1**) calibration range with a good correlation coefficient (0.990 or more), no intercept (b = 0), and with similar slopes (a). Calibration standards were added to blank urine at three levels: low, medium, and high level. Results of repeated measurements of these samples are given in **Table 1**. Repeatability gave satisfactory results. Recoveries are all between 90 and 115%. High recoveries indirectly prove that there is no ion suppression in the MS source when analyzing urine. The reproducibility is for most compounds close to expected relative standard deviations (RSDs) using the Horwitz equation (*32*). For methyl isothiocyanate mercapturic acid, RSDs for reproducibility are high, which could be due to poor adsorption on the C18 SPE column leading to loss while extracting the compounds from urine. The limit of quantification is on average 0.5 µM, and as specifications of the method were tested for the concentration range of 1.0-313 µM, the latter is considered to be the range shown to be reliable for analysis. This range roughly corresponds to the amounts of allyl isothiocyanate mercapturic acid found in 24 h urine after the consumption of allyl glucosinolate (sinigrin) present in, respectively, one leaf of one Brussels sprout and 200 g of Brussels sprouts. The stability of compounds **1-12** (except **7**, which was used as the internal standard) added to blank urine at medium level (on average 10.5 µM) is at room temperature 1 day, in the refrigerator several days, and in the freezer over 1 year.

The described SPE-HPLC-MS/MS method proved to be a valid method for the analysis of isothiocyanate mercapturic acids in urine. In blank urine from six volunteers, collected after refraining from eating cruciferous vegetables and condiments during 36 h, no isothiocyanate mercapturic acids could be found (<0.5 µM, **Figure 4A**). Phenyl isothiocyanate mercapturic acid was absent from all urines tested (data not shown).

Mercapturic Acids in Urine After Brassica Consumption. After eating a meal with 200 g of both broccoli (*Brassica oleracea* L. *italica*) and cauliflower (*Brassica oleracea* L. *botrytis* subvar. *cauliflora*), the isothiocyanate mercapturic acids **2** (133 µmol), **3** (4.7 µmol), **9** (11.0 µmol), **11** (0.9 µmol), and **12** (1.0 µmol) were found in 24 h urine (**Figure 4B**). The content of 4-methylsulfinylbutyl glucosinolate in broccoli is on average 80.7 µmol and in cauliflower is 63.8 µmol/100 g of fresh weight. Furthermore, cauliflower contains on average 27.9 µmol allyl glucosinolate/100 g of fresh weight (*33*). From the expected dose of 4-methylsulfinylbutyl glucosinolate (289 µmol), a large amount is recovered in the urine as its isothiocyanate mercapturic acid where only a minor amount of the expected dose of allyl glucosinolate (55.8 µmol) is recovered in the urine. The difference in relative excretion between the two compounds can be explained from the large variation in glucosinolate content in vegetables. A meal of mustard (*Brassica juncea* Coss.), garden cress (*Lepidium sativum* L.), water cress (*Nasturtium officinale* R.Br.), rocket (*Eruca sativa* Mill.) and winter radish (rettich, *Raphanus sativus var. alba* L.)(10 g each) and radish (*Raphanus sativus* L.) (90 g) resulted in the excretion in 24 h urine of compounds **2** (116 µmol), **3** (6.7 µmol), **9** (4.8 µmol), **11** (71.9 µmol), and **12** (51.7 µmol) (**Figure 4C**). The major glucosinolates (side chain mentioned) present in these condiments and salad crops are allyl for mustard, benzyl for garden cress, phenylethyl for water cress, 4-methylsulfinylbutyl and 4-methylthiobutyl for rocket, and 4-methylsulfinylbutenyl glucosinolate for both winter radish and radish which is reflected in the excreted isothiocyanate mercapturic acids. Breakdown products of the principal glucosinolate of winter radish and radish, 4-methylsulfinylbutenyl glucosinolate, were not measured with the here described method.

Biomarker of Isothiocyanate Intake. Proof of the health benefit potential of fruits and vegetables, and cruciferous vegetables in particular, is culminating, but the question remains, what are the responsible chemicals that make vegetables healthy? Because cruciferous vegetables differ from other vegetables in that they contain glucosinolates, these compounds might be the responsible bioactive phytochemicals. Glucosinolates themselves are not thought to diminish cancer risks. However, their breakdown products, isothiocyanates, have been proven (in *in vitro* and *in vivo* experiments) to modulate biotransformation enzyme activities resulting in reduced cancer risk (*7-9*). Glucosinolate intake is generally estimated using dietary questionnaires, which are not only subjective measures, but are not accurate because amounts of (cruciferous) vegetables in a whole meal are not easily estimated. Glucosinolates are thought not to be absorbed from the gut but partly converted into nitriles and isothiocyanates in the stomach, and to isothiocyanates

in the colon. Isothiocyanates are lipophilic and are readily absorbed from the gut. Spot urine cannot be used to quantify the uptake and excretion of isothiocyanates since the peak in excretion is between 3 and 6 h. Twelve hour urine collection contains the majority in isothiocyanate excretion and most isothiocyanates, eaten with common meals, are excreted within 24 h (data not shown).

Glucosinolate metabolites can be analytically measured in vegetables, and intake of isothiocyanates can thus be estimated. Because glucosinolates are only partly converted into isothiocyanates and interindividual kinetics differ, it is not feasible to estimate the exact intake of isothiocyanates through analysis of glucosinolates and isothiocyanates in vegetables. Myrosinase, which is still active in raw broccoli, is responsible for the breakdown of glucosinolates after chewing, resulting in an elevated isothiocyanate intake. Isothiocyanate conjugates, mostly mercapturic acids, quantitatively reflect isothiocyanate uptake but reflect glucosinolate intake only qualitatively. The amount of isothiocyanate conjugates excreted after eating raw broccoli is approximately three times greater than that after eating the same amount of cooked broccoli (*34*).

Indole glucosinolates do not yield isothiocyanates upon hydrolysis but are converted into indoles and thiocyanate ion (SCN^-). Measuring thiocyanate ion in urine can thus be a marker of indole glucosinolate intake and thus of cruciferous vegetable intake. High baseline concentrations in urine can be expected as thiocyanate ion is a metabolite from cyanide provided by cigarette smoke. Thiocyanate ion can be measured spectroscopically after ion-exchange cleanup (*35*) or can be measured using ion-exchange chromatography and suppressed conductivity detection. Isothiocyanate conjugate excretion can be measured using an assay which gives the sum parameter of isothiocyanates after a cyclocondensation reaction (*28*, *29*). This is a fast assay, but chemical specificity is not restricted to isothiocyanates (*28*), and it returns a sum parameter of isothiocyanates and conjugates. The limitations of these two assays are not encountered when analyzing individual isothiocyanate mercapturic acids.

In conclusion, SPE-HPLC-MS/MS isothiocyanate mercapturic acid analysis in urine is a specific, rugged, and validated method, which can be applied to measure isothiocyanate exposure. This biomarker reflects the dose of isothiocyanates absorbed after intake of isothiocyanates or after breakdown in the human gut of glucosinolates, and can thus be used to determine the health-promoting potential of cruciferous species in animal studies and clinical trials.

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Chapter 4

Association Between Consumption of Cruciferous Vegetables and Condiments, and Excretion in Urine of Isothiocyanate Mercapturic Acids

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ABSTRACT

A high intake of cruciferous vegetables is associated with a reduced risk of cancer and cardiovascular diseases. This protective effect has been linked to isothiocyanates, enzymatic hydrolysis products of glucosinolates. In this study, the metabolic fate of glucosinolates and isothiocyanates after ingestion of 19 different cruciferous vegetables was studied in three male subjects. After the consumption of 13 cruciferous vegetables (glucosinolate content, 0.01-0.94 mmol/kg) and six condiments (isothiocyanate content, 0.06-49.3 mmol/kg), eight different isothiocyanate mercapturic acids were determined in urine samples. Excretion levels after the consumption of raw vegetables and condiments were higher (bioavailability, 8.2-113%) compared to cooked vegetables (bioavailability, 1.8-43%) but the excretion rate was similar ($t_{½}$ = 2.1-3.9 h). Isothiocyanates in urine remain longer at a nonzero level after consumption of glucosinolates from cooked vegetables, as compared to raw vegetables and condiments, and maximal levels in urine were reached about 4 hours later. Isothiocyanate mercapturic acids can be used as a biomarker to reflect the active dose of isothiocyanates absorbed.

INTRODUCTION

Epidemiological studies indicate that consumption of fruits and vegetables is associated with a reduced risk of degenerative diseases such as cancer and cardiovascular diseases (*1,2*). In particular cruciferous vegetables, e.g. cabbages, kale, broccoli, Brussels sprouts, radish, mustard and cress, are possibly beneficial for human health (*3,4*). Since cruciferous vegetables differ from other vegetables in that they contain glucosinolates, these phytochemicals might be responsible for the health promoting effect (*5,6*). Cruciferous vegetables can be differentiated by the variety (**Table 1**) and amount of glucosinolates (*7*). One vegetable generally contains high amounts of two to five different glucosinolates (**Table 2**). In all cruciferous plants together some 120 different glucosinolates have been identified. There is a wide variation in the contents of glucosinolates in cruciferous vegetables and it is clear that processing, storage and cooking affects these components. For example broccoli is rich in 3-methylsulphinylpropyl glucosinolate (glucoiberin), whereas Brussels sprouts are rich in allyl and 2-hydroxy-3-butenyl glucosinolate (progoitrin). Garden and watercress, however, are rich in arylaliphatic glucosinolates (*8*). Unfortunately, there are no reliable tables of compositional data, which makes it difficult to conduct studies in populations (*9*). Therefore, we present an overview of the presence of glucosinolates in commonly consumed vegetables and condiments (**Table 2**).

Glucosinolates remain intact within the plant until the vegetable is processed, e.g. cutting or chewing. These processes release the enzyme myrosinase which hydrolyses glucosinolates into isothiocyanates and other breakdown products (**Figure 1**) (*8,10*). Glucosinolates that are not hydrolysed by myrosinase can be degraded, to a lesser degree, by microbes present in the human gut (*11*) but are likely not absorbed intact. After absorption, isothiocyanates are conjugated to glutathione and excreted into the urine as their corresponding mercapturic acids (**Figure 1**) as demonstrated in rats (*12*), guinea-pigs, rabbits (*13*), and humans (*14*). Mercapturic acids excreted in urine reflect the uptake of isothiocyanates, and thus the intake of glucosinolates which are present in cruciferous vegetables (*15*).

Table 1. Glucosinolates that are present in a wide range of vegetables and condiments

MW[a]	R =	rrf[b]	name	main dietary source
371.5	H_3C-	1.00	glucocapparin	capers
397.5	$H_2C{=}CHCH_2$-	1.00	sinigrin	cabbage, mustard
411.5	$H_2C{=}CH(CH_2)_2$-	1.11	gluconapin	Chinese cabbage
425.5	$H_2C{=}CH(CH_2)_3$-	1.15	glucobrassicanapin	Chinese cabbage
427	$H_2C{=}CHC(OH)HCH_2$-	1.09	progoitrin	Brussels sprouts, kale
441	$H_2C{=}CHCH_2C(OH)HCH_2$-	1.00	gluconapoleiferin	cabbage
461.6	$H_3CS({=}O)(CH_2)_3$-	1.07	glucoiberin	broccoli
459.6	$H_3CS(CH_2)_4$-	1.00	glucoerucin	rutabaga, turnip
475.6	$H_3CS({=}O)(CH_2)_4$-	1.07	glucoraphanin	cabbage, cauliflower
589.6	$H_3CS({=}O)(CH_2)_5$-	1.07	glucoalyssin	paksoi
463.5	$pHOC_6H_4CH_2$-	1.00	glucosinalbin	mustard
447.5	$C_6H_5CH_2$-	0.95	glucotropaeolin	garden cress
461.5	$C_6H_5(CH_2)_2$-	0.95	gluconasturtiin	water cress
486		0.29	glucobrassicin /	all cruciferous
516		0.20	neoglucobrassicin[c]	vegetables

[a] The molecular weight of each glucosinolate is calculated as its potassium salt in g/mol. [b] The relative response factor at 229 nm in water:acetonitrile (v:v, 9:1), relative to sinigrin (*17*). [c] Brassicins are 3-indolylmethyl compounds.

glucoraphanin — *thioglucosidase* / *rearrangement* → sulforaphane — *GSH conjugation* / *mercapturic acid pathway* → sulforaphane derived mercapturic acid

Figure 1. Example of the metabolism of glucosinolates; glucoraphanin is enzymatically hydrolysed into sulforaphane which is conjugated to glutathione (GSH) and further metabolised to sulforaphane mercapturic acid.

Table 2. Principal glucosinolates occurring in commonly consumed cruciferous vegetables[a]

	mmol glucosinolate / kg fresh weight (average (range))				
vegetable	sinigrin	gluconapin	glucobras-sicanapin	progoitrin	glucoiberin
broccoli		0.03 (0-0.06)		0.25 (0.20-0.31)	0.74 (0.01-3.3)
cauliflower	0.28 (0.01-1.6)				0.34 (0.01-3.3)
red cabbage	0.12 (0.02-0.26)	0.20 (0.05-0.30)		0.10 (0.04-0.14)	0.16 (0.05-0.31)
kohlrabi		0.11			0.19
Brussels sprouts	0.86 (0.04-3.9)	0.34 (0.01-2.2)		0.63 (0.01-3.0)	0.44 (0-1.5)
white cabbage	0.44 (0.04-1.6)	0.13		0.10	0.58 (0.05-2.8)
kale	0.97 (0.63-2.0)	0.21 (0-0.38)		0.70 (0.17-1.3)	0.12 (0-0.50)
Chinese cabbage		0.12 (0-0.59)	0.20 (0.03-0.62)	0.08 (0-0.44)	
rutabage/swede				0.63 (0.09-1.5)	
collards	0.21 (0.13-0.29)				0.39 (0.08-0.69)
savoy cabbage	0.53 (0.01-1.6)			0.07 (0-0.30)	0.11 (0.15-2.8)
turnip tops		0.61 (0.01-2.9)	0.43 (0.01-1.5)	0.52 (0-1.0)	

	glucoerucin	glucoraphanin	glucoalyssin	gluconasturtiin	indolyl glucosinolates
broccoli		0.81 (0.29-1.9)			0.67 (0.23-1.18)
cauliflower		0.64 (0.02-1.9)			0.59 (0.14-1.92)
red cabbage	0.02 (0.01-0.07)	0.55 (0.32-0.82)			0.73 (0.32-1.0)
kohlrabi					0.26
Brussels sprouts		0.08 (0-0.23)			2.31 (0.45-5.04)
white cabbage		0.1 (0-0.29)			0.5 (0.09-2.0)
kale					1.1 (0.67-1.7)
Chinese cabbage			0.13 (0.01-0.51)	0.15 (0.05-0.67)	0.41 (0.19-1.1)
rutabage/swede	0.20 (0.01-0.89)		0.08 (0-0.22)	0.14 (0.01-1.0)	0.42 (0.14-1.1)
collards					0.50 (0.44-0.70)
savoy cabbage					1.2 (0.7-2.0)
turnip tops	0.10 (0.01-0.29)			0.39 (0.02-1.2)	0.50 (0.12-1.1)

[a] Adapted from refs *8*, *28*, and *29*. Condiments: Garden cress; gluconasturtiin and glucotropaeolin present, horseradish; sinigrin (65-70) and gluconasturtiin (8.8-15), black mustard seed; sinigrin (39-175), brown mustard seed; sinigrin (1-43) and gluconapin (23-163), water cress; gluconasturtiin present.

To give more insight in the health effect of isothiocyanates, we determined the kinetic parameters for the individual isothiocyanates and the difference in bioavailability of isothiocyanates from raw and cooked vegetables. In literature, the sum of isothiocyanate conjugates is analysed, therefore no distinction can be made between different isothiocyanates from the same vegetable source. We developed methods for the analysis of individual isothiocyanates in milled, raw vegetables and condiments and for the analysis of individual isothiocyanate mercapturic acids in urine (*18*). The effect of cooking on the bioavailability and excretion kinetics is determined for raw and cooked vegetables.

In the current study we want to demonstrate a clear relation between the intake of glucosinolates and isothiocyanates from cruciferous vegetables, and the excretion in urine of their corresponding mercapturic acids.

Spot urine samples of three healthy volunteers were collected during 24 h after the consumption of 13 different cruciferous vegetables and six different cruciferous condiments. Seven of these vegetables were cooked and six different vegetables and six different condiments were eaten raw. Vegetables and condiments were analysed for their glucosinolate and isothiocyanate contents, and the urine samples were analysed for individual mercapturic acids. Absorption and excretion kinetics and the bioavailability were calculated.

MATERIALS AND METHODS

Subjects. Three apparently healthy, male subjects (31 ± 1 years, BMI of 21 ± 2 kg/m^2) were recruited from the Utrecht area (the Netherlands). Each subject gave written informed consent after being informed about the study, both verbally and in writing.

Experimental Design. Thirteen different vegetables and six different condiments were freshly obtained from a local grocer. Over a period of 9 months, each study substance was ingested on separate days at 12.30 p.m. as part of a complete warm meal (for amounts see **table 3**). The composition and amount of this meal was the same for each subject. No cruciferous vegetables were eaten for at least one day before each intervention day to make sure that all metabolites from glucosinolates and isothiocyanates were cleared from the body.

Food preparation. Vegetables that were consumed raw were freshly cut and served immediately. Cooking procedures were as follows. Brussels sprouts were cooked in boiling water for 10 min, white cabbage was stir fried for 10 min, sauerkraut was boiled for 20 min and subsequently heated in an oven for 10 min at 175 °C, green cabbage was stir fried for 20 min, curly kale was microwave cooked for 15 min at 1000 W, Chinese cabbage was stir fried for 10 min, and rutabaga was cooked in boiling water for 15 min.

Sampling Procedure. Shortly before serving, a representative sample of all vegetables (after cooking, where applicable) and condiments (raw) was taken for analysis of glucosinolates and isothiocyanates and was quickly frozen at < -18 °C. Spot urine samples

were separately collected during 24 h in clear polyethylene flasks for analysis of isothiocyanate mercapturic acids. Shortly before consumption of the vegetable a time zero urine sample was collected. After consumption of the vegetable, urine samples were collected at will and as often as possible. All samples were refrigerated immediately after collection and aliquot samples were stored frozen at < -18 °C.

Chemicals. Water was demineralised using an ELGA Option 7 Plus water purifier (Salm en Kipp, Breukelen, The Netherlands). Methanol, acetonitrile and dichloro methane for (solid phase) extraction and chromatography were HPLC grade. Sinigrin monohydrate, aryl sulphate sulphohydrolase (thioglucosidase) and 2-mercaptoethanol were from Sigma (Sigma-Aldrich, Zwijndrecht, The Netherlands). Methyl, allyl (2-propenyl), phenyl, benzyl and 2-phenylethyl isothiocyanate were purchased from Across Organics. All other chemicals were from Merck (Merck KGaA, Darmstadt, Germany) and of analytical grade. Erucin, sulforaphane, butenyl and pentenyl isothiocyanate were prepared as described (*16*). Ibervirin, iberin and erysolin were correspondingly prepared; methylthiopropylamine was converted into ibervirin using dipiridylthionocarbonyl and ibervirin was subsequently oxidised into iberin using *m*-chloroperbenzoic acid (MCPBA). Erysolin was prepared from sulforaphane by overnight oxidation using MCPBA. Methyl, allyl, 3-butenyl, 4-pentenyl, 4-methylthiobutyl, 4-methylsulfinylbutyl, phenyl, benzyl, and 2-phenylethyl isothiocyanate mercapturic acid were prepared as described (*16*).

Analysis of Glucosinolates in Vegetables. Glucosinolates were measured in extracts of vegetables after desulfation. Two different vegetable sample preparation protocols were used: (A) samples were snap-frozen in liquid nitrogen, freeze-dried and milled or (B) samples were snap frozen and milled in liquid nitrogen. A previous study showed only a slight difference in glucosinolate levels between these two different sample preparation protocols. Protocol A yielded lower levels of brassicins than protocol B but since levels of aliphatic and aromatic glucosinolates were not affected significantly, both protocols were used simultaneously. Samples were kept strictly frozen before extraction to prevent any myrosinase activity.

Glucosinolates were extracted using boiling methanol-water and trapped on quaternary amine solid phase extraction (SPE) columns (Mallinckrodt Baker B.V., Deventer, The Netherlands). Subsequently, glucosinolates were desulfated using aryl sulphate sulphohydrolase, and the corresponding desulfoglucosinolates were eluted with water.

Desulfoglucosinolates were separated on a Supelco Discovery C18 5 µm column (2.1 mm x 10 + 150 mm) with a water-acetonitrile (0.1% formic acid) gradient. Quantification was performed using diode-array detection and the relative response factors shown in **Table 1** (*17*). Sinigrin was used as an external standard. Linearity of analysis ranged from 0.5 to 500 µM desulfosinigrin and the valid range of analysis was 0.01 to 9.9 mmol/kg sinigrin.

Analysis of Isothiocyanates in Condiments. The condiments mustard, horseradish, garden cress, water cress, rocket and capers were eaten uncooked and were only analysed for their isothiocyanate content because glucosinolates were already converted into isothiocyanates, or would easily be converted upon thorough chewing. From garden cress, water cress, rocket, and capers only minimal sample material was available for freeze-drying, these samples could then easily defrost leading to loss of glucosinolates. Mustard and horseradish are products of crushing the seeds, respectively the root, of a plant and this crushing leads to complete conversion of glucosinolates to isothiocyanates.

Isothiocyanates were measured in extracts of condiments after conjugation to mercaptoethanol. Samples (2 to 5 g) were milled using an ultra-turrax after addition of an equal volume of water. Phenyl isothiocyanate (internal standard) and phosphate buffer (pH 7.0) were added before incubation with additional thioglucosidase. Isothiocyanates were extracted twice with 10 ml of dichloro methane. From the combined extracts, 10 ml was cleaned on Supelco Envi-Carb SPE columns and 1 ml of the eluent was pipetted into an HPLC crimpcap vial. To the vial, reagent was added (0.5 ml of 20 mM triethyl amine and 200 mM 2-mercaptoethanol in dichloro methane), the vial was capped and incubated at 30 °C for 60 minutes. Vials were decapped and dried under a stream of nitrogen and the residue was redissolved in 1 mL of acetonitrile:water 1:9 (v:v). Mercaptoethanol conjugates of isothiocyanates were separated on a Supelco Discovery C18 5 µm column (2.1 mm x 10 mm + 150 mm) with a water-acetonitrile gradient (**Figure 2**). Peak identification was performed using the retention times and the UV spectra obtained with diode-array detection. Quantification was performed at 271 nm with external standard calibration with phenyl isothiocyanate as internal standard. The performance of the method was accepted and characteristics were as follows. Linearity of analysis ranged from 0.1 to 100 µM, corresponding to a limit of detection of 0.01 mmol/kg fresh vegetable. The recovery of 0.25 µmol of 9 standards, added to 2 g of horseradish before extraction, was 94-103%.

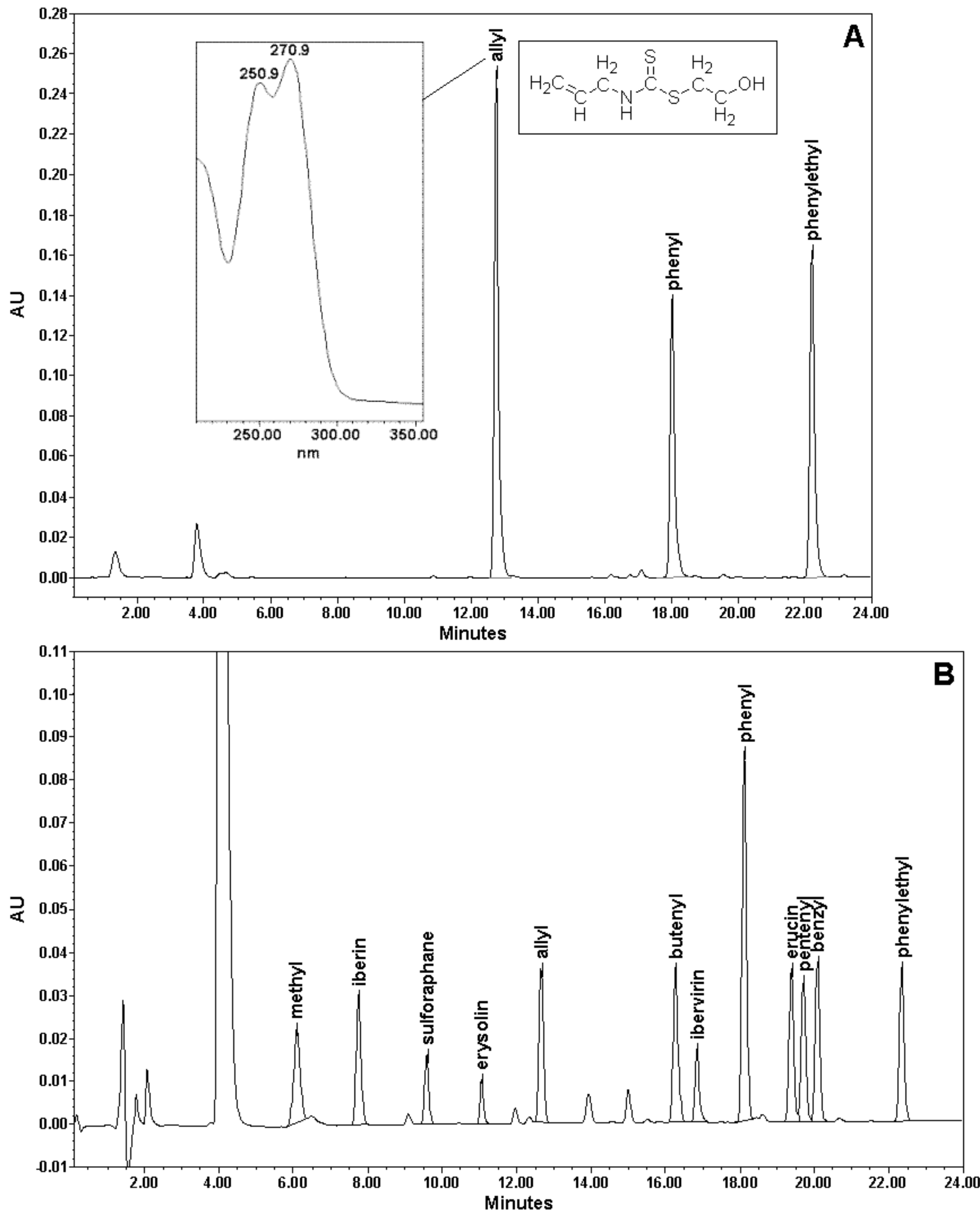

Figure 2. Typical chromatogram of a dichloromethane extract of horseradish containing (2-mercaptoethanol conjugates of) allyl and phenylethyl isothiocyanate. Inserted: UV spectrum and structural formula of S-(N-2-propenylthiocarbamoyl)-2-mercaptoethanol, the 2-mercaptoethanol conjugate of allyl isothiocyanate (**A**). Phenyl isothiocyanate was used as the internal standard. A typical chromatogram of 2-mercaptoethanol conjugates of a standard mixture of isothiocyanates (10 µM, **B**).

Analysis of Isothiocyanate Mercapturic Acids in Urine. Individual isothiocyanate mercapturic acids were determined in urine as described (*18*). Briefly, internal standard solution was added to urine, the samples were extracted on SPE columns and analysed on an HPLC-MS/MS system (LCQ ion-trap with electrospray ionization, Finnigan MAT). A widely used method of analysis measures the total of isothiocyanates and conjugates by cyclocondensation with a reagent which gives a sum result instead of results for individual isothiocyanates (*19,20*). Because of differences in biological activity between structurally different isothiocyanates we measured individual isothiocyanates in condiments and individual isothiocyanate mercapturic acids in urine.

Statistical and Pharmacokinetic Analysis. Results of urinary excretion were fitted to a one-compartmental model with the assumption of first-order isothiocyanate absorption and first-order isothiocyanate mercapturic acid excretion kinetics. For raw vegetables and condiments the initial dose (D_0) was taken as the amount of isothiocyanates, while for cooked vegetables D_0 was assumed to be the amount of glucosinolates. The bioavailability, F, was estimated by dividing the cumulative amount of a single isothiocyanate mercapturic acid excreted in 24 h by the consumed amount of isothiocyanate or glucosinolate.

For each isothiocyanate mercapturic acid, curves were fitted and visually checked for abnormalities. Excretion data were converted to their natural logarithm, the average excretion rate was plotted against the average time, the midpoint of the urine collection period, linearity was visually inspected and the slope (excretion rate constant, k_e) was calculated by linear regression. Glycosilated compounds like glucosinolates are generally poorly absorbed from the gut, glucosinolates from cooked vegetables first have to be converted in the colon into isothiocyanates. This will result in a delayed (lag-time) and slower absorption and therefore for cooked vegetables a first order kinetic model can not be used and the absorption rate constant (k_a) was not calculated. The k_a of isothiocyanates from raw vegetables was estimated from the intercept (i) using the formula: $e^{i} = \frac{(k_e * k_a * F * D_0)}{(k_a - k_e)}$ (*21*). From the analysis of metabolites in urine, as performed in this study, only a rough estimation of the absorption rate constant (k_a) can be made. Since the lag-time can not be calculated from urinary excretion data, the estimation of k_a includes the lag-time which will result in a lower apparent k_a. The data point of maximal excretion rate equals the time of maximal concentration in urine (t_{MAX}), t_{MAX} was not calculated from the fitted curve.

RESULTS

Vegetables and Condiments. Amounts of glucosinolates and isothiocyanates found in the consumed 13 vegetables and 6 condiments are presented in **Table 3**. The vegetables radish, broccoli, cauliflower, red cabbage, Pak Choi, and kohl rabi were eaten raw, glucosinolate levels ranged from 0.02 to 0.93 mmol/kg. In the vegetables Brussels sprouts, white cabbage, sauerkraut, green cabbage, kale, Chinese cabbage and rutabaga, that were eaten cooked, the glucosinolate levels after cooking ranged from 0.01 to 0.94 mmol/kg. The condiments mustard, horseradish, garden cress, water cress, rocket and capers were eaten uncooked, amounts of isothiocyanates varied from 0.06 to 49.3 mmol/kg. A typical chromatogram of the analysis of isothiocyanates in horseradish and of a mixture of isothiocyanates is shown in **Figure 2**.

Mercapturic Acids. Three volunteers consumed 13 different cruciferous vegetables and 6 different condiments and collected their urine in portions. Mercapturic acids of the following 8 isothiocyanates were measured in these urine portions: methyl, allyl, butenyl, pentenyl, benzyl, phenylethyl isothiocyanate, erucin, and sulforaphane. Not all possible mercapturic acids were analysed, e.g., the mercapturic acids from sulforaphene (radish) and iberin (broccoli and cabbages) were not determined in urine since reference compounds were not available. After consumption of the vegetables and condiments, 1-4 mercapturic acids per vegetable or condiment were found (**Table 3**). **Table 3** shows the relation between intake of isothiocyanates or glucosinolates and mercapturic acid excretion. Hydroxy alkenyl glucosinolates, like progoitrin, and indole glucosinolates, like glucobrassicin, are not hydrolysed into (stable) isothiocyanates, and metabolites were therefore not measured in urine. Isothiocyanate mercapturic acids were analysed in each individual spot urine sample and results were plotted as depicted in **Figure 3**. In urine samples collected shortly before consumption of the vegetable no isothiocyanate mercapturic acids were found. Each subject produced a sufficient number of spot urine samples to construct reliable excretion curves. Subject 1 produced on average (± SD) 3.5 ± 0.8 urine portions per 24 h; subject 2, 5.2 ± 0.9 and subject 3, 5.5 ± 1.8. In urine samples that were collected from 24 h until 48 h after ingestion only a subsequent 5% of isothiocyanate mercapturic acids was recovered so these samples were discarded.

Table 3. Isothiocyanate levels in six condiments and glucosinolate levels in 13 vegetables that were used for treatment with its biomarker found in urine

cruciferous vegetable, consumed amount and period (*genus*)	isothiocyanates[a]	mmol/ kg	found (n=3)[b]
capers, 10 g raw, august (*Capparis spinosa*)	methyl	0.34	3
garden cress, 7 g raw, august (*Lepidium sativum* L.)	benzyl	49.3	3
horseradish, 20 g raw, february (*Armoracia rusticana* G, M&S)	allyl	1.88	3
	phenylethyl	1.10	3
mustard, 9 g raw, june (*Brassica juncea* Coss.)	allyl	0.83	3
rocket, 14 g raw, august (*Eruca sativa* Mill.)	erucin	0.06	3
	sulforaphane	0.75	3
	2-OH-4-pentenyl [c]	2.77	ni
water cress, 10 g raw, september (*Nasturtium officinale* R.Br.)	phenylethyl	7.72	3
	unknown	±0.4	nd
	glucosinolates[a]		
radish, 50 g raw, june (*Raphanus sativus* L.)	glucoraphenin	0.93	nd
	gluconasturtiin	0.02	0
	indole	0.05	ni
broccoli, 162 g raw, june (*Brassica oleracea* L. *italica*)	glucoiberin	0.04	nd
	glucoerucin	<0.01	3, ne
	glucoraphanin	0.32	3
	indole	0.22	ni
cauliflower, 102 g raw, august (*B. oleracea* L. *botrytis cauliflora*)	glucoiberin	0.11	nd
	sinigrin	0.09	3
	glucoraphanin	0.02	3
	indole	0.18	ni
red cabbage, 100 g raw, august (*B. oleracea* L. *capitata* f. *rubra* Th.)	glucoiberin	0.03	nd
	sinigrin	0.03	3
	glucoerucin	<0.01	3, ne
	glucoraphanin	0.08	3
	gluconapin	0.06	3
	indole	0.09	ni
pak choi, 75 g raw, october (*Brassica campestris* L. *chinensis*)	glucoallysin	0.09	nd
	gluconapin	0.52	3
	glucobrassicanapin	0.44	3
		0.03	3
	gluconasturtiin	0.21	ni
	indole		
kohl rabi, 100 g raw, february (*Brassica oleracea* L. *gongylodes*)	glucoerucin	0.01	3
	glucoraphanin	0.01	3
Brussels sprouts, 200 g cooked, august (*B. oleracea* L. *gemmifera*)	glucoiberin	0.23	nd
	progoitrin	0.24	ni
	sinigrin	0.67	3
	glucoraphanin	0.07	3
	gluconapin	0.44	3
	indole	0.39	ni

Table 3. Continued.

cruciferous vegetable, consumed amount and period (*genus*)	glucosinolates[a]	mmol/ kg	found (n=3)[b]
white cabbage, 200 g cooked, jan. (*B. oleracea* L. *capitata* f. *alba* DC)	glucoiberin	0.42	nd
	progoitrin	0.09	ni
	sinigrin	0.59	3
	glucoerucin	<0.01	3, ne
	glucoraphanin	0.05	3
	gluconapin	0.10	3
	indole	0.26	ni
sauerkraut, 135 g cooked, september (*B. oleracea* L. *capitata* f. *alba*)	sinigrin/allylITC	<0.01	3, ne
green cabbage, 177 g cooked, january (*B. oleracea* L. *acephala*)	glucoiberin	0.86	nd
	progoitrin	0.05	ni
	sinigrin	0.94	3
	glucoraphanin	0.02	3
	gluconapoleiferin	0.07	nd
	indole	0.70	ni
(curly) kale, 107 g cooked, october (*B. oleracea* L. *acephala* var. *sab*)	glucoiberin	0.15	nd
	progoitrin	0.04	ni
	sinigrin	0.02	3
	glucoraphanin	0.02	3
	indole	0.07	ni
Chinese cabbage, 200 g cooked, october (*Brassica chinensis* L.)	progoitrin	0.06	ni
	glucoraphanin	0.02	3
	glucoallysin	0.09	nd
	gluconapin	0.03	3
	glucobrassicanapin	0.07	3
	gluconasturtiin	0.03	3
	indole	0.16	ni
rutabaga, 220 g cooked, january (*Brassica napus* L. *napobrassica*)	glucoiberin	0.01	nd
	progoitrin	0.13	ni
	glucoerucin	0.02	3
	gluconapoleiferin	0.03	nd
	glucoallysin	0.04	nd
	gluconasturtiin	0.11	3
	indole	0.17	ni

[a]Condiments were analysed for their isothiocyanate content because glucosinolates were already converted into isothiocyanates, or would easily be converted upon thorough chewing. [b]Isothiocyanate mercapturic acid was found in 24 h urine of all volunteers, ni = no isothiocyanate is formed after hydrolysis of the glucosinolate, nd = not determined, ne = not expected. [c]This isothiocyanate was tentatively assigned 2-hydroxy-4-pentenyl.

Kinetics and Bioavailability. The calculated k_a from vegetables and condiments that were consumed raw was, on average, 2.0-3.0 h^{-1}. The excretion rate constant (k_e) ranged from 0.18 to 0.33 h^{-1} ($t_{½}$ = 2.1-3.9 h) for both raw and cooked vegetables (**Table 4**), indicating that the same route of excretion was followed.

In **Figure 3**, the average excretion rates of two different isothiocyanate mercapturic acids in urine after the consumption of 4 different vegetables by subjects 1, 2 and 3 are plotted against the average time (midpoint of the collection period). Sulforaphane mercapturic acid was excreted after consumption of cooked Chinese cabbage and raw broccoli and allyl isothiocyanate mercapturic acid was excreted after consumption of cooked Brussels sprouts and mustard. As shown in **Figure 3D** and **3B**, respectively, the average t_{MAX} of allyl isothiocyanate from mustard and of sinigrin derived allyl isothiocyanate from Brussels sprouts was 3.1 h and 8.5 h, respectively. With Brussels sprouts only the glucosinolate sinigrin is ingested, while with mustard only the allyl isothiocyanate. After consumption of raw vegetables, isothiocyanate mercapturic acids appeared fast in urine (average t_{MAX} = 4h) with high excretion levels (average F = 8.2-113%). Due to metabolic reduction of conjugates of sulforaphane into conjugates of erucin, F values of up to 464% were found. For cooked vegetables, isothiocyanate mercapturic acids were excreted several hours after consumption (average t_{MAX} = 6h) and the concentration in urine was lower (average F = 1.8-43%) than for raw vegetables.

The concentration of isothiocyanates in urine was increased for a longer time after consumption of cooked vegetables, compared to raw vegetables and condiments, because of prolonged absorption (**Figure 3)**. The total amount of isothiocyanate conjugates, however, was lower after consumption of cooked vegetables, compared to raw vegetables and condiments. The bioavailability of allyl isothiocyanate from mustard is similar for all three subjects and is over 100% (**Table 4**).

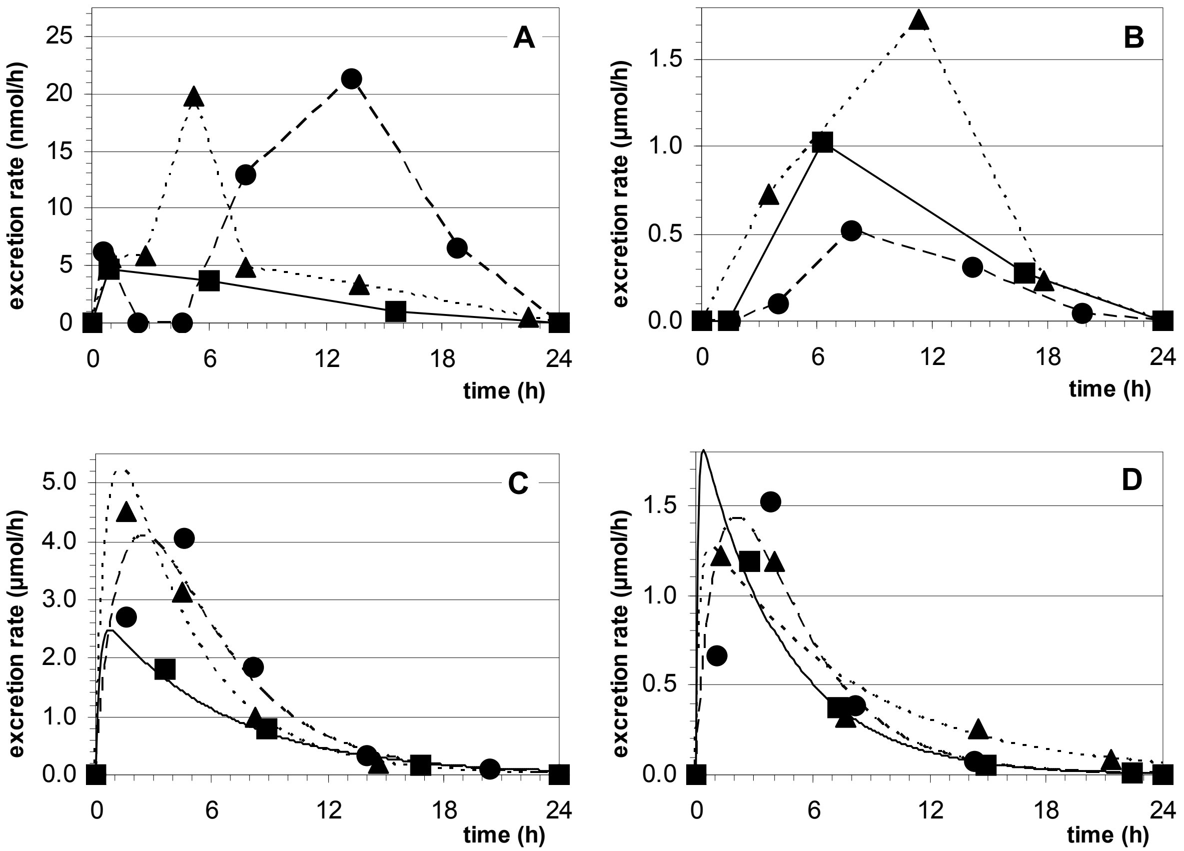

Figure 3. Average excretion rates for persons 1 (—■—), 2 (– • –), and 3 (--▲--) for sulforaphane mercapturic acid after consumption of 200 g of cooked Chinese cabbage (**A**, raw data in nmol/h) and 200 g of cooked Brussels sprouts (**B**, raw data in µmol/h) and for allyl isothiocyanate mercapturic acid after consumption of 200 g of raw broccoli (**C**, fitted curve) and 9 g of mustard (**D**, fitted curve).

Table 4. Kinetics of isothiocyanates from raw and cooked crucifers, measured as their derived isothiocyanate mercapturic acids in spot urine samples from three human volunteers

raw cruciferous vegetables	excreted mercapturic acid	k_a (h^{-1}) avg (SD)	k_e (h^{-1}) avg (SD)	t_{MAX} (h) avg (SD)	F (%) avg (SD)
mustard	allyl	5.2 (4.7)	0.21 (0.08)	3.1 (0.6)	123 (14)
horseradish	allyl	1.3 (0.4)	0.31 (0.13)	2.7 (0.8)	96 (8.3)
	phenylethyl	1.1 (0.5)	0.28 (0.15)	2.7 (0.8)	95 (6.6)
garden cress	benzyl	0.8 (0.5)	0.22 (0.07)	5.3 (1.6)	14 (7.8)
water cress	phenylethyl	1.2 (1.2)	0.27 (0.07)	4.6 (2.5)	50 (23)
rocket	erucin	2.5 (2.0)	0.24 (0.08)	4.1 (2.0)	.
	sulforaphane	2.0 (1.3)	0.19 (0.04)	4.1 (2.0)	72
	combined[b]				94 (28)
capers	methyl	1.2 (0.7)	0.25 (0.06)	4.8 (3.7)	71 (41)
radish	none measured				
broccoli	erucin[c]			3.7 (0.8)	29 (11)
	sulforaphane	2.4 (2.2)	0.22 (0.05)	3.7 (0.8)	50 (13)
cauliflower	allyl	2.6 (1.7)	0.21 (0.01)	2.9 (1.4)	113 (44)
	erucin[c]			2.9 (1.4)	43 (40)
	sulforaphane	2.1 (0.8)	0.23 (0.07)	2.9 (1.4)	81 (44)
red cabbage	allyl	1.7 (0.5)	0.20 (0.07)	3.6 (1.1)	46 (25
	butenyl	1.7 (0.9)	0.28 (0.06)	3.6 (1.1)	24 (7.5)
	erucin[c]			4.4 (1.4)	25 (2.1)
	sulforaphane	2.2 (1.7)	0.24 (0.04)	3.6 (1.1)	71 (11)
pak choi	butenyl	2.0 (0.4)	0.27 (0.05)	2.2 (0.2)	8.2 (3.1)
	pentenyl	1.5 (0.3)	0.33 (0.07)	2.2 (0.2)	8.2 (3.1)
kohl rabi	erucin	0.5 (0.3)	0.22 (0.06)	3.7 (0.6)	66 (29)
	sulforaphane	1.6 (1.7)	0.18 (0.01)	3.7 (0.6)	108 (65)
cooked cruciferous vegetables	**excreted mercapturic acid**	**k_a (h^{-1}) avg (SD)**	**k_e (h^{-1}) avg (SD)**	**t_{MAX} (h) avg (SD)**	**F (%) avg (SD)**
Brussels sprouts	allyl	-[a]	0.26 (0.11)	8.5 (2.6)	9.3 (5.8)
	butenyl		0.28 (0.14)	8.5 (2.6)	3.3 (2.3)
	sulforaphane		0.23 (0.07)	8.5 (2.6)	5.2 (2.3)
white cabbage	allyl	-	0.31 (0.14)	5.2 (0.8)	12 (9.0)
	butenyl		0.29 (0.10)	5.2 (0.8)	12 (8.5)
	erucin[c]			5.2 (0.8)	16 (16)
	sulforaphane		0.30 (0.13)	5.2 (0.8)	21 (13)
sauerkraut	allyl (*F in µmol*)	-	0.25 (0.06)	3.8 (0.5)	*0.8 (0.3)*
green cabbage	allyl	-	0.27 (0.06)	7.4 (2.0)	7.3 (6.7)
	sulforaphane		0.20 (0.06)	7.4 (2.0)	4.3 (2.4)
(curly) kale	allyl	-	0.33 (0.04)	6.8 (1.6)	43 (44)
	sulforaphane		0.29 (0.11)	6.8 (1.6)	18 (19)
Chinese cabbage	butenyl	-	0.19 (0.02)	5.3 (0.7)	4.2 (4.1)
	pentenyl		0.23 (0.01)	5.3 (0.7)	1.8 (1.6)
	sulforaphane		0.18 (0.05)	5.3 (0.7)	3.6 (2.2)
	phenylethyl		0.25 (0.05)	5.8 (0.5)	4.1 (1.5)
rutabaga	erucin	-	0.19 (0.05)	7.8 (1.1)	5.2 (1.0)
	phenylethyl		0.18 (0.03)	7.8 (1.1)	1.8 (0.2)

[a] Absorption rate constants for isothiocyanates derived from glucosinolates in cooked vegetables were not calculated from urine data since these data do not follow first order absorption kinetics. Furthermore, the conversion of glucosinolates into isothiocyanates in the colon results in a lag-time in absorption. [b]Excretion of erucin and sulforaphane mercapturic acids were combined because of metabolic interconversion. [c]Erucin was not taken in (<0.01 mmol/kg) but erucin mercapturic acid was excreted because of metabolic conversion from sulforaphane. Erucin mercapturic acid is expressed as % of the dose of glucoraphanin.

DISCUSSION

This study shows that isothiocyanate mercapturic acids in urine reflect the dose of glucosinolates and isothiocyanates absorbed after a meal containing cruciferous vegetables (n=3). After the consumption of 13 different vegetables and 6 condiments on separate days, a selection of 8 isothiocyanate mercapturic acids was found in the urine portions collected for 24 h. The results of this study show that it is possible to make a distinction between cabbages by analyzing the glucosinolate pattern since each crucifer contains a number of glucosinolates with a different side chain and varying amount. Our results show that isothiocyanate mercapturic acids in urine reflect the pattern of side chains and amounts of glucosinolates present in the vegetable or condiment that was consumed. We confirmed the finding that condiments contain less different glucosinolates but in higher concentrations than vegetables. All mercapturic acids that were detected were derived from isothiocyanates that were expected from the consumed crucifer, with some minor inconsistencies that will be discussed later. Mercapturic acids can not easily be validated as a biomarker of intake of a specific cruciferous vegetable because of the variation in glucosinolate content in different vegetable subspecies. Nevertheless, this study clearly shows that mercapturic acids can be used as a marker of the active dose of isothiocyanates that is absorbed.

Bioavailability. In this study we show that there are substantial differences in bioavailability, absorption and excretion kinetics between glucosinolates from cooked vegetables, and isothiocyanates from raw vegetables and condiments (**Table 4**). The amount of isothiocyanates absorbed from cooked vegetables was 2-6 times lower than from (thoroughly chewed) raw vegetables. This difference can not be attributed to loss of glucosinolates due to cooking since the bioavailability was calculated relative to the content of glucosinolates present in cooked vegetables. The bioavailability of isothiocyanates from condiments was almost 100%. The only exceptions were benzyl isothiocyanate from garden cress, and to a lesser extent phenylethyl isothiocyanate from water cress, which seem difficult to liberate from the plant cells by chewing since the bioavailability was on average 14% and 50%, respectively. Butenyl and pentenyl isothiocyanate from raw pak choi were also poorly absorbed, the bioavailability was 8% on average. The absorption of butenyl and pentenyl isothiocyanate from cooked Chinese cabbage was 4 and 2% on average, respectively, which is low as expected for cooked vegetables. The overall average bioavailability for raw vegetables was 61% (range 8.2-113%) and for cooked vegetables was 10% (range 1.8-43%).

This indicates that from raw crucifers complete uptake of isothiocyanates is possible and that, apparently, after cooking only 10% is bioavailable. Conaway et al. (*22*) showed that after the consumption of fresh and cooked broccoli, 32% and 10% of the dose of isothiocyanates was excreted in 24 h urine, respectively. They (*22*) measured metabolites in the urine by cyclocondensation with 1,2-benzene-dithiol. This is a generic method which results in a sumparameter for all isothiocyanates so kinetic results for individual isothiocyanates could not be calculated. The bioavailability values were comparable to our results.

Hydrolysis of glucosinolates to isothiocyanates is dependent on the enzyme myrosinase which is inactivated upon cooking of the vegetable. The thioglucosidase enzyme which is present in the colon is less effective. When eating crucifers the intake thus consists of glucosinolates as well as isothiocyanates. The variation in bioavailability between the three volunteers can partly be explained by the difference in intensity of chewing (see e.g. garden and water cress) and by the difference in colonic microflora. The difference in activity of the colonic microflora between subjects could result in interindividual variation where one subject could metabolise 3 times more glucosinolates into isothiocyanates than another subject (*11*). The colonic microflora could also have a negative effect on the proportion of isothiocyanates available for absorption, as demonstrated by Rouzaud et al. (*23*). They found that in rats harbouring a whole human faecal flora, compared to germ-free rats, the administered dose of benzyl glucosinolate was completely converted into non-isothiocyanate metabolites.

Metabolism. Isothiocyanates are conjugated in a phase II reaction to glutahione, catalysed by glutathione S-transferase, followed by conversion to the mercapturic acid. Phase II biotransformation is a fast reaction and therefore phase I biotransformation was not expected, the metabolic rate constant (k_m) was assumed to be zero. The cellular enzyme beta-lyase converts a mercapturic acid into a free thiol compound by cleavage of the N-acetyl-cysteine group, leaving the sulfur. Beta-lyase activity is present in liver and kidney but is not a logical step in metabolism and was not determined. Oxidation of sulfur in e.g. erucin derived mercapturic acid could occur but was not expected since conjugation to glutathione is fast.

Interestingly, mercapturic acids from both erucin and sulforaphane were found after consumption of rocket, broccoli, red and white cabbage although broccoli, red and white cabbage did not contain glucoerucin (<0.01 mmol/kg). These results show that the reduction of sulfinyl, changing sulforaphane into erucin, takes place. This is also confirmed

in rats by Kassahun et al. (*24*) who showed that 12% of a dose of sulforaphane was excreted as erucin instead of sulforaphane mercapturic acid. Oxidation of sulphur in erucin also takes place, since after administration of erucin, 67% of the dose was excreted as sulforaphane mercapturic acid (*24*).

Kinetics. In our study, in which vegetables were eaten during lunch, the lagtime of the appearance of isothiocyanate mercapturic acid in urine was 4 h on average. Isothiocyanate conjugates in the body reach higher levels but are faster excreted after consumption of raw vegetables and condiments. The results show that levels of isothiocyanate conjugates in the body were longer at non-zero levels after cooked vegetables were consumed.

Ye et al. (*25*) described the pharmacokinetics of broccoli sprout isothiocyanates in humans also using a cyclocondensation sumparameter assay (described in bioavailability). They reported that an average of 58% of the ingested dose of isothiocyanates was excreted in urine collected for 8 h with an elimination half-life of 1.8 h (*25*). The difference between our study and the study of Ye et al. is that we found somewhat longer elimination half-lifes, 2-4 h on average. Shapiro et al. (*26*) showed that the excreted cyclocondensation product was related to the glucosinolate/isothiocyanate profiles administered. Furthermore, consumption of graded doses of isothiocyanates resulted in the rapid excretion of 42% of urinary conjugates with first-order kinetics with an excretion rate constant of approximately 0.42 h^{-1}, which is a faster than the excretion rate we found, 0.18-0.33 h^{-1}. Moreover, the conversion of glucosinolates was negligible after bowel microflora were reduced (*26*). Further studies of Shapiro et al. (*27*) showed even a higher bioavailability, measured by excreted isothiocyanate conjugates in 72 h urine. From broccoli sprouts that were treated with myrosinase, 80% of the dose of isothiocyanates was excreted, and 12% of the dose of glucosinolates from cooked broccoli sprouts was excreted as isothiocyanate conjugates. They (*27*) also conducted dose-response experiments but no further kinetic data was obtained.

In conclusion, isothiocyanate mercapturic acids excreted in urine reflect the intake of glucosinolates from cooked vegetables and of isothiocyanates from raw vegetables and condiments. Interindividual variation occurs and might be explained by differences in the extent of chewing, absorption, conversion, colonic degradation and/or metabolism. Urinary isothiocyanate mercapturic acids can be used as a biomarker to reflect the active dose of isothiocyanates absorbed.

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Chapter 5

Potency of Isothiocyanates to Induce Luciferase Reporter Gene Expression via the Electrophile-responsive Element from Murine Glutathione S-transferase Ya

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ABSTRACT

Isothiocyanates are electrophiles that are able to induce phase II biotransformation enzyme gene expression via an electrophile-responsive element (EpRE) in the gene regulatory region. To study the potency of different isothiocyanates to induce the expression of EpRE-regulated genes, a Hepa-1c1c7 luciferase reporter cell line was constructed and exposed to structurally different isothiocyanates. The reporter cell line, EpRE(mGST-Ya)-LUX, was constructed using the EpRE from the regulatory region of the mouse glutathione S-transferase Ya gene. Isothiocyanates containing a methyl-sulphur side chain, e.g. sulforaphane, showed a lower EC50 (0.8-3.2 µM) and a comparable induction factor (17-22.4) compared to the structurally different isothiocyanates containing an alkyl or aromatic side chain, e.g. allyl and phenylethyl isothiocyanate (EC50 3.9-6.5 µM, induction factor 17.5-23). After 24 h of exposure, on average (± SD) 23 ± 5 % of the isothiocyanate was found in the cells and 77 % in the cell medium. Isothiocyanates prove to be strong inducers of electrophile-responsive element-mediated gene expression at physiological concentrations. The here described luciferase reporter cell line is a suitable assay to measure the potency of compounds to induce EpRE-regulated gene expression.

INTRODUCTION

The human diet contains a number of ingredients that were reported to protect against several types of cancer. For example, a large number of epidemiological studies have shown that the consumption of vegetables and fruits is inversely associated with the risk of cancer (*1*). Data from seven prospective cohort studies and 87 case-control studies showed that especially consumption of Brassica (cruciferous) vegetables (all cabbage and kale species, as well as broccoli, cauliflower, turnip and radishes are part of this family) reduce the risk of cancer (*2*). The cancer-protective properties of cruciferous vegetables are attributed to the fact that they are a unique source of glucosinolates which, upon chewing or cutting, are converted into isothiocyanates (*3*).

One of the mechanisms that has been proposed in the chemopreventive action of vegetables and fruits, is induction of Phase II detoxifying enzymes, such as glutathione S-transferases (GSTs), NAD(P)H:quinone oxidoreductase 1 (NQO1) and superoxide dismutase (SOD)(*4*). These enzymes are capable of detoxifying strong electrophiles such as quinones and reactive oxygen species. GST conjugates glutathione to certain electrophiles including metabolites of hydrophobic compounds like polycyclic aromatic hydrocarbons. All eukaryotes possess several GST iso-enzymes, both in the cytosol and bound to membranes. The expression of phase II biotransformation enzyme genes plays a critical role in the sensitivity of cells to many toxic compounds (*5*). The chemical induction of Phase II enzymes was found to be often under control of a common transcription enhancer element, called antioxidant-responsive (ARE) or electrophile-responsive element (EpRE)(*6*).

The core sequence of nucleotides of the EpRE is GTGACnnnGC (*7*). This element was identified in the upstream regions of, amongst others, the human glutathione S-transferase pi gene, the NQO1 gene, the heavy subunit of the human glutamylcysteine synthetase gene, and the rat and mouse glutathione S-transferase Ya gene. Nevertheless, the EpREs from the various enzymes and species differ outside the core sequence according to the consensus sequence: TMAnnRTGAYnnnGCRwww (*8*). However, a recent mutation study (*9*) revealed a novel functional EpRE (5'-gagTcACaGTgAGtCggCAaaatt-3') that was slightly different form the previously published consensus sequence. Transcription factor Nrf2 is essential for both constitutive expression of NQO1 and its induction by sulforaphane (*9*). Nrf2 constitutively associates with the NQO1 EpRE in low amounts. Under uninduced conditions, Nrf2 interacts with Keap1 and is therefore subject to a more rapid proteasomal degradation than it is in oxidatively stressed cells where this interaction is counteracted (*10*).

Direct adduct formation between isothiocyanates and Keap1 has been demonstrated, and is hypothesised to lead to Nrf2 activation (*11*) just as has been found for other electrophilic Nrf2 activators (*12*). Especially remarkable is the diversity of chemical structures among transcription inducers acting through the EpRE (*8, 13*).

To study the induction potential of not only purified compounds but also extracts from vegetables, or complete diets, and for instance new functional foods, two stable EpRE-LUX reporter gene cell lines were developed recently (*13*). These reporter cell lines allow to screen food components for the ability to modulate EpRE-regulated gene expression without interference by other signal transduction pathways.

In the present paper, we studied the potency of 9 structurally different isothiocyanates to induce luciferase reporter gene expression using the EpRE(mGST-Ya)-LUX reporter cell line. Isothiocyanates are a category of compounds known to be inducers of EpRE-controlled gene transcription. The dose dependency of the response was tested and it was investigated whether different compounds have intrinsically similar or different induction potencies. Each of the tested isothiocyanates is typical for one or several cruciferous vegetables or condiments (see **Table 1**). The fraction of isothiocyanate present in the cell after 24 h of exposure was measured to gain insight into the internal cellular exposure and to determine whether the cellular uptake is different for the tested isothiocyanates.

MATERIALS AND METHODS

Chemicals. Methyl, allyl, benzyl and phenylethyl isothiocyanate were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Ibervirin, iberin, erucin, sulforaphane and erysolin were synthesised as described before (*14, 15*). In short, dibromobutane was added to phthalimide potassium salt. The obtained *N*-(bromobutyl)-phthalimide was added to a solution of sodium methylmercaptide yielding *N*-(methylthiobutyl)-phthalimide. Hydrazinolysis yielded methylthiobutylamine, which was distilled (b.p. 87 °C at 18 mBar). Methylthiopropylamine was purchased from Acros (Geel, Belgium). Methylthiopropylamine and methylthiobutylamine were converted to their corresponding isothiocyanate (ibervirin and erucine) by adding di-2-pyridyl thionocarbonate. Through distillation, ibervirin (b.p. 165 °C at 15 mBar) and erucine (b.p. 136 °C at 7 mBar) were purified. Ibervirin and erucin were oxidised to iberin and sulforaphane, respectively, using *meta*-chloroperbenzoic acid with a short reaction time, erysolin was obtained from erucine after overnight oxidation.

Table 1. Structures of the isothiocyanate compounds used in this study. Isothiocyanates are present in a wide range of vegetables and condiments and contain a variable side chain.

nr	MW[a]	name	main dietary source
1	73.12	methyl isothiocyanate	capers
2	99.15	allyl isothiocyanate	cabbage, mustard
3	149.22	benzyl isothiocyanate	garden cress
4	163.24	phenylethyl isothiocyanate	water cress
5	147.26	ibervirin	*Iberis*[b]
6	163.26	iberin	(green) cabbage
7	161.28	erucin	rocket, rutabaga, turnip
8	177.29	sulforaphane	cabbage, broccoli
9	193.29	erysolin	*Erysimum*[b]

[a] The Molecular Weight of each compound is indicated in g/mol. [b] Botanical plants, not consumed as part of a normal diet.

Purity and concentration of the stock solutions was confirmed by high performance liquid chromatography (HPLC) analysis and was found to be > 99 % for all synthesised compounds except for erysolin which contained 14 % of sulforaphane. Dimethyl sulfoxide (DMSO) was purchased from Janssen Chimica (The Netherlands) and toluene-3,4-dithiol was purchased from Fluka (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands).

Construction and transfection of an EpRE-regulated luciferase reporter gene vector. Two stable EpRE-LUX reporter gene cell lines were developed recently (*13*). In short, to construct the electrophile-responsive reporter vector pTI(mGST-Ya-EpRE)Luc+, a double-strandend TI oligonucleotide (containing a TATA-box and basic initiator) was inserted in the pSP72 plasmid, from which a suitable TI containing restriction fragment was excised. This fragment was inserted in the pGL3-Basic plasmid (Promega) and a double-stranded synthetic oligonucleotide containing the electrophile-responsive element from the regulatory region of the mouse glutathione S-transferase (mGST-Ya-EpRE) was inserted in this pGL3-Basic-TI construct. The obtained vector was transfected into mouse Hepa-1c1c7 cells and stable clonal cell lines were designated EpRE(mGST-Ya)-LUX.

Exposure. For the luciferase induction experiments, EpRE(mGST-Ya)-LUX cells were seeded in white 96-wells plates with clear flat bottoms (Corning Incorporated, Cambridge, USA) in 100 µl of medium at a density of 10,000 cells/well, and were seeded in parallel in 24-wells plates in 400 µl of medium at a density of 40,000 cells/well, and allowed to attach for 24 h. The cells in the white 96-wells plates were used for the luciferase induction assay. Medium and cells in the 24-wells plates were used for chemical analysis. One day after seeding, the medium was replaced with αMEM containing the chemicals of interest. All test compounds were freshly dissolved in DMSO with always a final concentration of DMSO in the medium of 0.3 %. An equivalent amount of the solvent (DMSO) was added to control cells. To determine the EC_{50} of induction, methyl, allyl, benzyl, or phenylethyl isothiocyanate, ibervirin, iberin, erucin, sulforaphane or erysolin was added to the medium separately in duplicate wells at 5-7 concentrations (0.3-60 µM final concentration).

Luciferase induction assay. Following a 24 h incubation in white 96 well plates, cells were harvested and luciferase expression was measured using a luminometer (Labsystems Luminoscan RS) as follows. Cells were washed once with 100 µl of 0.5 x phosphate buffered saline (PBS, Life Technologies ltd., Paisley, Scotland) followed by the addition of 30 µl of low salt buffer (LSB, 2 mM dithiothreitol, 2 mM 1,2,-diaminocyclohexane-N,N,N',N'-tetra acetic acid, 10 mM Tris pH 7.8). Cells were lysed by incubation on ice for 15 minutes and subsequent freezing at –80°C for at least 1 h. After thawing, shaking, and equilibrating at room temperature, the plates were mounted in the luminometer and upon injection of 100 µl of flash mix (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 2.0 mM dithiothreitol, 470 µM luciferine, 5.0 mM ATP) per well, luciferase activity was immediately determined and expressed as Relative Light Units (RLUs). Before

measuring the next well, 100 µl of 0.2 M NaOH was injected to quench the remaining signal in the well, thus preventing signal contamination between neighbouring wells. Induction factors were subsequently calculated relative to solvent controls.

Isothiocyanate levels in cells and culture medium. Isothiocyanates in cell medium in 24-wells plates were analysed directly after addition to the culture medium and after 24 h of exposure, and in addition in cell lysate prepared after 24 h exposure, at two levels (10 and 30 µM). After removal of the culture medium (400 µl) for further analysis, cells were washed once with 600 µl of 0.5 x phosphate buffered saline (PBS), followed by the addition of 200 µl of low salt buffer (LSB). Cells were lysed by incubation on ice for 15 min and subsequent freezing at –80°C for at least 1 h. Culture medium and PBS were combined and the concentration of isothiocyanate and its conjugates was determined, LSB containing cell lysate was analysed similarly. The percentage of isothiocyanate in the cell was calculated as the amount present in cells (LSB) divided by the total amount found in medium (culture medium + PBS) and in the cells, as measured after 24 hours of exposure.

Isothiocyanates and their conjugates react quantitatively with toluene-3,4-dithiol, yielding 5-methyl-1,3-benzodithiole-2-thione and the corresponding free amine. This is a modification of the method described by Zhang et al. (*16*), using toluene-3,4-dithiol instead of 1,2-benzenedithiol for its higher boiling point and therefore less unpleasant odour. To 300 µl of medium or cell lysate sample in an 1.5 ml HPLC vial, 600 µl of reagent (1.25 ml of toluene-3,4-dithiol dissolved in 1 l of methanol) and 300 µl of buffer (6.5 ml of formic acid and 50 ml of triethylamine dissolved in 1 l of methanol) were added, and the vial was capped and heated for 60 min at 65 °C. After clearance by centrifugation, 100 µl of the supernatant was injected on a Nucleosil C18 column (150 x 4.6 mm, 3 µm particle size) using a Waters 2690 HPLC and the reaction product, 5-methyl-1,3-benzodithiole-2-thione, was quantified at 365 nm with a photo-diode array (PDA) detector. The limits of detection and quantification were 0.1 and 1 µM, respectively.

Data processing. A sigmoidal dose-response equation with variable slope ($Y = B + \frac{T - B}{1 + 10^{(\log EC_{50} - X) * HillSlope}}$) was fitted to the data using Graphpad Prism software (Graphpad Prism) to obtain EC_{50} values. ClogP was calculated using shareware (Daylight Chemical Information Systems at www.daylight.com) (**Table 2**).

RESULTS

Luciferase induction. The induction of luciferase reporter gene expression via the electrophile responsive element (EpRE) was dose-dependent for all compounds tested. The EC_{50} was different and the maximum induction factor was comparable for structurally different isothiocyanates. We show that methyl sulphur alkyl isothiocyanates, like sulforaphane, display a lower EC_{50} value (0.8-3.2 μM) and an induction factor comparable to isothiocyanates containing an alkyl or aromatic side chain, like allyl and phenylethyl isothiocyanate (EC_{50} 3.9-6.5 μM; **Figure 1** and **Table 2**). We found induction factors for methyl sulphur alkyl isothiocyanates ranging from 17-22.4, where erysolin showed a lower induction factor of 11.7. Isothiocyanates containing an alkyl or aromatic side chain displayed an induction factor of 17.5-23, with the exception of methyl isothiocyanate, which gave an induction factor of 7.1. The deviating results for erysolin and methyl isothiocyanate could not be explained by quenching of the luciferase signal, since no quenching by isothiocyanates (up to 30 μM) of the activity of purified luciferase was found (data not shown). Exposure of Hepa-1c1c7 cells to isothiocyanates at a concentration of 30 μM during 24 h led to visible cytotoxicity for most compounds, and a concentration of 100 μM led to complete cell death for all compounds. Still, induction factors were higher at 30 μM than at 10 μM (**Figure 1**).

Isothiocyanate levels. The amount of isothiocyanates present in medium and in cells after 24 h of exposure was analysed. Roughly three quarters (77%) was found in cell medium and one quarter (23%, with an SD of 5%) was found inside the cells (**Table 2**). Analysis of isothiocyanates in cell medium immediately after the addition to the cells confirmed the presence of the expected amount of compound. The volatility of the tested isothiocyanates is different, as can be concluded from their boling points as listed in **Table 2**, but no relation with the response was found.

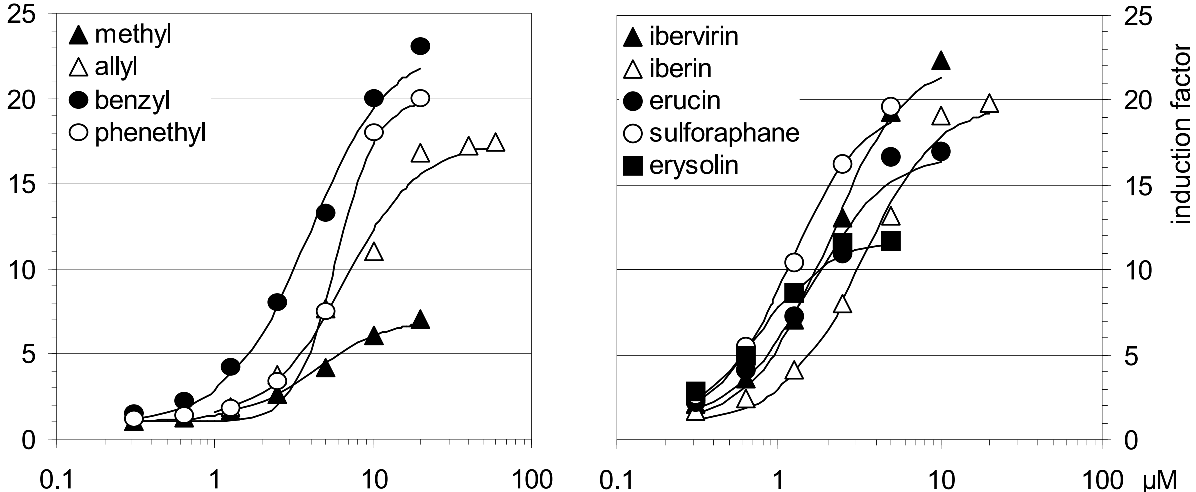

Figure 1. Induction of electrophile-responsive element (EpRE)-controlled luciferase reporter gene expression by 9 isothiocyanates, expressed as the induction factor relative to a solvent control.

Table 2. Results of luciferase induction, chemical analysis of the distribution of isothiocyanate between cell and culture medium, and physical parameters of the isothiocyanates tested.

isothiocyanate	induction factor	EC_{50} (µM)	average amount in cells	ClogP (o/w) [a]	boiling point in °C (reduced pressure in hPa)
1 methyl	7.1	4.3	28 %	1.17	119
2 allyl	17.5	6.5	22 %	1.94	152
3 benzyl	23.0	3.9	24 %	3.20	257
4 phenylethyl	20.0	5.8	16 %	3.26	140 (15)
5 ibervirin	22.4	2.0	23 %	1.94	165 (15)
6 iberin	19.9	3.2	31 %	0.17	unknown
7 erucin	17.0	1.6	16 %	2.37	136 (16)
8 sulforaphane	19.7	1.2	26 %	0.15	165 (0.2)
9 erysolin	11.7	0.8	21 %	0.03	unknown

[a] Calculated (log) partition coefficient octanol / water.

DISCUSSION

Hepa-1c1c7 mouse hepatoma cells transfected with the constructed luciferase reporter gene plasmid pTI(mGST-Ya-EpRE)LUX can be used to study electrophile-responsive element (EpRE)-mediated induction of gene expression by compounds. The performance of the assay for isothiocyanates was established by measuring the EC_{50}, induction factor, possible quenching of the luciferase signal and cytotoxicity of different isothiocyanates. We found dose-dependent luciferase induction for all isothiocyanates that were tested.

Structurally different isothiocyanates displayed different EC_{50} values. Methyl-sulphur alkyl isothiocyanates, like sulforaphane, resulted in lower EC_{50} values compared to isothiocyanates with an alkyl or aromatic side chain, like allyl and benzyl isothiocyanate. A comparable maximal response (induction factor) and shape of the dose-reponse curves was found, with the exception of methyl isothiocyanate and erysolin.

Care should be taken, because these compounds are reactive and could quench luciferase activity. Another limitation of the assay would be the cytotoxicity of the test compounds. Although 30 μM of isothiocyanate led to visible cytotoxicity, the induction factor was nevertheless higher than at 10 μM. Although a certain level of cell death was observed at 30 μM, the induction of luciferase expression is apparently sustained at a level which still exceeds the level at 10 μM. The cytotoxicity of all compounds is confirmed by Tawfiq et al. (*17*) who found that glucosinolates at 30 μM were not toxic to Hepa-1c1c7 cells, but isothiocyanates and nitriles, at 30 μM, were.

Isothiocyanates are readily dissolved in culture medium and do not stick to the (plastic) wall of the well. This is supported by the finding that the major part of isothiocyanate was found in culture medium (data not shown). The added amount of isothiocyanates, whether chemically free or bound to the cell or otherwise, is entirely detected by the applied analysis method, because the reagent on which this method is based, toluene-3,4-dithiol, reacts with all isothiocyanates and conjugates (*16*).

Isothiocyanates are moderately lipophilic as can be seen from their corresponding ClogP (**Table 2**). Iberin, sulforaphane, and erysolin are relatively hydrophilic (ClogP 0.03-0.17) whereas the other isothiocyanates are not (ClogP 1.17-3.26). Isothiocyanates are liquid (oil) at room temperature, with the exception of methyl isothiocyanate and erysolin which are solid. No correlation was found between the ClogP of the isothiocyanates and the EC_{50} ($r^2 = 0.54$), but from the data a trend can be seen where more hydrophilic isothiocyanates yield a lower EC_{50}.

It seems that induction of EpRE-controlled gene expression by isothiocyanates is optimal at 10 μM and that at a low concentration (1 μM) expression is slightly induced. The

amount of glucosinolates, the precursor of isothiocyanates, in vegetables and condiments ranges from several micromoles to 5 millimoles/100 g fresh material (*15*). With a meal rich in cruciferous vegetables, 1-100 µmol of a single isothiocyanate is ingested. Assuming a volume of distribution of 25 l, the resulting concentration of isothiocyanate (free and conjugated) in the human body will then be approximately 0.04-4 µM. Excretion rates are high ($t_{½}$ = 2.1-3.9 h, *15*) so when data from this murine reporter gene plasmid transfected in mouse hepatoma cells is applicable to humans, this indicates that sometimes, but not after every meal, physiologically active doses of isothiocyanates are ingested. In the small or large intestine, concentrations reached upon oral intake can be even higher. If for instance 200 g of broccoli is consumed, containing 1 millimole/100 g of isothiocyanates, the concentration in the gut will be approximately 2 mM, assuming a gut volume of 1 l.

In conclusion, it seems that structurally different isothiocyanates have different EC_{50} values and comparable induction factors. Isothiocyanates prove to be strong inducers of electrophile-responsive element-mediated gene expression at physiological concentrations. The constructed EpRE(mGST-Ya)-LUX luciferase reporter cell line is a suitable assay to study EpRE-regulated gene expression.

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Chapter 6

Bioavailability and Kinetics of Sulforaphane in Humans After Consumption of Cooked Versus Raw Broccoli

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ABSTRACT

The aim of this study was to determine the kinetics and effects of the supposed anticarcinogen sulforaphane, the hydrolysis product of glucoraphanin, from raw and cooked broccoli. Eight men consumed 200 g of crushed broccoli, raw or cooked, with a warm meal in a randomised, free living, open cross-over trial. Higher amounts of sulforaphane were found in blood and urine when broccoli was eaten raw (bioavailability of 37%) versus cooked (3.4%, p=0.002). Absorption of sulforaphane was delayed when cooked broccoli was consumed (peak plasma time 6 h) versus raw broccoli (1.6 h, p=0.001). Excretion half-lives were comparable, 2.6 and 2.4 h on average for raw and cooked broccoli, respectively (p=0.5). From this study (n=8) it can not be concluded whether polymorphisms of the glutathione S-transferase (GST)-M1, T1, P1 and NAT2 genes have an effect on the calculated kinetics of sulforaphane. Furthermore, no effect of raw versus cooked broccoli on biomarkers in urine and plasma was found, iPF2-alpha (p=0.7), 8-oxodG (p=0.11) and GST-alpha (p=0.3). This study gives complete kinetic data and shows that consumption of raw broccoli results in faster absorption, higher bioavailability and higher peak plasma amounts of sulforaphane, compared to cooked broccoli.

INTRODUCTION

Epidemiological studies indicate that consumption of fruits and vegetables is associated with a reduced risk of degenerative diseases such as cancer and cardiovascular diseases (*1*). In particular cruciferous vegetables, e.g. cabbages, kale, broccoli, Brussels sprouts, radish, mustard and cress, are expected to be beneficial for human health (*2, 3*). Cruciferous vegetables contain glucosinolates, which are not present in other vegetables. These phytochemicals might therefore be responsible for the protecting effect (*4*). Broccoli contains high amounts of glucoraphanin which, upon chewing, is enzymatically hydrolysed by myrosinase into the corresponding isothiocyanate, sulforaphane, and other breakdown products (*5*). Myrosinase is present in all cruciferous vegetables. Glucosinolates that are not hydrolysed can be degraded by thioglucosidase activity of microbes present in the human gut (*6*), but are not likely to be absorbed intact. Isothiocyanates that are absorbed will be conjugated to glutathione, further metabolised to mercapturic acids, and subsequently excreted in the urine. Mercapturic acids present in urine reflect the uptake of isothiocyanates, and thus the intake of glucosinolates from cruciferous vegetables (*7, 8*).

Sulforaphane has been shown to have anticarcinogenic properties in animal experiments. The proposed mechanism of action is by inhibition of carcinogen activating phase 1 biotransformation enzymes, induction of phase 2 detoxification enzymes, anti inflammation, and induction of apoptosis (*9*). Glutathione S-transferases (GSTs) are phase II enzymes that catalyse the biotransformation and elimination of numerous xenobiotics. GST-α prevails in the liver and leaks from the liver at a constant rate. An increase in GST-α can therefore be measured in the blood and it can be used as a biomarker of phase 2 enzyme induction. Isoprostanes like 8-iso prostaglandin F2α (iPF2-α) are produced in vivo by a non-enzymatic free-radical-induced peroxidation of polyunsaturated fatty acids. iPF2-α is an arachidonic-acid-derived isoprostane that has been identified in human urine and proposed as a specific biomarker of lipid peroxidation (*10*). Furthermore, increased excretion of iPF2-α in cigarette smokers reflects the increased risk for cardiovascular disease (*11*). Oxidation of another endogenous molecule, DNA, is considered a pathogenic event in many cancers (*12*). As a biomarker of oxidative DNA damage, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) is used.

A large degree of variability in susceptibility to diseases like cancer could be explained by genetic polymorphisms in, e.g., GST-mu, pi and theta and N-acetyltransferse 2 (NAT2, *13, 14*).

Studies showed that allele frequencies of metabolic genes are randomly distributed within ethnic and geographical groups. More than half of the Caucasian population lacks the GST-μ enzyme, which is responsible for the biotransformation of xenobiotics.

In this study we want to demonstrate the differences in bioavailability and kinetics of the isothiocyanate sulforaphane and the effect on biomarkers after the consumption of raw versus cooked broccoli. Eight healthy, moderately smoking men consumed broccoli in a cross-over design. Sulforaphane in raw and glucoraphanin in cooked broccoli was determined and metabolites were measured in urine (sulforaphane mercapturic acid) and blood (sulforaphane conjugates). The following biomarkers of effect were analysed; GST-α in blood and iPF2-α and 8-oxodG in urine. Furthermore, polymorphisms in acetylation (NAT2) and glutathione conjugation (GSTM1, GSTT1 and GSTP1) were determined to establish the effect on sulforaphane elimination and excretion kinetics. We used smoking volunteers since they respond better to a presumable 'health promoting' intervention, a change in biomarkers of effect is more apparent.

MATERIALS AND METHODS

Subjects. Apparently healthy, smoking, adult (aged 18-60 year) male volunteers were recruited from the Utrecht-Zeist area (The Netherlands). Inclusion criteria were among other things: A regular (Dutch) food pattern, good health determined with medical history, physical examination and clinical laboratory analysis of blood and urine; having a smoking habit of more than 10 cigarettes per day and a body mass index (BMI) between 19 and 27. Ten volunteers were recruited and eight subjects completed the entire intervention period. One subject was missing at day 1 and could not be followed up. Another subject was eliminated from the study because of illness that was not related to the study. Characteristics of the remaining eight volunteers were: age, 34 ± 13 years and BMI, 25.0 ± 1.8 kg/m² (average ± SD). Their self-reported smoking was 10-20 cigarettes per day, which can be categorised as moderate smoking. We used smoking volunteers since a change in biomarkers of effect is more apparent.

Each subject gave written informed consent after being informed about the study, both verbally and written. The study was performed according to ICH (International Conference on Harmonization of Technical Requirements of Registration of Pharmaceuticals for Human Use) guidelines for Good Clinical Practice, and was approved by an external Medical Ethical Committee. The study was conducted at the Department of Physiological Sciences of TNO Nutrition and Food Research, Zeist, The Netherlands.

Study design. Volunteers consumed 200 g of crushed broccoli, raw or microwave cooked, together with a warm meal on 2 separate days, in the morning, with 1 day of wash out in between in a randomised, free living, open cross-over trial. Volunteers were not fasted before consumption of this meal. The warm meal consisted of a fixed amount of a meat burger and mashed potatoes (days 3 and 5). Volunteers refrained from eating crucifers on the days before treatment (days 1, 2 and 4).

Diets. Broccoli was obtained from a local greengrocer's shop (Zeist, The Netherlands) on day 2 and stored at 4°C. On days 3 and 5, 1 kg of raw broccoli was crushed with a blender and incubated at room temperature for 2 hours prior to serving to the volunteers. One kg of broccoli was microwave cooked at 1000W, crushed with a blender and immediately served. Samples were taken on both days and frozen at -20°C for later analysis of glucosinolates and isothiocyanates.

Urine and blood collection. Urine samples were collected during 24 h in one flask on days 2 and 4 and spot urine samples were collected during 24 h in separate flasks on days 3 and 5. All urine samples were kept refrigerated and were frozen on the same day. On both treatment days, blood samples were collected from five of the eight subjects, on 13 time points during 12 h, in vacutainer tubes with EDTA as anticoagulant. Whole blood was divided into portions, remaining whole blood was centrifuged to obtain plasma and all samples were stored frozen on the same day.

Genotyping. Genomic DNA was extracted from whole blood using the QIAamp 96 DNA isolation blood kit (Qiagen,Inc.), diluted to a concentration of 20 ng/µL, and stored at 4°C until analysis. A multiplex PCR was performed to determine presence or absence of the GSTM1 and GSTT1 genes simultaneously (*15*) and the GSTP1 polymorphism A313G was determined (*16*). The N-acetyl transferase 2 (NAT2) gene was amplified primarily, followed by 3 nested PCRs to amplify the regions with possible pointmutations, T341C, G590A, and A803G + G857A combined (*17*).

Chemical analyses. ***Glucosinolates*** were determined according to a modified method (*8*). Briefly, glucosinolates were extracted, trapped on solid phase extraction columns and desulfated. The corresponding desulfoglucosinolates were eluted with water, measured using high-performance liquid chromatography (HPLC) coupled to a diode-array detector (DAD) and determined using relative response factors.

Isothiocyanates were conjugated with 2-mercaptoethanol and determined by HPLC-DAD (*8*). ***Isothiocyanate mercapturic acids*** in urine were measured as described before (*18*). Briefly, internal standard was added to urine and the samples were purified on solid-phase extraction columns. Eluates were analysed using reversed phase HPLC coupled to an LCQ ion trap mass spectrometer (MS/MS, Finnigan-Thermo Quest, Breda, The Netherlands). 4-Methylsulfinylbutyl isothiocyanate mercapturic acid was prepared as described (*19*). ***Sulforaphane conjugates*** in plasma were measured with reversed phase HPLC-MS/MS. Phenyl isothiocyanate was added as internal standard and the sample was added to n-butanethiole diluted in methanol and incubated at 50°C for 2 h. The n-butanethiole conjugates of sulforaphane and phenyl isothiocyanate were measured on a Sciex-API3000 triple quadrupole mass spectrometer (Applied Biosystems, Breda, The Netherlands). ***Creatinine*** was measured in urine to verify the collection of urine and in plasma and urine combined to calculate creatinine clearance. Creatinine was measured enzymatically on a Hitachi 911. ***Liver damage markers*** in plasma of five volunteers, aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), gamma-glutamyltransferase (γ-GT) and albumin, were measured enzymatically on a Hitachi 911. ***Nicotine and cotinine*** in urine were measured using HPLC-MS/MS. Urine samples were centrifuged and deuterated internal standards were added. Chromatographic separation and detection of nicotine and cotinine was performed using HPLC coupled to an ion trap MS/MS using Atmospheric Pressure Chemical Ionisation. ***GST-α*** in plasma was measured using an enzyme-linked immunosorbent assay (ELISA) according to the method of Biotrin (Dublin, Ireland). Levels of GST-α at t=0 and 0.25h on days 3 and 5 were averaged (baseline) and levels at t=10 and 12h were averaged on days 3 and 5 separately, to calculate the effect of treatment. ***iPF2-α*** (8-isoprostaglandin F2alpha) in urine was measured according to a modified method (*10*). Briefly, internal standard was added to an aliquot of urine and the sample was centrifuged. iPF2-α was derivatised and subsequently analysed using gaschromatography - Negative Chemical Ionisation mass spectrometry. ***8-OxodG*** (8-oxo-7,8-dihydro-2'-deoxoguanosine) in urine was measured using LC-LC-MS/MS. Urine was centrifuged and filtrated and internal standard, 8-hydroxy-2-deoxyguanosine-O^{18} (kindly provided by NIST, USA) was added to the sample. Fractions from the first HPLC separation were collected, evaporated and 8-oxodG was separated and detected using a second HPLC coupled to an Ultima triple quadrupole mass spectrometer using electrospray ionization. Amounts of iPF2-α and 8oxodG excreted on days 2 and 4 were averaged (baseline) and amounts on days 3 and 5 were used to calculate the effect of treatment.

Statistical and pharmacokinetic analysis. Plasma concentrations of sulforaphane conjugates and urinary excretion of sulforaphane mercapturic acid were fitted to a one-compartmental model with the assumption of first-order absorption and excretion kinetics. The elimination rate constant (k) was calculated from the natural logarithm of plasma amounts of sulforaphane conjugates plotted versus time, and expressed as $t_{½}$ (0.693/k). The absorption rate constant (k_a) was calculated using the intercept and slope of the same plot with the method of residuals. Using the trapezoid method, the area under the plasma concentration versus time curve (extrapolated to infinite) was calculated. The lagtime was determined graphically from the curve of plasma amounts of sulforaphane conjugates plotted versus time. The bioavailability, F, was estimated by dividing the cumulative amount of sulforaphane mercapturic acid excreted in urine in 24 h by the consumed amount of glucoraphanin for cooked broccoli or sulforaphane for raw broccoli. The excretion rate constant (k_e) was calculated from the natural logarithm of absolute amounts of excreted sulforaphane mercapturic acid per hour plotted versus time, and expressed as $t_{½}$ (0.693/k_e). Statistical significance of the difference between the kinetic parameters, after consumption of raw versus cooked broccoli, was calculated with a two-sided, paired Student's t-test. Biomarkers were calculated as average ± standard deviation and were analysed (t-test) by comparing the results áfter intervention with raw versus cooked broccoli; baseline values were not used for the analysis and are only given as information.

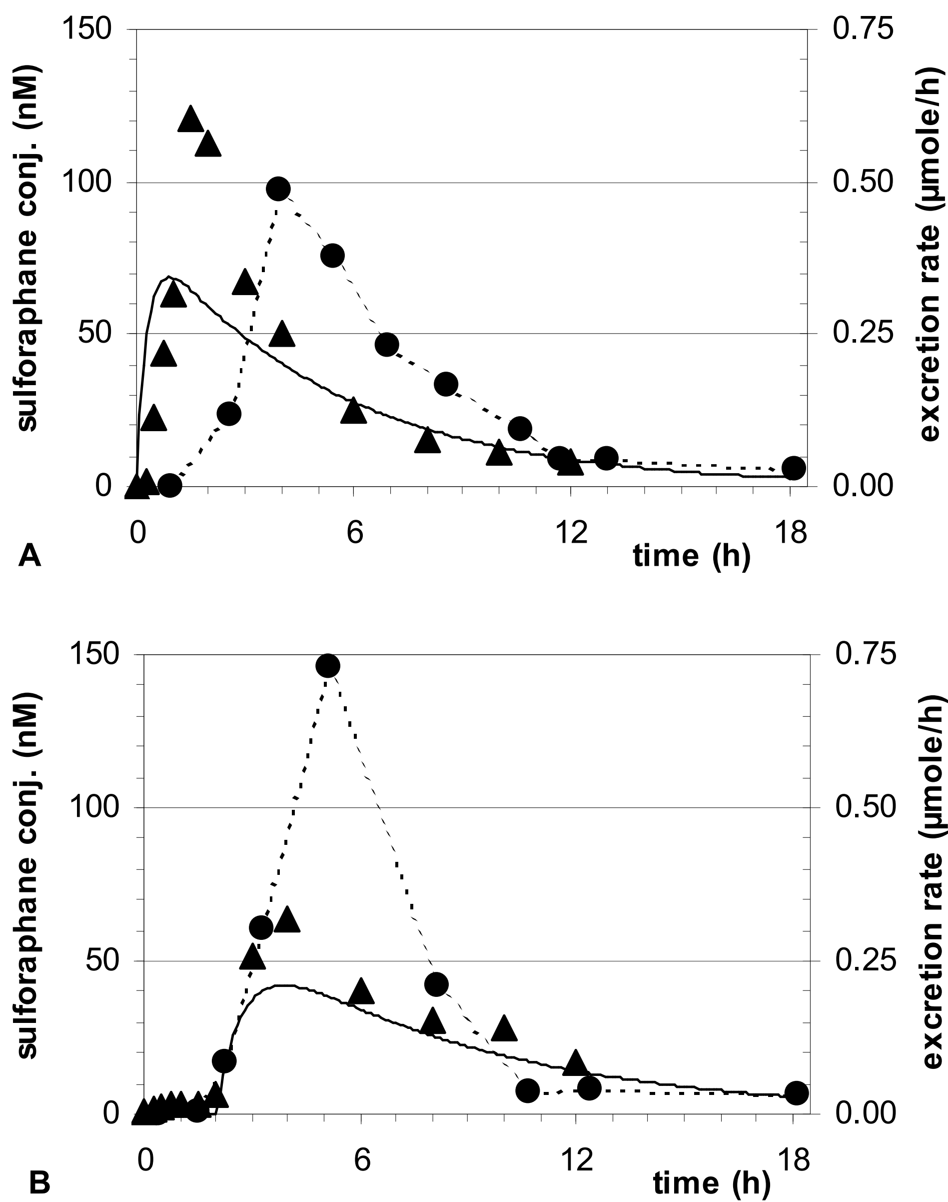

Figure 1. Typical uptake and excretion curves from volunteer three. Amount of sulforaphane conjugates in blood (▲; data, –; kinetic fit) and excretion of its mercapturic acid in urine (• with dashed line; data) after consumption of 200 g of broccoli which was crushed raw (A) or cooked and crushed (B).

RESULTS

Subjects. Liver function markers (enzymes) in plasma were with one exception all within the normal ranges. There was no significant difference in ASAT, ALAT, γ-GT and albumin levels (p=0.3, 0.7, 0.4, and 0.6, respectively) between both days of intervention as determined with a two-sided, paired Student's t-test. On average 11.3 (range 9-16) portions of urine / 24h were produced at both days of intervention with amounts of 0.1-0.5 L.

Diets. The glucoraphanin content of the cooked broccoli serving (200g) was 61.4 µmole on average for both days of intervention (319 and 296 µmole/kg, respectively). Cooked broccoli also contained glucoiberin (27 µmole/kg), and glucobrassicins (508 µmole/kg in total). The raw broccoli serving (200g) contained 9.92 µmole of sulforaphane on average, 48 and 51 µmole/kg for both days of intervention, respectively. No other isothiocyanates were detected.

Chemical analyses. Cumulative creatinine concentration in urine correlated with time (R^2 = 0.993-0.9996) for all volunteers confirming the collection of all urine. There was no significant (p=0.3) difference in creatinine levels between both days of intervention as determined with a two-sided, paired Student's t-test, and corrected creatinine clearance was on average 124 (range 80-147) ml/min/1.73m², indicating a healthy excretion (**Table 2**). Excretion of nicotine and cotinine was constant over time with cumulative amounts of on average 1.6 and 2.5 (range 0.2-4.7 and 0.5-5.3) mg/24h urine, respectively. There was no significant difference in nicotine and cotinine levels (p=0.2 and 0.3, respectively) between both days of intervention as determined with a two-sided, paired Student's t-test, indicating unchanged, moderate smoking.

Kinetics. Typical results for the absorption and excretion curves are depicted in **Figure 2**. The results of plasma sulforaphane conjugate kinetic parameters and of mercapturic acid excretion are depicted in **Tables 1** and **2**, respectively. When calculated using the amount of excreted sulforaphane mercapturic acid, the bioavailability of sulforaphane from raw broccoli (F=37%) is 11 times higher than from cooked broccoli (F=3.4%), which is statistically significant (p=0.002). The same difference is apparent from the amounts of sulforaphane conjugates in blood where a corrected difference in AUC of 10.2 (times) can be calculated. When raw broccoli was consumed, the AUC was 1.7 times higher than when cooked broccoli was consumed, and the content of glucoraphanin in cooked broccoli (61.4 µmole) is 6 times higher than the content of sulforaphane in raw broccoli (9.92 µmole). The peak plasma time is significantly different, 1.6 h when raw broccoli is consumed versus 6 h for cooked broccoli.

Table 1. Plasma sulforaphane conjugate pharmacokinetic secondary parameter summary after a single dose of 200 g of broccoli, either raw or cooked, was consumed [a]

person nr.	peak concentration (C_{max} in nM) raw	cooked	peak time (t_{max} in h) raw	cooked	lagtime (t in h) raw	cooked	$AUC_{0-\infty}$ (nM*h) [b] raw	cooked	absorption rate constant (k_a) raw	cooked	elimination half-life ($t_{½}$ in h) raw	cooked
1	160	19.0	1	6	0	4	535	147	5.9	1.3	3.7	4.3
2	77.2	17.9	3	8	0	2	438	195	1.1	0.2	2.8	4.0
3	121	63.6	1.5	4	0	2	470	485	3.4	1.3	3.7	4.6
4	75.6	22.7	1.5	6	0	1.5	510	233	2.9	0.3	4.0	4.2
5	78.5	31.1	0.75	6	0	1	523	371	7.3	0.4	5.0	6.0
average	103	31	1.6	6	0	2.1	495	286	4.1	0.7	3.8	4.6
SD	37	19	0.9	1.4	0	1.1	40	139	2.5	0.5	0.8	0.8
p t-test	0.01		0.001		0.01		0.04		0.03		0.01	

[a]Calculated from sulforaphane conjugate amounts measured in blood. [b]$AUC_{0-\infty}$ is the sum of AUC_{0-12} (measured amounts) and $AUC_{12-\infty}$ (extrapolated from t=12).

The peak plasma concentration of sulforaphane is 20 times higher when raw broccoli is consumed, compared to cooked broccoli, relative to the intake. The variation in absorption rate between volunteers is large, the relative standard deviation (RSD) was 61% and 71% for raw and cooked broccoli, respectively. The variation in AUC is however only 8% (RSD) for raw broccoli and 49% for cooked broccoli. The elimination of sulforaphane from blood is slower compared to the excretion in urine alone, elimination (excretion) half-lives are 3.8 (2.6) and 4.6 (2.4) h for raw and cooked broccoli, respectively.

Genotype. Two subjects were slow acetylators as determined with NAT2 genotyping, all other subjects were heterozygous in NAT2 (**Table 2**). Furthermore, three subjects lacked GST M1 and one subject lacked GST P1 as well. Another polymorphism was found in one subject who lacked GST T1. The reference value for slow NAT in a European population is 59% (*20*). The reference values for GSTM1-1 null, GSTP1 null and GSTT1-1 null are 48% in a European population (*21*), 11.7% in a Caucasian population (*21*) and 10% in a European population (*22*), respectively. The reference value for both GSTM1 null and GSTT1 null together is 10.4% in a Caucasian population (*13*). From these reference values, more subjects were expected to be slow acetylators (59% instead of 25%) but GST

polymorphism frequencies (37.5%, 12.5% and 12.5%, respectively) are in accordance with these reference values.

Biomarkers. The average baseline value of GST-α was 6.1 ± 1.9 µg/L plasma. No statistically significant difference in GST-α increase was found between consumption of raw (8.0 ± 4.2 µg/L) and cooked (6.6 ± 1.6 µg/L) broccoli (p=0.3). The moderately smoking subjects (10-20 cigarettes per day) in this study excreted on average 289 (range 186-568) ng iPF2-α per 24 h corresponding to 169 pg iPF2-α / mg creatinine. After consumption of cooked broccoli, excretion of iPF2-α was not statistically significant (p=0.7) increased (311 ± 57 ng/24h) compared to raw broccoli (306 ± 58 ng/24h). The lipid oxidation marker iPF2-α was excreted at a constant rate since the correlation (R^2) with time was 0.98-0.999 for all volunteers. The baseline amount of 8-oxodG excreted in urine was 7.8 ± 2.8 µg/24 h on average. After consumption of cooked broccoli, excretion of 8-oxodG was not statistically significant (p=0.11) increased (9.2 ± 2.6 µg/24h) compared to raw broccoli (8.3 ± 2.1 µg/24h).

Table 2. Urinary sulforaphane mercapturic acid pharmacokinetic secondary parameter summary after a single dose of 200 g of broccoli, either raw or cooked, was consumed [a]; with genotypes

person nr.	creatinine clearance (ml/m²/min)	bioavailability (F in %)		peak time (t_{max} in h)		excretion half-life ($t_{½}$ in h)		genotype			
									GST		
		raw	cooked	raw	cooked	raw	cooked	NAT2	M1	P1	T1
1	85	21	3.0	5.0	6.0	2.1	1.3	slow	null	null	
2	81	62	1.7	3.0	5.0	2.4	2.8				null
3	68	37	5.8	4.5	5.5	2.5	1.6	slow	null		
4	76	21	3.3	4.0	5.5	2.5	2.7				
5	81	37	3.1	4.5	4.0	2.6	2.4				
6	66	69	3.2	5.0	6.0	2.4	3.0				
7	46	30	2.4	5.5	7.5	3.1	1.7				
8	68	20	3.2	7.5	7.0	3.3	4.0		null		
average	71	37	3.4	4.9	5.8	2.6	2.4				
SD	12	19	1.0	1.3	1.1	0.4	0.9				
p t-test		0.002		0.03		0.5					

[a] Calculated from excreted sulforaphane mercapturic acid in urine.

DISCUSSION

The kinetics of isothiocyanate absorption and excretion have been described in several publications. In this study we used LC-MS techniques to increase sensitivity and, mostly, specificity compared to earlier studies. In addition, the accuracy of the data gave us the possibility to study the kinetics of sulforaphane from raw and cooked broccoli in detail, e.g. the parameters AUC and t_{max}. The concentration of sulforaphane conjugates in blood and sulforaphane derived mercapturic acid in urine were used for calculation of the kinetics.

Intake. The sulforaphane content of raw broccoli was lower than the glucoraphanin content of cooked broccoli, 9.92 and 61.4 µmole, respectively. It seems that the conversion from glucosinolate to isothiocyanate was incomplete, or another reaction occurred. No glucoraphanin (<10 µmole/kg) was detected after crushing and incubation, indicating that glucoraphanin was completely converted. The pH of the crushed raw broccoli was 7, at which pH, in theory, isothiocyanates are the only metabolites from glucosinolates. However, broccoli might contain additional enzymes that inhibit the conversion, or convert glucoraphanin into other hydrolytic products like thiocyanate and nitrile (*23*). These products have a different bioactivity than sulforaphane and are not known to induce phase II enzymes.

Kinetics. The mercapturic acid pathway is the major route of elimination of isothiocyanates. Minor routes of excretion could be defecation, exhalation, and perspiration. From the ingested amount of glucoraphanin and sulforaphane from cooked and raw broccoli, 3.4% and 37% was recovered in urine as sulforaphane mercapturic acid, respectively. This is comparable with a similar study in which these values for bioavailability were 10 and 32%, respectively (*24*). Possible explanations for these low recoveries are that glucoraphanin is not entirely converted into sulforaphane or that sulforaphane is not entirely absorbed from the gut. It might also be the case that it is excreted via other routes or it is metabolised into non-isothiocyanate metabolites, which is confirmed by Combourieu *et al*. (*25*). They found that besides the slow and incomplete conversion of glucoraphanin into sulforaphane in the colon, sulforaphane is further degraded into its amine. Indeed, several studies show that less than 100% recovery was obtained for dosed glucosinolates or isothiocyanates. For example, pure benzyl isothiocyanate from garden cress administered to humans was recovered for 54% as mercapturic acid in urine (*26*) and phenylethyl isothiocyanate from watercress, after chewing, was recovered for 47% (*27*). The recovery of isothiocyanates from 350g of cooked watercress was 1.2-7.3% and from 150g of uncooked

watercress was 17.2-77.7% (*28*). Cumulative excretion of dithiocarbamates after consumption of homogenates of boiled broccoli sprouts was 12% and after myrosinase treatment of the homogenate, the cumulative excretion was 80% (*29*). In a recent study, allyl isothiocyanate from mustard was recovered for 68% as mercapturic acid in urine and sinigrin from cooked and raw cabbage was recovered for 15% and 37%, respectively (*30*). However, in another study we showed that after consumption of several raw cruciferous vegetables more or less than 100% of the ingested isothiocyanate was recovered in urine as mercapturic acid (*8*). From that study it seems that the vegetable matrix is an important factor determining the bioavailability. In general, bioavailability of isothiocyanates is higher from condiments like crushed horseradish than from vegetables like cabbage.

Glutathione conjugates of sulforaphane are the means of transport of this bioactive substance through the body. After ingestion of glucoraphanin from cooked broccoli and sulforaphane from raw broccoli, peak concentrations of sulforaphane conjugates in blood were maximal after on average 6 and 1.6 h, respectively. This difference in peak time indicates that the absorption of sulforaphane after consumption of cooked broccoli was delayed, on average, by approximately 4.5 h. Furthermore, if glucoraphanin was fully hydrolysed into sulforaphane by myrosinase in crushed raw broccoli, the amount of bioactive sulforaphane conjugates in blood would be 10, instead of only 1.7, times higher than after consuming cooked broccoli. When trying to produce more healthy cruciferous vegetables it is therefore not only important to selectively increase glucoraphanin but also to control the co-factors responsible for incomplete conversion of the glucosinolate into the isothiocyanate, as already stated by Faulkner *et al.* (*23*). The elimination half-lives of sulforaphane from blood after consumption of cooked and raw broccoli were 4.6 and 3.8 h, respectively. These values are higher than expected from the excretion half-lives of sulforaphane mercapturic acid in urine, 2.4 and 2.6 h, respectively, probably because of a prolonged absorption from the gut, in particular after the consumption of cooked broccoli.

Effect of genotype. From this study (n=8) it can not be concluded whether individuals who are slow acetylators as determined with NAT2, and/or lack GST M1, T1 and/or P1 eliminate or excrete sulforaphane slower, or have higher AUC's or F's than other individuals. From literature the lower excretion of isothiocyanates in relation to different polymorphisms is apparent and it can also be concluded that the risk for cancer is altered by these polymorphisms (*13, 31, 32, 33*).

In another trial with broccoli, comparing GSTM1-null subjects with GSTM1-positive subjects, three parameters (AUC, k_e and F) were slightly, but statistically significant, higher (*34*). However, in a recent study (*35*) there were no substancial differences in ITC levels among genotypes.

Effect on biomarkers. The amount of GST-α measured in the blood represents the induction in the liver only if there is no liver damage. There was no liver damage as measured by liver function markers in blood: ASAT, ALAT, γ-GT and albumin. GST-α was increased to the same extent by raw and cooked broccoli. Average amounts in plasma after treatment are comparable to another Brassica intervention trial in which male non-smokers consumed cooked Brussels sprouts (*36*). The importance of GST-α in health is demonstrated by clinical studies, which have established an association between decreased glutathione S-transferase activity and increased risk for colorectal cancer (*37*).

Isoprostanes, like iPF2-α excreted in urine, are biomarkers for lipid oxidation and are elevated in smokers and patients with atherosclerosis, hypertension or hypercholesterolaemia. Baseline levels of iPF2-α were comparable to other studies (*11, 38*). Although a decrease in oxidative damage could be expected in this study (*39*), another intervention study with flavonoids in onions and tea also found no statistically significant effect on plasma F2-isoprostane levels (*40*). The DNA adduct that is most abundantly formed by oxidative damage to DNA is 8-oxodG. The difference between the two treatments was not statistically significant but it seems that oxidative damage or repair of oxidative damage was increased by cooked broccoli. Contradicting results were also found in a study with Brussels sprouts where a decrease in 8-oxodG was found in four out of five males but not in (five) females (*41*).

CONCLUSIONS

Higher amounts of sulforaphane conjugates in blood and sulforaphane derived mercapturic acid in urine were found when broccoli was eaten raw (bioavailability of 37% on average) versus cooked (3.4%, p=0.002). Absorption of sulforaphane was delayed (by 2.1 h) when cooked broccoli was consumed and the peak plasma time was 6 h on average versus 1.6 h for raw broccoli (p=0.001). Excretion half-lives were comparable, 2.6 and 2.4 h on average for raw and cooked broccoli, respectively (p=0.5). Althoug consumption of raw broccoli resulted in faster absorption, higher bioavailability and higher peak plasma amounts of sulforaphane, compared to cooked broccoli, no effect on biomarkers in urine and plasma was found; iPF2-alpha (p=0.7), 8-oxodG (p=0.11) and GST-alpha (p=0.3). Furthermore, from this study (n=8) it can not be concluded whether polymorphisms of the glutathione S-transferase (GST)-M1, T1, P1 and NAT2 genes have an effect on the calculated kinetics of sulforaphane. In future research, care should be taken that glucoraphanin is also hydrolysed into other metabolites when crushing broccoli, reducing the amount of sulforaphane.

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Chapter 7

Summary, general discussion and conclusions

INTRODUCTION

Consumption of fruits and vegetables is associated with a reduced risk of degenerative diseases such as cancer and cardiovascular disease, as indicated by several studies (*1*). In particular cruciferous vegetables, e.g. cabbages, broccoli, mustard and cress, are expected to be beneficial for human health (*2*). Cruciferous vegetables contain glucosinolates, which are not present in other vegetables. These phytochemicals might therefore be responsible for the protecting effect (*3*). Glucosinolates are hydrolysed by myrosinase, which is released by cutting and chewing, or by enzymes present in the colon (*4,5*). One of the breakdown products are isothiocyanates that have been shown to have anticarcinogenic properties in animal experiments. Isothiocyanates are not direct antioxidants but the proposed mechanism of action is by inhibition of carcinogen activating phase 1 biotransformation enzymes, induction of phase 2 detoxification enzymes, anti inflammation, and induction of apoptosis (*6*). Isothiocyanates are absorbed intact and transported through the body as glutathione conjugates. To discriminate between different glucosinolate containing vegetables and to study the effect of isothiocyanates, the uptake can be estimated by its metabolites excreted in urine, mercapturic acids (*7*).

Measuring the effect of a complex matrix like a vegetable is difficult. To simplify this, biomarkers can be used to measure the intake of a vegetable or of a specific compound and to measure the effect in a short period of time. There are many structurally different glucosinolates present in the diet, which have different kinetics depending on physicochemical parameters. This, and the effect of cooking vegetables on the bioavailability and kinetics of isothiocyanates can be determined with conjugates measured in blood and urine. With these biomarkers, hopefully, the question which of these glucosinolates yield the most active isothiocyanate, in the highest amount per gram vegetable can be answered. Also, the amount of cruciferous vegetables that should be consumed for a safe but health promoting effect could be determined.

SUMMARY

The work in this thesis describes the bioavailability, kinetics and effects of isothiocyanates from cruciferous vegetables. To obtain reference compounds for isothiocyanate intake, at first, mercapturic acids of the most important isothiocyanates were chemically synthesised. In up to four synthesis steps commercially not available isothiocyanates were prepared by adding the corresponding alkyl bromide to phthalimide potassium salt. The obtained N-alkyl-phthalimide was hydrazinolysed yielding the alkyl

amine, which subsequently was reacted with thiophosgene yielding the isothiocyanate with a total yield of 16%. In a fifth step of synthesis, isothiocyanates were conjugated to N-acetyl-L-cysteine, yielding the mercapturic acid in a typical yield of 77%. Methods of synthesis of twelve mercapturic acids, derived from saturated and unsaturated aliphatic and aromatic isothiocyanates, are described (**chapter 2**).

These compounds were used to develop a method of analysis for the determination of isothiocyanate mercapturic acids in urine, using liquid chromatography coupled to tandem mass-spectrometry (**chapter 3**). Analytes from urine were purified using solid phase extraction prior to chromatography. Sensitive and specific detection of low levels of isothiocyanate mercapturic acids in urine was obtained with ion-trap mass spectrometry. The method proved valid and rugged for the parameters tested. The range of reliable analysis was 1.0-310 μM in urine, corresponding to the amount of allyl isothiocyanate mercapturic acid found in 24 h urine after the consumption of allyl glucosinolate (sinigrin) present in, respectively, one leaf of one Brussels sprout and 200 g of Brussels sprouts.

To determine bioavailability and excretion kinetics of glucosinolates and its derived isothiocyanates in humans, 3 volunteers consumed 19 different raw and cooked vegetables and condiments (**chapter 4**). Results showed that excretion levels of mercapturic acids were higher after the consumption of raw vegetables and condiments (bioavailability 8.2-113%) compared to cooked vegetables (bioavailability 1.8-43%). Maximal concentration levels of isothiocyanates in urine were reached about 4 hours later after consumption of cooked vegetables, compared to raw vegetables and condiments but the excretion rate was similar ($t_{½}$ = 2.1-3.9 h). To study the contribution of the colonic microflora to the conversion of glucosinolates into isothiocyanates an *in vitro* experiment was performed. Using the TNO gastro-intestinal model (TIM 2) the bioavailability and bioconversion of sinigrin and allyl isothiocyanate (from Brussels sprouts) in the large intestine was determined. With different individual human microflora, 10-30% of sinigrin was converted into allyl isothiocyanate (not described in this thesis, *8*).

The effect of isothiocyanates was studied *in vitro*, where human enzyme activity, or actually a part of the genes coding for a phase 2 metabolism enzyme, was used. Isothiocyanates are indirect antioxidants and induce phase 2 metabolism enzymes via an electrophile responsive element (EpRE) that is present in the promotor region of the genes coding for these enzymes. The induction of the EpRE *in vitro* by isothiocyanates is described (**chapter 5**). The induction factor differs between the various isothiocyanates.

Isothiocyanates containing a methyl-sulfur side chain (like sulforaphane, EC_{50} value 1.2 µM) are more potent inducers than the aliphatic and aromatic isothiocyanates tested (e.g. allyl isothiocyanate, EC_{50} value 6.5 µM). The estimated concentrations of individual isothiocyanates in the body after consumption of cooked cabbage may amount to 0.04-4 µM and might thus in some cases reach these EC_{50} values voor EpRE induction, and represent biologically active concentrations. Another group of phytochemicals that induce phase 2 enzymes are flavonoids, antioxidants present in tea, red wine and apples. However, no consistent relation between EpRE induction and flavonoid antioxidant potential was observed pointing to differences in induction mechanism between these various chemoprotective compounds (not described in this thesis, *9*).

The effect of cooking broccoli on the absorption and excretion kinetics of sulforaphane, derived from glucoraphanin, was studied in a trial with eight smoking men (**chapter 6**). The cooked broccoli serving contained 61 µmoles of glucoraphanin but the same amount of raw broccoli contained only 9.9 µmoles of sulforaphane, due to conversion into other metabolites. The effect on biomarkers of protection and oxidative damage, glutathione S-transferase alpha (GST-α), 8-isoprostaglandin F2alpha (iPF2-α) and 8-oxo-7,8-dihydro-2'-deoxoguanosine (8-oxodG), was also determined. Higher levels of sulforaphane were found urine when broccoli was eaten raw (bioavailability of 37%) versus cooked (3.4%). The area under the blood-concentration curve was higher when broccoli was eaten raw (0.50 µM·h, dose of 9.9 µmol sulforaphane) versus cooked (0.29 µM·h, dose of 61 µmol glucoraphanin). No effect on GST-α, iPF2-α and 8-oxodG was found. Also, no effect of polymorphisms of the glutathione S-transferase (GST)-M1, T1, P1 and N-acetyl transferase (NAT)-2 genes on the absorption and excretion kinetics of sulforaphane was found.

The use of isothiocyanate mercapturic acids as a biomarker of cruciferous vegetable intake was validated using urine obtained from a study performed by Fowke et al. (*10*). In this single armed trial with 34 women, behavioural intervention facilitated daily cruciferous vegetable intake among participants. Several mercapturic acids were determined, sulforaphane correlated best with vegetable intake (Spearman correlation 0.49, $p<0.01$, unpublished results).

A BIOMARKER FOR CRUCIFEROUS VEGETABLE INTAKE?

Glucosinolate intake is generally estimated using dietary questionnaires, which are not only subjective measures, but are not accurate because amounts of (cruciferous) vegetables in a whole meal are not easily estimated. Glucosinolate metabolites can be analytically measured in vegetables, and intake of isothiocyanates can thus be estimated.

Because glucosinolates are only partly converted into isothiocyanates and interindividual kinetics differ, it is not feasible to estimate the exact intake of isothiocyanates through analysis of glucosinolates and isothiocyanates in vegetables. Myrosinase, which is still active in raw broccoli, is responsible for the breakdown of glucosinolates after chewing, resulting in an elevated isothiocyanate intake. Isothiocyanates are excreted in the urine but spot urine cannot be used to quantify the uptake and excretion of isothiocyanates since the peak in excretion is between 3 and 6 h. Twelve hour urine collection contains the majority in isothiocyanate excretion and most isothiocyanates, eaten with common meals, are excreted within 24 h.

Isothiocyanate conjugates, mostly mercapturic acids, quantitatively reflect isothiocyanate uptake and reflect glucosinolate intake at least qualitatively. Furthermore, excreted mercapturic acids reflect the pattern of side chains of glucosinolates present in the vegetable or condiment that was consumed. The bioavailability and kinetics are subject to variation, which might be explained by differences in the extent of chewing, absorption, conversion, colonic degradation and/or metabolism. Nonetheless, isothiocyanate mercapturic acids can be used as a biomarker to reflect the active dose of isothiocyanates absorbed.

Indole glucosinolates do not yield isothiocyanates upon hydrolysis but are converted into indoles and thiocyanate ion (SCN^-) instead. Measuring thiocyanate ion in urine can thus be a marker of indole glucosinolate intake and thus of cruciferous vegetable intake. High baseline concentrations in urine can be expected as thiocyanate ion is a metabolite from cyanide provided by cigarette smoke. Thiocyanate ion can be measured using ion-exchange chromatography and suppressed conductivity detection. To measure isothiocyanates and their corresponding mercapturic acids in physiological fluids a sum parameter assay can be used. The assay utilises a vicinal dithiol reagent and measures the reaction product spectrophotometrically (*11,12*). Chemical specificity of the cyclocondensation reaction is not restricted to isothiocyanates and the sum parameter does not give any information about which kind of crucifer has been consumed. The limitations of these two assays are not encountered when analyzing individual isothiocyanate mercapturic acids. Therefore, a chromatographic method using mass spectrometry (MS/MS) detection was developed and used.

Our results show that isothiocyanate mercapturic acids in urine reflect the pattern of side chains and amounts of glucosinolates present in the vegetable or condiment that was consumed. We confirmed the finding that condiments contain less different glucosinolates but in higher concentrations than vegetables.

BIOAVAILABILITY AND KINETICS OF ISOTHIOCYANATES

This thesis shows that there are substantial differences in bioavailability, absorption and excretion kinetics of isothiocyanates between cooked vegetables, and raw vegetables and condiments. The fraction of isothiocyanates absorbed from cooked vegetables was lower than from (thoroughly chewed) raw vegetables. The uptake of isothiocyanates from condiments was almost complete. This indicates that from raw crucifers complete uptake of isothiocyanates is also possible and that, apparently, after cooking only a small amount is available.

Hydrolysis of glucosinolates to isothiocyanates is dependent on the enzyme myrosinase which is inactivated upon cooking of the vegetable. The thioglucosidase enzyme which is present in the colon is less effective. When eating crucifers the intake thus consists of glucosinolates as well as isothiocyanates. Variation in bioavailability can partly be explained by differences in intensity of chewing and by differences in colonic microflora (*8*). The colonic microflora could also convert isothiocyanates into non-isothiocyanate metabolites which has a negative effect on the amount available for absorption (*13*).

Research by Verkerk et al. (*14*) shows that microwave treatment of cabbage resulted in glucosinolate levels exceeding the total content of the untreated cabbage material. This might be due to increased extractability of glucosinolates or is caused by enzymatic or chemical conversions and is especially the case for indole-glucosinolates (*14,15*). Conventional cooking results in the loss of almost all glucosinolates in the boiling water but this thesis found that consumption of raw cruciferous vegetables can result in total recovery of the glucosinolates as isothiocyanate mercapturic acids in the urine. It is therefore concluded from this thesis that cruciferous vegetables should preferably be consumed raw and chewed thoroughly.

Isothiocyanates are conjugated to glutahione in a phase II reaction, catalysed by glutathione S-transferase, followed by conversion to the mercapturic acid. When glucoraphanin is hydrolysed into sulforaphane in the colon there is no first-pass effect from the liver, e.g. oxidation or reduction into erucin. Interestingly, mercapturic acids from both erucin and sulforaphane were found after consumption of crucifers that contain glucoraphanin but not glucoerucin. These results show that the reduction of sulfinyl, changing sulforaphane into erucin, takes place. Oxidation of sulphur in erucin also takes place, since after administration of erucin, part of the dose was excreted as sulforaphane mercapturic acid (*16*). Sulforaphane is transported in the blood as conjugates and accumulates in cells by up to several hundred-fold over the extracellular concentration, primarily by conjugation with intracellular glutathione (*17*). The accumulated sulforaphane,

however, is rapidly exported by multidrug resistance associated protein-1 (MRP-1) and P-glycoprotein-1 (Pgp-1), mainly as its glutathione conjugate (*18*). These export pumps therefore decrease the time in which sulforaphane could be effective.

Isothiocyanates accumulate in different tissues which depends on the side chain. Benzyl and phenethyl isothiocyanate accumulate in lung tissue because of their relative higher hydrophobicity and volatility. Sulforaphane, however, does not seem to be targeted towards a specific tissue. The excretion kinetics also depend on the side chain but the food matrix largely determines the absorption and excretion. This thesis shows that isothiocyanate conjugates reach higher levels but are faster excreted after consumption of raw vegetables and condiments, compared to cooked vegetables. Isothiocyanates from cooked vegetables could have a prolonged effect since levels of isothiocyanate conjugates in the body were longer at non-zero levels, but the levels might be too low for a physiological effect.

The pharmacokinetics of broccoli sprout isothiocyanates in humans have been described by Ye et al. (*19*), they used the cyclocondensation sumparameter assay (*11*, *12*). They reported that an average of 58% of the ingested dose of isothiocyanates was excreted in urine collected for 8 h with an elimination half-life of 1.8 h (*19*). The difference between our study and the study of Ye et al. is that we found somewhat longer elimination half-lifes, 2-4 h on average. Shapiro et al. (*20*) showed that the excreted cyclocondensation product was related to the glucosinolate/isothiocyanate profiles administered. Furthermore, consumption of graded doses of isothiocyanates resulted in the rapid excretion of 42% of urinary conjugates with first-order kinetics with an excretion rate constant of approximately 0.42 h-1, which is faster than the excretion rate we found, 0.18-0.33 h-1. Moreover, the conversion of glucosinolates was negligible after bowel microflora were reduced (*20*). Further studies of Shapiro et al. (*21*) showed even a higher bioavailability, measured by excreted isothiocyanate conjugates in 72 h urine. From broccoli sprouts that were treated with myrosinase, 80% of the dose of isothiocyanates, and 12% of the dose of glucosinolates from cooked broccoli sprouts was excreted as isothiocyanate conjugates. They (*21*) also conducted dose-response experiments but no further kinetic data was obtained. In our study, in which vegetables were eaten during lunch, the lagtime of the appearance of isothiocyanate mercapturic acid in urine was 4 h on average.

Isothiocyanate mercapturic acids excreted in urine reflect the intake of glucosinolates from cooked vegetables and of isothiocyanates from raw vegetables and condiments. There are substantial differences in bioavailability, absorption and excretion kinetics of isothiocyanates between cooked vegetables, and raw vegetables and condiments.

Interindividual variation occurs and might be explained by differences in the extent of chewing, absorption, conversion, colonic degradation and/or metabolism.

HOW TO STUDY EFFECTS ON HEALTH?

The role of isothiocyanates in human health could be measured by relevant biomarkers which detect potential benefits relating to target functions of the body. Several stages of biomarkers can be distinguished in research. Food is consumed and (non-)nutrients are absorbed, the exposure to food compounds is reflected by biomarkers of intake. The positive effect on health can be measured by biomarkers of (intermediate) effect.

In this thesis, these biomarkers were developed for the intake and effect of isothiocyanates and their relation was determined. It is shown that isothiocyanate mercapturic acids in urine reflect the dose of glucosinolates and isothiocyanates absorbed after a meal containing cruciferous vegetables. It is possible to make a distinction between cabbages by analyzing the glucosinolate pattern since each crucifer contains a number of glucosinolates with a different side chain and varying amount.

Several biomarkers of effect were determined in blood and urine from volunteers who consumed broccoli. Although high levels of sulforaphane were found in blood and urine, no effect was found on biomarkers of protection and oxidative damage. 8-Oxo-dG was measured in urine as a marker of oxidative DNA damage, which is a potential mutagenic lesion (*22*). This biomarker may be used in population comparisons to measure oxidised DNA base formation, but individual disease risks can not be calculated with oxidised DNA levels. Intraindividual variability is high and affected by factors such as health, diet, exercise and stress. Some oxidised lesions are mutagenic and may be involved in carcinogenesis, but the link with cancer in humans is thin. Besides 8-oxo-dG, other oxidised DNA bases should be considered as additional markers to assess oxidative DNA damage (*22*). Another biomarker which was measured in urine is iPF2-α, a marker of lipid peroxidation. It is a useful marker but it is a minor pathway and there is a large range even in healthy people (*22*). An elevation in isoprostanes is found in diabetes and, to a lesser degree, in smokers. As a more specific marker of vascular function, oxidation of LDL can be used since it is a major risk factor.

As a biomarker of protection, GST-α was measured in plasma. It is an accurate index for induction of GST in the liver when there is no hepatocellular damage. Glutathione can react directly with reactive oxygen species via a sensitive SH-group, a reaction which is catalysed by GST. Glutathione can also be inactivated by disulphide formation, which can be reversed by GST-α. Thus, an up-regulation of GST-α may lead to a reduced damage of

reactive oxygen species and increased levels of active glutathione. Regulation of gene transcription was measured using an in vitro assay with the electrophile responsive element (EpRE) from GST. The chemical induction of phase II enzymes, such as GST, was found to be mediated by this common transcription enhancer element. This assay allows to screen food components for the ability to modulate EpRE-regulated gene expression.

CONCLUSIONS

Methods to determine the bioavailability, kinetics and effects of isothiocyanates from cruciferous vegetables were developed and applied in this thesis. The bioavailability of isothiocyanates from cooked vegetables is lower than from vegetables and condiments when eaten raw. On average 60% of the isothiocyanates present in vegetables and condiments is absorbed and excreted when eaten raw and only 10% when eaten cooked. Excretion kinetics are comparable when, for example, broccoli is eaten cooked or raw but absorption kinetics differ markedly. Absorption of isothiocyanates from cooked vegetables is delayed and lowered because cooking inactivates the enzyme responsible for the conversion of glucosinolates into isothiocyanates. This conversion is therefore limited to chemical decomposition and to the activity of the colonic microflora, with a lower yield. In urine, isothiocyanate mercapturic acids reflect the total active dose of isothiocyanates absorbed. Mercapturic acids can therefore be used as biomarkers of the intake of glucosinolates and isothiocyanates, and to a lesser degree as biomarkers of cruciferous vegetable intake. Combined with biomarkers of protection or oxidative damage the effect of eating vegetables, and more specific of isothiocyanates, on health was determined but a relation was not found. Future studies could enrol more subjects, last longer, stratify on genotype, administer broccoli with higher amounts of glucoraphanin and, upon crushing, with more sulforaphane, and measure more various biomarkers. Enzyme induction via the electrophile responsive element showed that the relative potency of isothiocyanates with a methyl-sulfur side chain, like sulforaphane, is higher than other aliphatic and aromatic isothiocyanates.

This thesis shows that more isothiocyanates are absorbed after the consumption of raw cruciferous vegetables, compared to cooked vegetables. It is not feasible to consume all crucifers raw, some are not edible since the plant matrix needs to be softened by cooking. This cooking should be kept to a minimum in time, amount of boiling water and microwave power, to retain as much active compounds as possible. Also, to protect health, more often crucifers should be consumed and in larger portions.

Cabbages and broccoli are favorite since they contain high amounts of sulforaphane, which is the most promising isothiocyanate (lowest EC_{50} on the EpRE). Since cruciferous vegetables contain several isothiocyanates which act synergistically, concentrations might thus reach these EC_{50} values for EpRE induction, and represent biologically active concentrations. After the consumption of raw crucifers, in particular condiments, a sharp rise in isothiocyanates in the blood is observed followed by a rapid decline. When cooked vegetables are consumed, longer lasting, lower concentrations of isothiocyanates in the body are observed so more data should be generated on the effect of these lower levels. Furthermore, an increase in glucosinolate content of the vegetables also increases the intake. This could be achieved by genetic modification of the plants or by selective plant breeding.

Future studies could use the kinetics and biomarkers described in this thesis to study the health potential of isothiocyanates from cruciferous vegetables.

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SAMENVATTING

Introductie

De consumptie van groente en fruit wordt in meerdere studies geassocieerd met een verlaagde kans op ziektes zoals kanker en hart en vaatziektes (1). In het bijzonder koolsoorten, waaronder witte kool, rode kool, Chinese kool, broccoli, mosterd, tuinkers en rucola, zijn naar verwachting gezond voor de mens (2). Koolsoorten bevatten glucosinolaten, stoffen die niet voorkomen in andere groenten. Daarom zijn deze actieve plantstoffen wellicht verantwoordelijk voor het beschermende effect van koolsoorten (3). Glucosinolaten worden gehydrolyseerd door myrosinase dat vrijkomt bij het snijden van- en kauwen op de groenten of door enzymen in de dikke darm (4,5). Eén van de afbraakproducten van glucosinolaten zijn isothiocyanaten waarvan in dierproeven is bewezen dat ze kunnen beschermen tegen het ontstaan van kanker. De voorgestelde werkingsmechanismen voor dit effect van isothiocyanaten zijn i) remming van fase 1 biotransformatie-enzymen (deze activeren carcinogene stoffen), ii) versnelling van fase 2 biotransformatie enzymen (deze detoxificeren giftige stoffen), iii) ontstekingsremming en iv) stimulering van apoptose (6). Isothiocyanaten worden opgenomen en getransporteerd door het lichaam als glutathionconjugaten. Om de werking van diverse glucosinolaat bevattende koolsoorten en het effect van de uit de glucosinolaten gevormde isothiocyanaten te kunnen bepalen, is het nodig dat de opname van de isothiocyanaten gekwantificeerd kan worden. Dit is mogelijk door in de urine de metabolieten van de isothiocyanaten te meten: de isothiocyanaat-mercaptuurzuren (7). Zie figuur 1.

Om iets te kunnen zeggen over de mogelijk gezondheidsbevorderende effecten van de inname van koolsoorten en dus van isothiocyanaten is het nodig parameters te meten die iets zeggen over de biologische beschikbaarheid van de isothiocyanaten vanuit een complexe matrix als groente. Om dit te kunnen doen worden biomarkers gebruikt. Met biomarkers voor biobeschikbaarheid kan de inname van een specifieke component en daarmee van de betreffende groente gemeten worden. Onder biobeschikbaarheid wordt verstaan dat deel van de opgegeten stof dat in het bloed komt en actief kan zijn. Er zijn qua structuur veel verschillende glucosinolaten aanwezig in de voeding die een verschillende kinetiek vertonen, afhankelijk van hun fysisch-chemische eigenschappen. Onder kinetiek wordt verstaan de opname, verdeling, omzetting en uitscheiding van een stof in de tijd. Deze kinetiek en het effect van koken van groente op de biobeschikbaarheid en kinetiek van isothiocyanaten kan bepaald worden aan de hand van conjugaten (metabolieten) die gemeten worden in bloed en urine.

Deze conjugaten dienen als biomarker waarmee antwoord kan worden gegeven op de vraag welke glucosinolaten de meest actieve isothiocyanaten opleveren, met de hoogste hoeveelheid per gram groente. Tevens zou dan de hoeveelheid kool vastgesteld kunnen worden die gegeten moet worden om de gezondheid te bevorderen.

glucoraphanin
thioglucosidase
rearrangement
sulforaphane
GSH conjugation
mercapturic acid pathway
sulforaphane derived mercapturic acid

Figuur 1. Voorbeeld van het metabolisme van glucosinolaten. Glucoraphanine wordt enzymatisch gehydrolyseerd tot sulforafaan, dat op zijn beurt geconjugeerd wordt met glutathion (GSH) en in de mercaptuurzuur-route verder wordt gemetaboliseerd tot sulforafaan mercaptuurzuur.

Samenvatting

Dit proefschrift beschrijft de biobeschikbaarheid, kinetiek en effecten van isothiocyanaten uit koolsoorten. Als eerste zijn mercaptuurzuren van de meest belangrijke isothiocyanaten chemisch gesynthetiseerd om te dienen als referentiestof voor de analyse. Deze isothiocyanaat-mercaptuurzuren zijn gebruikt als een biomarker voor de inname van isothiocyanaten. In maximaal vier synthesestappen zijn (commercieel niet verkrijgbare) isothiocyanaten gemaakt door het corresponderende alkylbromide toe te voegen aan kalium-phthalimide. Het verkregen N-alkyl-phthalimide is gehydrazinolyseerd wat het alkylamine opleverde. Het amine is met thiofosgeen omgezet in het isothiocyanaat met een totale opbrengst van 16%. In een vijfde synthesestap zijn isothiocyanaten geconjugeerd met N-acetyl-L-cysteine. Dit leverde het isothiocyanaat-mercaptuurzuur op met een typische opbrengst van 77%. De methoden voor de synthese van 12 isothiocyanaat-mercaptuurzuren worden beschreven in hoofdstuk 2.

Deze gesynthetiseerde referentiestoffen zijn gebruikt om een analysemethode op te zetten voor de bepaling van isothiocyanaat-mercaptuurzuren in urine met behulp van

vloeistofchromatografie en tandem-massaspectrometrie (hoofdstuk 3). Isothiocyanaat-mercaptuurzuren zijn uit de urine geïsoleerd met ‘solid phase extraction’. Met ‘ion-trap’ massaspectrometrie is gevoelige en specifieke detectie bereikt van lage gehalten isothiocyanaat-mercaptuurzuren in urine. De analysemethode als geheel is valide en robuust bevonden. Het concentratiebereik waarbinnen gemeten kan worden is 1,0-310 µM. Dit komt overeen met de hoeveelheid allylisothiocyanaat-mercaptuurzuur, gevonden in 24-uurs urine na de consumptie van allyl glucosinolaat (sinigrine) aanwezig in respectievelijk één blaadje van één spruitje (1,0 µM) en 200 gram spruiten (310 µM).

Drie vrijwilligers hebben 19 verschillende rauwe en gekookte groenten en specerijen gegeten om de biobeschikbaarheid en excretiekinetiek te bepalen van glucosinolaten en de afgeleide isothiocyanaten (hoofdstuk 4). Uit de resultaten blijkt dat de excretie van isothiocyanaat-mercaptuurzuren hoger was na consumptie van rauwe groenten en specerijen (biobeschikbaarheid van 8,2-113%) in vergelijking met gekookte groenten (biobeschikbaarheid van 1,8-43%). De piekconcentratie van isothiocyanaat-mercaptuurzuren in urine was na consumptie van gekookte groenten vier uur later dan na consumptie van rauwe producten. De halfwaardetijd was gelijk (2,1-3,9 uur). Geconcludeerd werd dat de biobeschikbaarheid van isothiocyanaten uit glucosinolaten groter is indien de groenten rauw gegeten worden dan wanneer ze worden gekookt.

In aanvullende experimenten, niet beschreven in dit proefschrift, werd de bijdrage van darmbacteriën aan de omzetting van glucosinolaten in isothiocyanaten bestudeerd met een *in vitro*-systeem. In dat onderzoek werd de biobeschikbaarheid en bioconversie van sinigrine en allylisothiocyanaat uit spruitjes bepaald met het TNO-maagdarmkanaalmodel (TIM 2). Met verschillende individuele menselijke microflora werd 10-30% sinigrine omgezet in allylisothiocyanaat in 36 uur (8). Geconcludeerd werd dat het verschil in biobeschikbaar allylisothiocyanaat als gevolg van verschillen in de individuele microflora groot is.

In verdere experimenten werd onderzoek gedaan naar het effect van isothiocyanaten op de activiteit van fase 2-biotransformatie-enzymen, één van de mogelijke mechanismen via welke isothiocyanaten hun beschermende werking tegen kanker zouden kunnen uitoefenen (hoofdstuk 5). Isothiocyanaten induceren de expressie van dit soort enzymen via een electrofiel responsief element (EpRE) dat zich in de promotorregio van het gen bevindt dat codeert voor deze enzymen. De mate van inductie werd gemeten met behulp van een *in vitro*-reporter gen assay. De mate van inductie van de genexpressie van deze fase 2 biotransformatie-enzymen via de EpRE varieert tussen de verschillende isothiocyanaten.

Isothiocyanaten met een methyl-zwavel-zijketen (waaronder sulforafaan, EC_{50} = 1,2 μM) zijn meer potent dan alifatische en aromatische isothiocyanaten (bijvoorbeeld allyl-isothiocyanaat, EC_{50} = 6,5 μM). Na een maaltijd rijk aan koolsoorten is het gehalte van een enkel isothiocyanaat in het lichaam 0,04-4 μM en kunnen dus in sommige gevallen biologisch actieve concentraties bereikt worden.

In een studie met acht mannelijke rokers is het effect van het eten van rauwe en gekookte broccoli op de opname- en excretiekinetiek van sulforafaan bestudeerd (hoofdstuk 6). De maaltijd met 200 gram gekookte broccoli in deze studie bevatte 61 μmol glucoraphanine maar dezelfde hoeveelheid rauwe broccoli (200 gram) bevatte slechts 9,9 μmol sulforafaan. Na hydrolyse van glucoraphanine is in de rauwe broccoli blijkbaar slechts 16% sulforafaan gevormd en 84% andere stoffen. Er zijn hogere gehaltes sulforafaan in urine gevonden na consumptie van rauwe broccoli (biobeschikbaarheid 37%) dan na gekookte broccoli (3,4%). In bloed werd een hoger gehalte sulforafaanconjugaten gemeten na consumptie van rauwe broccoli (0,50 μM·h, dosis van 9,9 μmol sulforafaan) in vergelijking met gekookt (0,29 μM·h, dosis van 61 μmol glucoraphanine). Het effect op biomarkers van bescherming en van oxidatieve schade is ook nagegaan door het meten van de activiteit van glutathion-S-transferase-alfa (GST-α), en de gehalten aan 8-isoprostaglandine F2-alfa (iPF2- α) en 8-oxo-7,8-dihydro-2'-deoxoguanosine (8-oxodG). Er is geen effect van de broccoli-opname op deze biomarkers gevonden. Polymorfisme in de genen van glutathion-S-transferase-M1, -T1 en -P1 en in de N-acetyl-transferase (NAT)-2-genen veranderde de opname- en excretiekinetiek van sulforafaan niet.

De toepassing van isothiocyanaat-mercaptuurzuren als biomarker voor de inname van koolsoorten is gevalideerd aan de hand van urine uit een studie van Fowke et al. (9). In deze studie met 34 vrouwen is door middel van gedragsinterventie de dagelijkse consumptie van koolsoorten verhoogd. Meerdere isothiocyanaat-mercaptuurzuren zijn gemeten en sulforafaan correleerde het best met de groente-inname (Spearman correlatie 0,49, $p<0,01$).

Conclusies

In dit proefschrift zijn methoden beschreven voor de bepaling van de biobeschikbaarheid, de kinetiek en een aantal mogelijke biomarkers voor de biologische effecten van isothiocyanaten uit koolsoorten. De biobeschikbaarheid van isothiocyanaten uit gekookte groenten is lager dan uit groenten en specerijen die rauw gegeten zijn. Gemiddeld 60% van de isothiocyanaten uit groenten en specerijen wordt opgenomen en uitgescheiden nadat ze rauw zijn opgegeten terwijl dit slechts 10% is na koken. De uitscheidingskinetiek

uitscheidingskinetiek van isothiocyanaten uit rauwe en gekookte groente is vergelijkbaar maar de kinetiek van opname verschilt significant. De opname van isothiocyanaten uit gekookte groenten is vertraagd en verlaagd omdat door het koken het enzym dat glucosinolaten omzet in isothiocyanaten geïnactiveerd wordt. Deze omzetting is daardoor beperkt tot de chemische afbraak en tot de omzetting door bacteriën in de dikke darm, met een lagere opbrengst. Isothiocyanaat-mercaptuurzuren in urine reflecteren de totale actieve dosis van opgenomen isothiocyanaten. Isothiocyanaat-mercaptuurzuren gemeten in de urine zijn daarom bruikbaar als biomarker voor de inname van glucosinolaten en isothiocyanaten. Het effect op de gezondheid van de inname van groenten, meer specifiek van isothiocyanaten, is in dit proefschrift bepaald met behulp van mercaptuurzuren (biomarker van blootstelling) en met biomarkers die een maat zijn voor de beschermende werking. Er werd geen verschil gevonden in effect op biomarkers voor mogelijk beschermende mechanismen tussen consumptie van rauwe en gekookte broccoli. Tips voor toekomstige studies zijn: het gebruik van meer proefpersonen, een langere blootstellingsduur, stratificeren op genotype, meer (verschillende) biomarkers gebruiken, gebruik van broccoli met een hoger gehalte glucoraphanine en zorgen dat na malen van rauwe broccoli meer sulforafaan gevormd wordt en de stof niet wordt afgebroken. De relatieve mate van enzyminductie via het electrofiel responsieve element (EpRE) is hoger met isothiocyanaten die een methyl-zwavelzijketen bevatten, zoals sulforafaan, dan die met een alifatische of aromatische zijketen.

Dit proefschrift toont aan dat een grotere hoeveelheid isothiocyanaten wordt opgenomen na consumptie van rauwe koolsoorten dan na inname van gekookte koolsoorten. Niet alle koolsoorten kunnen rauw gegeten worden, sommige moeten gekookt worden om de celstructuur zachter te maken. Er moet dan kort gekookt worden in weinig water en bij voorkeur in de magnetron op laag vermogen om zoveel mogelijk glucosinolaten vast te houden. Om de gezondheid te beschermen zouden koolsoorten vaker en in grotere hoeveelheden gegeten moeten worden. Kool en broccoli zijn favoriet want deze bevatten veel sulforafaan en dit is het meest veelbelovende isothiocyanaat omdat deze isothiocyanaat de laagste EC_{50} voor EpRE gemedieerde geninductie liet zien. Na het eten van rauwe koolsoorten en in het bijzonder specerijen, wordt een snelle toename van isothiocyanaten in het bloed waargenomen gevolgd door een snelle afname. Na het eten van gekookte groenten worden lagere concentraties waargenomen maar is de concentratie isothiocyanaten wel gedurende langere tijd verhoogd.

Gerelateerd aan de resultaten van het onderzoek met de EpRE kan geconcludeerd worden dat de concentraties in het lichaam na eten van gekookte koolsoorten een factor 1-100 onder de EC_{50} liggen en dat dus alleen in sommige gevallen biologisch actieve concentraties bereikt worden. Er is meer onderzoek nodig naar het biologische effect van deze lage concentraties isothiocyanaten. Verhogen van de hoeveelheid glucosinolaten in de groenten en specerijen zal de inname verhogen. Dit kan worden bereikt door genetische modificatie of door selectief kweken.

Toekomstige studies kunnen de in dit proefschrift beschreven nieuwe methoden voor het meten van de biobeschikbaarheid en kinetiek van isothiocyanaten gebruiken om het gezonde effect van isothiocyanaten uit koolsoorten te bestuderen.

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CURRICULUM VITAE

Martijn Vermeulen was born on 9th June, 1972, in De Bilt, The Netherlands. In his first year he moved to Montfoort where he followed junior education. In 1984 he passed secondary school, Atheneum, at the Dr. F.H. de Bruyne Lyceum in Utrecht and started the BSc study in Laboratory Education in Utrecht. During this study he focussed on analytical chemistry and worked for 11 months at TNO Nutrition and Food Research Institute in Zeist (Zeist has it all). The graduation-project at TNO taught him that tea has many varieties, many ways of preparation and a lot of different polyphenols. He received his license in Radiation Hygiene level 5B in 1994 in Utrecht. He finished his BSc in Analytical Chemistry in January 1995. At TNO he realised that chemical analysis should have a purpose and that nutrition was an interesting application. He therefore followed the study Human Nutrition, later called Nutrition and Health, at Wageningen Agricultural University, later referred to without agriculture. In September 1998, he obtained his MSc degree 'with distinction' in Human Nutrition (and Health) with main topics in nutrition, food chemistry, toxicology and molecular biology. He was then appointed for five years as a PhD student on the project 'Biomarkers of vegetable intake' at TNO in Zeist. As part of this project, he collaborated with research groups from the Department of Organic Synthesis at Nijmegen University, and with the Department of Microbiology at INRA, France. He received his license as Laboratoy Animal Scientist in 2002 in Utrecht. At TNO he received training in managing projects on a commercial basis. Later, this knowledge and his skills in analytical chemistry were also put into practice at NOTOX in 's Hertogenbosch where he worked for one year as study director in vitro metabolism. Since June 2006, he is working as a study director for Nutrient and Biomarker Analysis at TNO in Zeist.

LIST OF PUBLICATIONS

M. Vermeulen, I.W.A.A. Klöpping-Ketelaars, R. van den Berg, W.H. J. Vaes. Bioavailability and kinetics of sulforaphane in humans after consumption of cooked versus raw broccoli. J. Agric. Food Chem. 2008, 56(22), 10505–10509.

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A-M.J.F. Boerboom, M. Vermeulen, H. van der Woude, B.I. Bremer, Y.Y. Lee-Hilz, E. Kampman, P.J. van Bladeren, I.M.C.M. Rietjens, and J.M.M.J.G. Aarts. Newly constructed stable reporter cell lines for mechanistic studies on electrophile-responsive element-mediated gene expression reveal a role for flavonoid planarity. Biochem. Pharmacol. 2006, 72, 217-226.

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Mijn eerste jaar als AIO bracht ik door op de Universiteit van Nijmegen bij de groep Organische Chemie. Binne Zwanenburg, Gordon Chittenden, Henk Regeling en andere collega’s bedankt voor de hulp bij de organische synthese van isothiocyanaten en van mercaptuurzuren.

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Overview of completed training activities

	ECTS credits
Discipline specific activities	
Courses	
Laboratory animal science, Universiteit Utrecht, 2002	4.2
Nutrigenomics Masterclass, VLAG, Wageningen, 2001	0.9
Mechanism-based pharmacokinetics and -dynamics, LACDR, Leiden, 2000	0.3
Chemistry and biochemistry of antioxidants, VLAG, Wageningen, 2000	2.0
Hands-on LC-MS training, TNO Pharma, Zeist, 2000	1.4
Meetings	
Inflammatory and oxidative cell death, Seoul, South Korea, 2003 (oral presentation)	1.1
Eurofeda, Functional effects of dietary antioxidants, Cambridge, UK, 2002 (poster)	1.7
Eurofoodchem XI, Dietary antioxidants - health, Norwich, UK, 2001 (poster)	1.7
Functional Foods 2000, Leatherhead Food RA & BV IPI, The Hague, 2000	0.9
Workshop on antioxidants, NVVL/KNCV, Wageningen, 1999	0.3
Food and Cancer Prevention III, Norwich, United Kingdom, 1999 (poster presentation)	1.7
Biomarkers 99 Diet & health, Jena, Germany, 1999 (poster presentation)	1.4
NWO Nutrition, Papendal, The Netherlands, 1998-2004	0.9
General courses	
VLAG PhD week, Nijmegen, 2000	1.1
Project management, Leeuwendaal advies BV, Arnhem, 2001	0.9
Practical implementation of Good Laboratory Practice, Cygnus, Zeist, 2001	0.6
Scientific journalism, KNCV, Utrecht, 2000	1.2
Farmaco kinetics, Leiden/Amsterdam Center for Drug Research, Oss, 2000	1.2
Time management, Leeuwendaal advies bv, Maarssen, 2000	0.9
Customer oriented business, DOOR, Noordwijkerhout, 2000	0.3
Employability, Employability company, Zeist, 1999	0.3
Optionals	
Preparation of PhD research proposal	6.0
Nijmegen University PhD-student discussion groups, 1998-1999 (weekly)	1.5
TNO Zeist PhD-student discussion groups, 1998-2004 (yearly)	1.5
Total	***34***

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