IN VITRO GASTROINTESTINAL STABILITY OF GLUCOSINOLATES AND THEIR DEGRADATION PRODUCTS FROM SELECTED PLANTS OF THE ORDER BRASSICALES

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Dani doktorata biotehničkog područja
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Brassicaceae family

- Vegetables that are present in the daily diet
- Contains sulfur compounds (glucosinolates)
- Glucosinolates are natural organic compounds (secondary plant metabolites)
- In plant tissues, the glucosinolates are present at the same time as the enzyme myrosinase
- Myrosinase is distributed in myrosin cells that do not contain glucosinolates

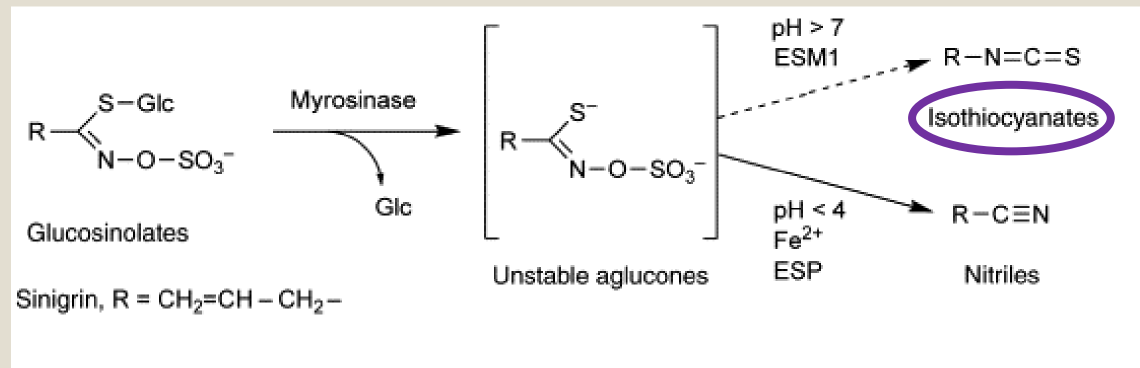


Figure 1. Degradation of glucosinolates

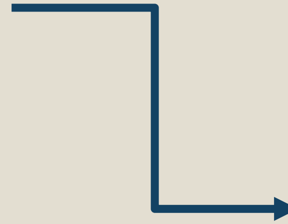
- The isothiocyanates have been repeatedly studied for their anticancer effects

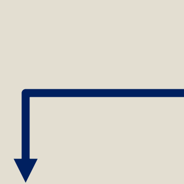
Isothiocyanates

- Anticancer activity
- Antimicrobial activity
- Antifungal activity
- Antioxidant activity

The experimental part…

Extraction of glucosinolates





Identification of glucosinolate present in various mustards using UHPLC-DAD-MS/MS

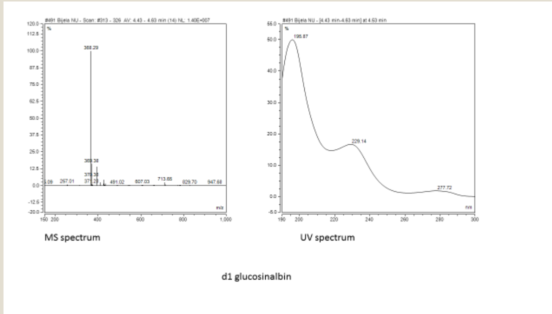
Table. GLS contents in various mustard seeds (*Sinapis alba*, *Brassica juncea*, and *Brassica nigra*)

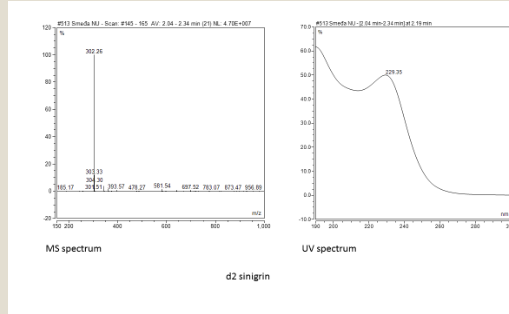
Glucosinolates present in mustard seeds	GLS content (μmol/g DW)		
	S. alba	*B. juncea*	*B. nigra*
Glucosinalbin	142.33	/	/
Sinigrin	/	53.56	12.40
Gluconapin	/	/	42.30
4-hydroxyglucobrassicin	/	1.00	1.60
Total content of glucosinolates	142.33	54.56	56.30

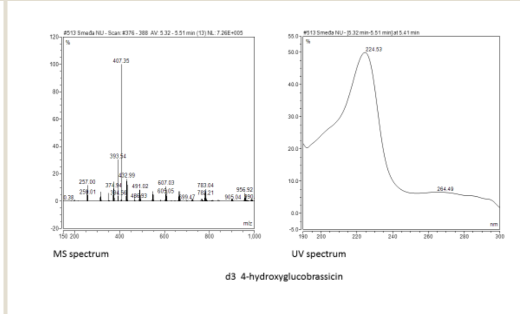
GLS glucosinolate
DW dry weight

White mustard (*Sinapis alba* L.)

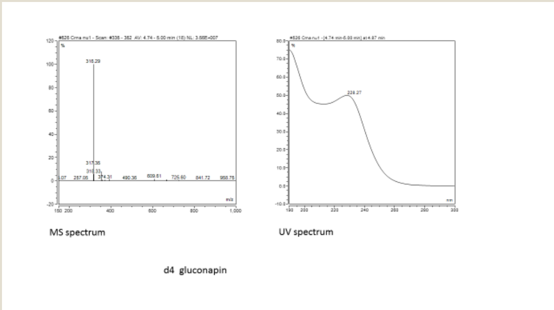
Brown mustard (*Brassica juncea* L.)

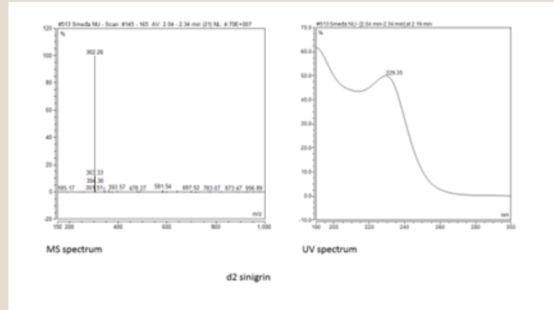


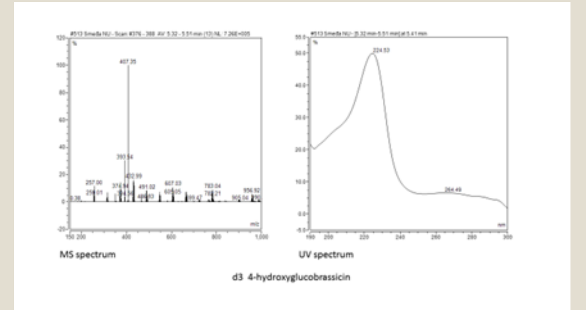




Black mustard (*Brassica nigra* L.)







Obtaining essential oil by microwave-assisted distillation (MAD)

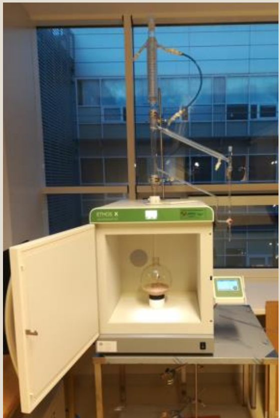


Fig. Obtaining essential oil by microwave-assisted distillation (MAD)

Fig. GC-MS/MS analysis

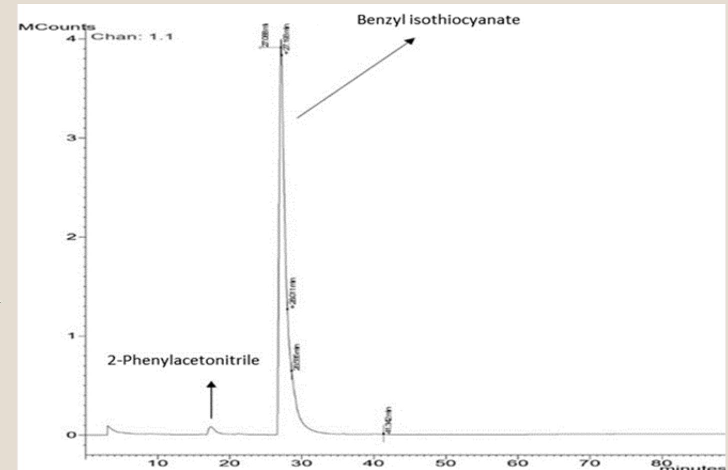


Fig. Chromatogram of volatile compounds in essential oil of *Tropaeolum majus* L. *altum* seeds

Content of benzyl isothiocyanate in essential oil of *T. majus* L. seeds = 97.81%

GC-MS analysis of essential oils from mustards

Brown mustard

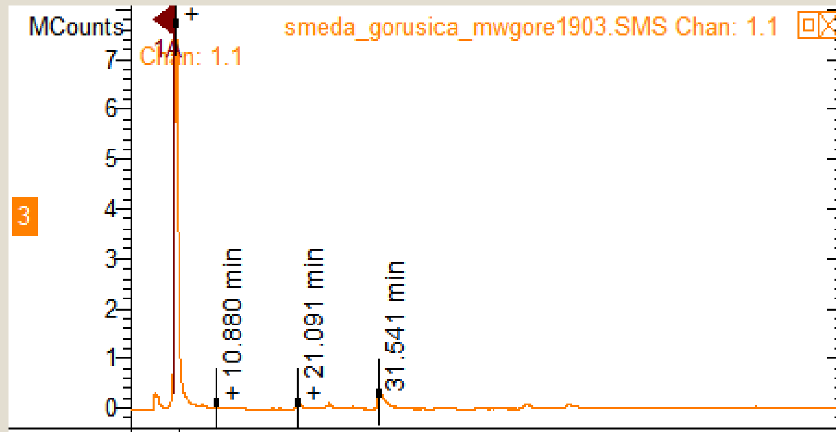


Fig. Chromatogram of volatile compounds in essential oil of *Brassica juncea* L. seeds

Black mustard

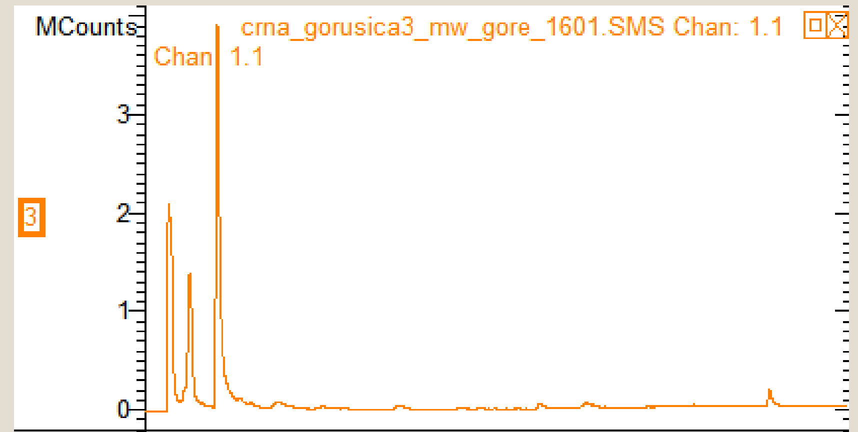


Fig. Chromatogram of volatile compounds in essential oil of *Brassica nigra* L. seeds

Content of allyl isothiocyanate in *B. juncea* L. seeds 91.07%

Content of allyl isothiocyanate in *B. nigra* L. seeds 16.51%

Proteolytic activity of human gastric and duodenal juices

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In vitro digestion of bovine and caprine milk by human gastric and duodenal enzymes

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Abstract

In vitro digestion was performed by human proteolytic enzymes on bovine and caprine individual milks. Two types of caprine milk were investigated: with high and low contents of α_{S1}-casein (CN). In addition the influence of heating of the milk on digestion was examined. The digestion was performed in two steps using human gastric and duodenal juice. Protein and peptide profiles were studied by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF). Caprine milk proteins were digested faster than bovine milk proteins. This was confirmed by the degradation profile obtained for both cows' and goats' milk, and was most evident for β-lactoglobulin. Comparing the digestion of milk protein from two groups of goats, high and low in α_{S1}-CN content, respectively, did not show significant differences. Heat treatment of milk had a strong and significant effect on the level of digestion. Raw milk was degraded faster than the heat-treated milk, and the effect of heating was different for bovine and caprine milk.
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Keywords: Digestion; Human proteolytic enzymes; Caprine milk; Bovine milk; Genetic polymorphism; Heat treatment

1. Introduction

Milk proteins provide a major dietary source for humans, supplying amino acids for the synthesis of proteins and other nitrogen-containing compounds (Munro, 1969; Millward & Pacy, 1995; Young & Pellet, 1989). In addition, some of these proteins contain bioactive peptides released by hydrolysis that may affect the human health. These effects include mineral binding, growth factors, blood pressure reduction (Tomé & Debabbi, 1998) and protective properties against different micro-organisms and viruses (Meisel & Schlimme, 1996; Pihlanto & Korhonen, 2003). The nutritional efficiency of milk proteins clearly depends on the content of essential amino acids that is delivered during the digestion of the proteins, and the absorption in the gut of amino acids and peptides released (Bos, Gaudichon, & Tomé, 2000).

There has recently been an increased attention on cows' milk allergy, particularly among infants (Paupe, Paty, de Blic, & Scheinmann, 2001; Sampson, 2004). As a result, alternative sources for milk have been asked for. This has lead to an increasing interest in and demand for caprine and equine milk. Milk from the goat differs from that of the cow in the composition of many components, which may influence the digestibility of the milk. The composition and structure of the fat, for instance, is quite different in both types of milk. Goats' milk contains smaller fat globules and higher amounts of short-chain fatty acids. The naturally emulsified fat of goats' milk is, from a human health standpoint, much easier to digest (Haenlein, 1992).

Also, the protein composition and structure of milk of these animals differ, again with possible consequences for the digestibility. Although the general distribution of

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2.3. Human gastric and duodenal enzymes

Human proteolytic enzymes were obtained in the activated state by collecting human gastric (HGJ) and duodenal juice (HDJ) according to Holm, Hanssen, Krogdahl, and Florholmen (1988). All gastric and duodenal enzymes used in this study were obtained from one person. In brief, a three-lumen tube enabled both simultaneous instillation of saline in the duodenum, and aspiration of gastric and duodenal juice from the volunteer. Saline (100 mL h^{-1}) was instilled close to the papilla of Vater to stimulate the production of proteolytic enzymes, and duodenal juice was aspirated some 18 cm distally. Aspirates were collected on ice and frozen in aliquots. Before further use the individual samples of HGJ and HDJ were mixed into two batches to avoid differences in enzyme activity between the samples.

Proteolytic activity in the HGJ was assayed according to Sanchez-Chiang, Cisternas, and Ponce (1987). The pepsin activity was measured with bovine haemoglobin at pH 3.0 as a substrate. In HDJ the concerted action of proteases and peptidases named "Total proteolytic activity" was assayed at pH 8.0 with CN as substrate according to Krogdahl and Holm (1979). The reactions were stopped after 20 min of incubation by addition of 10% TCA. After centrifugation, absorbance at 280 nm of the trichloroacetic acid soluble hydrolysis product was used as measure of proteolysis. One unit (U) of enzyme activity is defined as the amount of enzyme that gives an absorbance of 1.0 at 280 nm in 20 min at 37 °C.

2.4. Digestibility assay—pH drop method

A modified digestibility assay, in vitro protein digestion (AOAC Official Method 982.30; Rasco, 1994), was performed in two steps, using HGJ and HDJ. The procedure developed to mimic a "normal digestion" in the human gastro-intestinal tract consisted of two incubation periods, imitating both the human stomach and the duodenum. Each period lasted 30 min at 37 °C. Previous results showed that no new peptides were produced with an extended reaction time (unpublished results). First, 10 mL of skimmed milk acidified to pH 2.5 with 2 M HCl were incubated with 50 μL (0.4 U) HGJ. Then the pH was adjusted to 7.5 with 1 M NaOH, 400 μL (13.0 U) HDJ was added and the mixture was incubated again with continuous stirring. The change in pH in the milk during the degradation with HDJ was measured every minute, and the corresponding pH curves were plotted. Aliquots (0.5 mL) were also taken out for gel electrophoresis at different times during the incubation. To stop the proteolytic reactions, samples were put on ice, frozen and freeze dried.

The assay was performed with individual milk samples from eight animals of each group: two groups of goats, and one group of cows. Each sample was run in duplicate, and results are presented as the average of all 16 measurements within each group.

2.5. Gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to evaluate the protein profile after each step of hydrolysis (PhastSystem™, Pharmacia Laboratory Separation Division, Amersham Biosciences, Uppsala, Sweden). The assay was performed according to standard protocols (Laemmli, 1970), using 20% acrylamide gels (PhastSystem™ Homogeneous 20 gels, Amersham Biosciences). The molecular mass markers used were the low molecular weight standard kit (LMW Calibration kit, Amersham Biosciences). Staining was performed according to standard procedure (Amersham Biosciences). Gels from SDS-PAGE were scanned, and the amount of protein was quantified by analysis using Image Master 1D quantification software (Amersham Biosciences). The amount of protein was divided with the total protein content in the milk (see Section 2.2), in order to be able to compare results between different milk samples.

Genetic variants of the α_{S1}-CN from individual goats were determined by IEF using ultra thin (0.3 mm) urea containing polyacrylamide gels according to a modified method of Erhardt (1989) (Devold et al., 2000; Vegarud et al., 1989). A mixture of ampholytes was chosen in order to give a maximum resolution of the caprine α_{S1}-CN-complex; Ampholine pH 3.5–5.0, Pharmalyte pH 4.2–4.9 and Pharmalyte pH 5.0–6.0 (Amersham Biosciences) were used in the ratio 3:4:1. Coomassie Brilliant Blue R-250 was used for staining. The different α_{S1}-CN-variants of goats were identified according to lyophilised CN samples from goats known to be strong or lacking the α_{S1}-CN in the milk (kindly provided by Prof. F. Grosclaude, INRA, France).

2.6. Statistics

Student's *t* tests (two-sample, assuming equal variances) were run to compare the protein and CN contents in the different types of goats' milk (assuming one-tail alternative). Differences were considered significant when p values were less than 0.05, here and in the following analyses. For the digestion studies the drop in pH during the first 5, 10 and 30 min of hydrolysis with HDJ were studied. The drop in pH was modelled as dependent on the groups of goats and cows (in proc GLM of SAS) using the model:

$$\text{Drop in pH} = \text{mean} + \text{milk group} + \text{error}. \quad (1)$$

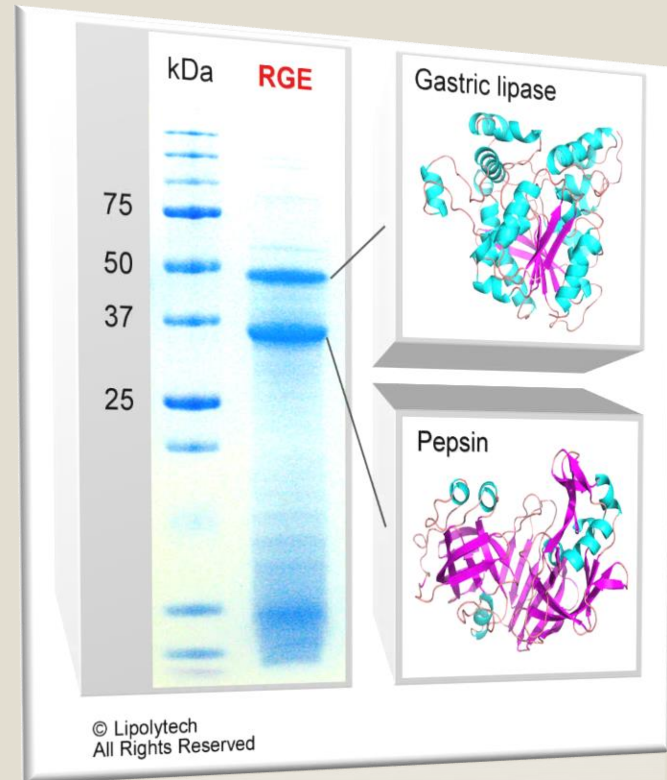
Milk group was either from goats lacking the α_{S1}-CN (G0), from goats expressing the α_{S1}-CN (GS) or from cows. The term "error" is the effect of each of the eight individuals in a group, in addition to random error. Significances of pair-wise milk-type comparisons, and the contrast of goat versus cow were estimated.

When comparing the effect of heat treatment on mixtures of milk samples from the eight individual animals

Collection of human digestive juices

- Human digestive juices (gastric and duodenal) were aspirated from healthy volunteers.
- The initial fraction of aspired juice was discarded from each healthy volunteer and the remaining amount was collected in a sterile tube, which was centrifuged to remove mucus and cell debris.
- Prior to further analysis, all human gastric and duodenal juices (HGJ and HDJ) were mixed in order to avoid differences in enzymatic activity.

- The lipase activity present in rabbit gastric extract (RGE) has already been well characterized and compared to that of human gastric lipase under conditions mimicking gastric digestion.



- The absorbance of digestive juices was measured spectrophotometrically.

SDS-PAGE ELECTROPHORESIS

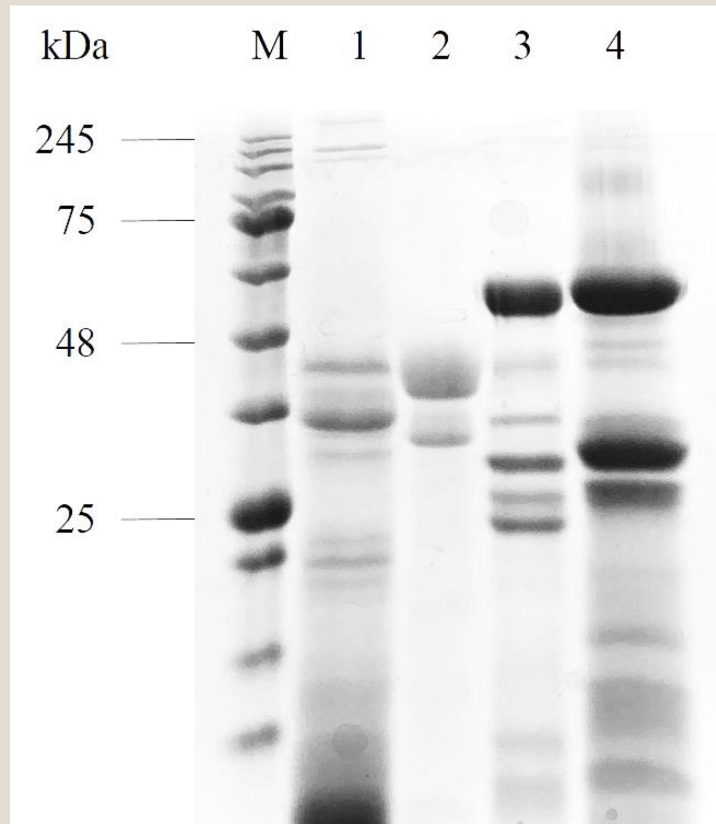
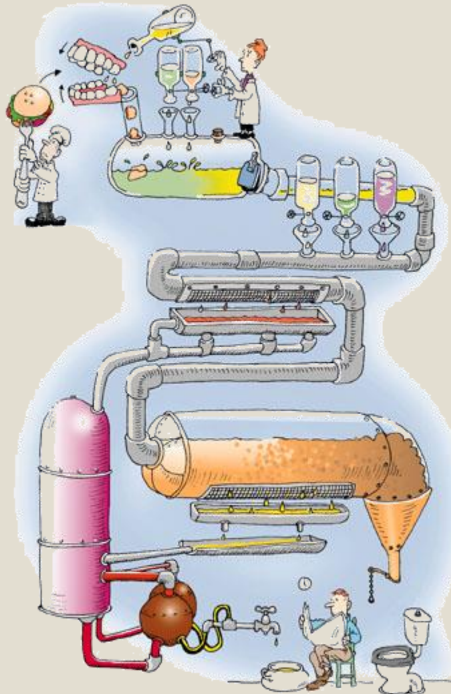


Figure 1. Quality control by SDS-PAGE analysis of commercial and human digestive juices: M-Prestained Protein Marker VI (Applichem); 1-Rabbit gastric fluid (RGF); 2-Human gastric juice (HGJ); 3-Simulated intestinal fluid (SIF); 4-Human duodenal juice (HDJ).

For SDS-PAGE analysis, 10 µL of protein sample was mixed with sample buffer (6X) and denatured at 95°C for 5 min. Samples were analyzed on previously prepared 12% SDS-PAGE gels with pre-stained protein marker VI (AppliChem) as molecular weight reference. Gels were immersed in glycine buffer and electrophoresis was performed at 100 V for 1 hour. The gels were stained overnight with staining solution (0.5 g Coomassie bb R-250, 800 mL methanol, 140 mL acetic acid and water up to 2 L) and destained next day in mQH_2O. Gel images were taken by ChemiDoc MP Imaging System (Bio-Rad) and analysed with Image Lab software (Bio-Rad).

In vitro digestion method



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A standardised static *in vitro* digestion method suitable for food – an international consensus†

M. Minekus, M. Alminger, P. Alvito, S. Ballance, T. Bohn, C. Bourlieu, F. Carrière, R. Boutrou, M. Corredig, D. Dupont, C. Dufour, L. Egger, M. Golding, S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. Macierzanka, A. Mackie, S. Marze, D. J. McClements, O. Ménard, I. Recio, C. N. Santos, R. P. Singh, G. E. Vegarud, M. S. J. Wickham, W. Weitschies and A. Brodkorb

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INFOGEST static in vitro simulation of gastrointestinal food digestion

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Developing a mechanistic understanding of the impact of food structure and composition on human health has increasingly involved simulating digestion in the upper gastrointestinal tract. These simulations have used a wide range of different conditions that often have very little physiological relevance, and this impedes the meaningful comparison of results. The standardized protocol presented here is based on an international consensus developed by the COST INFOGEST network. The method is designed to be used with standard laboratory equipment and requires limited experience to encourage a wide range of researchers to adopt it. It is a static digestion method that uses constant ratios of meal to digestive fluids and a constant pH for each step of digestion. This makes the method simple to use but not suitable for simulating digestion kinetics. Using this method, food samples are subjected to sequential oral, gastric and intestinal digestion while parameters such as electrolytes, enzymes, bile, dilution, pH and time of digestion are based on available physiological data. This amended and improved digestion method (INFOGEST 2.0) avoids challenges associated with the original method, such as the inclusion of the oral phase and the use of gastric lipase. The method can be used to assess the endpoints resulting from digestion of foods by analyzing the digestion products (e.g., peptides/amino acids, fatty acids, simple sugars) and evaluating the release of micronutrients from the food matrix. The whole protocol can be completed in ~7 d, including ~5 d required for the determination of enzyme activities.

Introduction

The worldwide prevalence of diet-related diseases has been on the increase for the past few decades[1]. Large-scale human intervention trials have been used to correlate diet with the health of different demographic groups. However, to understand the physiological response to specific foods, it is necessary to follow the complex digestive processes within the human digestive tract in more detail. This can be achieved with invasive procedures, such as aspiration from the stomach[2] or small intestine[3] or with less invasive imaging technologies (e.g., magnetic resonance imaging[4]) and wireless

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Advantage: Although in vivo models give the most accurate results, in vitro models are a cheaper and useful alternative to in vivo models!

In vitro digestion method of glucosinolates

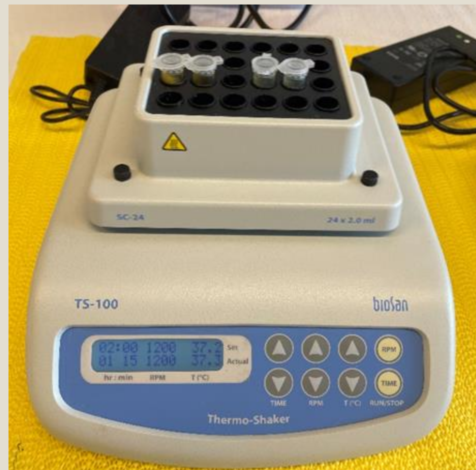
GASTRIC DIGESTION

- imitation of pH in the stomach of an adult
- pH 2,5
- Duration of gastric phase: 30 min

INTESTINAL DIGESTION

- imitation of pH in the intestines of an adult
- pH 6,5–7
- Duration of intestinal phase: 120 min

Samples with commercial digestive juices were prepared according to the method described by Brodkorb et al. (2019).



$$\text{Bioaccessibility (\%)} = (\text{sample}_{\text{concentration after digestion}} / \text{sample}_{\text{concentration before digestion}}) \times 100\% \quad (1)$$

Desulfatation of glucosinolates

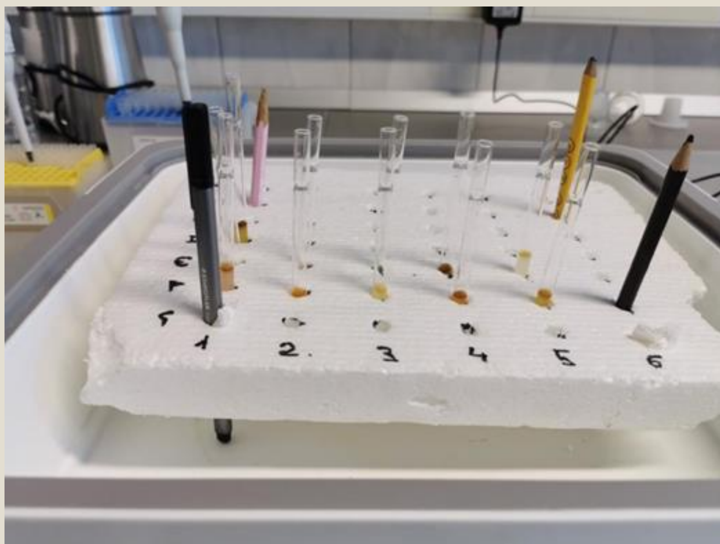


Fig. Prepared columns for extraction of desulfoglucosinolates

4. Extraction of Glucosinolates

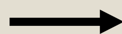
1. Weigh freeze-dried and finely-ground plant material (usually 50.0-100.0 mg of dry weight; the final glucosinolate concentrations in the extract should be in the range of the reference curve) to the nearest 0.1 mg in 2-mL, labeled, round-bottom reaction tubes. Add two small metal balls (3 mm in diameter) as boiling retardants to each tube.
   NOTE: The protocol can also be applied to fresh, flash-frozen materials, that have been ground under liquid nitrogen and kept frozen until extraction. Increase the amount of material weighed for extraction and the percentage of MeOH in the extraction liquid to 85% to compensate for dilution by the water in the materials[19].
2. Pipette 1 mL of 70% MeOH into each tube and vortex briefly. Close the tubes and seal them with safety caps before placing them as quickly as possible into a hot water bath (90-92 °C) for a few minutes (~5 min), until the 70% MeOH just boils. Caution: Wear safety goggles during this step!
3. Place the sample tubes in an ultrasonic bath for 15 min. Meanwhile, take the sulfatase and the five sinigrin reference samples out of the freezer to thaw them at RT.
4. After ultra-sonication, centrifuge the sample tubes at 2,700 x g in a benchtop centrifuge for 10 min at RT; a pellet should form in each tube. Add the supernatants to the labelled columns and pipette the five reference samples onto separate columns.
   1. While pipetting the supernatants, keep the tip well over the pellet to avoid pipetting plant materials. Note that when dried samples are used, the supernatant volume will be less than 1.0 mL.
5. Add 1 mL of 70% MeOH to the remaining pellets in the sample tubes and vortex the tubes before placing them in an ultrasonic bath for 15 min. Centrifuge the tubes again, as in step 4.4, and add the supernatants to the respective columns; due to the properties of the column material, the negatively charged sulfate group of the glucosinolates will be specifically retained on the column.
6. Wash the columns with the extracts in three sequential steps.
   1. Pipette 2 x 1 mL of 70% MeOH onto each column. Wait for the column to run dry before adding the next 1 mL; this will remove more apolar compounds from the extracts (*e.g.*, chlorophyll).
   2. Flush out the MeOH by adding 1 mL of ultrapure water to each column.
   3. Pipette 2 x 1 mL of 20 mM NaOAc buffer to each column to create the optimal conditions for the sulfatase reaction.
7. Take the rack with the columns out of the waste tray and dry the feet of the rack with a tissue. Place the rack over the block with vials and labeled tubes. Make sure that each column tip is in the corresponding, labeled, 2-mL tube (see **Figure 1**).
8. Add 20 µL of sulfatase solution to the columns. Ensure that the sulfatase reaches the surface of the column material. Pipette 50 µL of NaOAc buffer onto each column to flush down the sulfatase. Cover the columns with aluminum foil and let stand overnight.
   NOTE: Due to the activities of the sulfatase, the sulfate group will be removed, releasing the desulfoglucosinolates from the column so that they can elute with the water. For a detailed description, see Crocoll *et al.*[19].
9. The next day, elute the desulfoglucosinolates by pipetting 2 x 0.75 mL of ultrapure water onto each column. When all columns have run dry, lift the column rack and remove it from the reaction tubes.
   1. Cap the tubes (make sure that there are holes in the caps) and freeze them in liquid nitrogen or in a -80 °C freezer for 30 min. Freeze-dry the samples for 12-24 h (depending on the number of samples and the capacity of the freeze-drier) to remove all water.
10. After freeze-drying, re-dissolve the residue in an exact volume (usually 1.0 mL) of ultrapure water. Transfer the samples and the five sinigrin references to labeled HPLC vials. Keep the samples in a refrigerator (4 °C) for up to two weeks or a freezer (-20 °C) for up to one year before analyzing them with HPLC.
11. Let the glass columns dry under the hood overnight and dispose them when dry. Recover the metal balls from the sample tubes used in step 4.5 for reuse and put the tubes in the waste disposal bin.

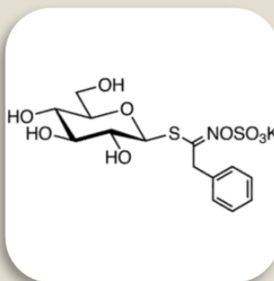
Table. Bioaccessibility of GSLs from various mustard seeds after two-phase *in vitro* and *ex vivo* digestion methods

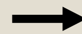
Digestion phases	**Bioaccessibility (%)**					
	Various mustard seeds					
	S. alba 1	*B. juncea* 2	*B. juncea* 3	*B. nigra* 2	*B. nigra* 3	*B. nigra* 4
In vitro gastric phase	74.94	63.51	97.00	88.52	83.07	79.39
In vitro intestinal phase	54.04	35.36	75.00	23.59	14.65	28.49
Ex vivo gastric phase	85.58	93.38	~100.00	97.24	84.38	~100.00
Ex vivo intestinal phase	80.26	41.44	35.00	41.18	15.25	64.48

1 Glucosinalbin
2 Sinigrin
3 4-Hidroxyglucobrassicin
4-Gluconapin

In vitro gastrointestinal stability of isothiocyanates







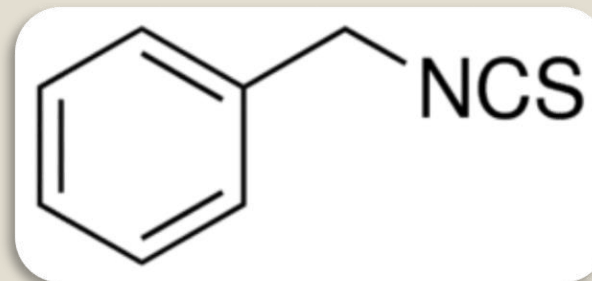


Table. Stability of benzyl isothiocyanate (BITC) from *Tropaeolum majus* L. *altum* essential oil after *in vitro* and *ex vivo* digestion methods

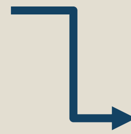
	Concentration (mg/ml)	Stability, (%)	Overall stability (%)*
after gastric phase (*in vitro*)	1.62	97.57	69.44
after intestinal phase (*in vitro*)	1.19	71.17	
after gastric phase (*ex vivo*)	1.22	73.47	40.34
after intestinal phase (*ex vivo*)	0.91	54.90	

*Overall stability was calculated by multiplying percentages of gastric and intestinal phases.

Table. Stability of pure benzyl isothiocyanate (BITC) after *in vitro* and *ex vivo* digestion methods

	Concentration (mg/ml)	Stability, (%)	Overall stability (%)*
after gastric phase (*in vitro*)	1.41	79.23	55.75
after intestinal phase (*in vitro*)	1.25	70.36	
after gastric phase (*ex vivo*)	1.29	72.24	46.74
after intestinal phase (*ex vivo*)	1.15	64.70	

*Overall stability was calculated by multiplying percentages of gastric and intestinal phases



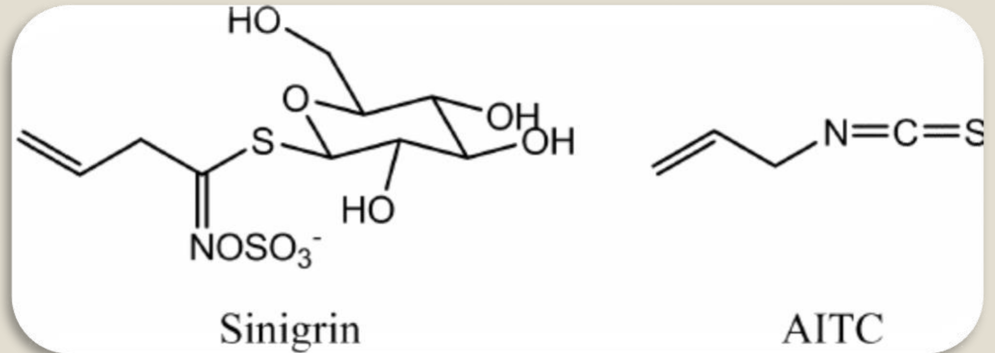


Table. Concentration of allyl ITC before and after *in vitro* and *ex vivo* digestion methods

Concentration of allyl ITC	Stability (%)
After gastric phase (*in vitro*)	85.14
After intestinal phase (*in vitro*)	54.87
After gastric phase (*ex vivo*)	86.27
After intestinal phase (*ex vivo*)	30.20

ITC isothiocyanate

The content of ITCs that remains after digestion and is available for the absorption is important due to the various biological activities mentioned above!!!

Conclusion

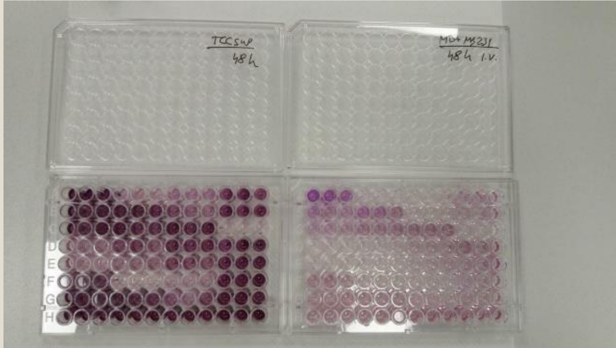
- Understanding the absorption routes of GSLs and their degradation products in the human body is of great importance, due to the biological properties of their breakdown products, especially ITCs.

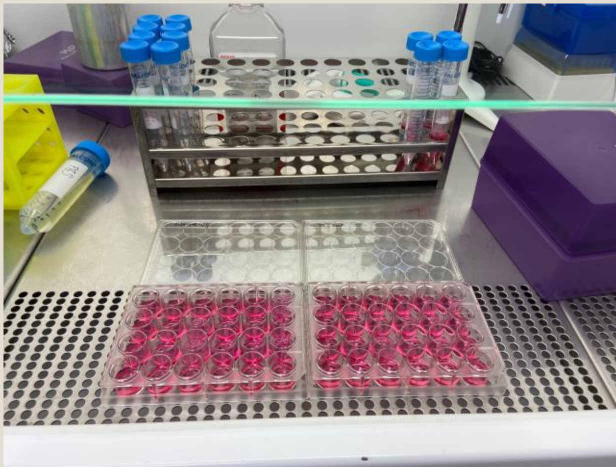
- The GSLs and ITCs contents were significantly reduced after the *in vitro* and *ex vivo* intestinal phases compared to the *in vitro* and *ex vivo* gastric phases.

- In addition, differences in pH and enzyme activity, concentrations of electrolytes, duration of digestion phases may also considerably alter results.

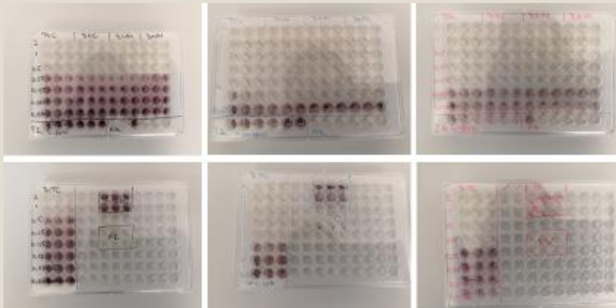
- Although in vivo models give the most accurate results, *in vitro* models are a cheaper and useful alternative to *in vivo* models!

Continuation of experiments…





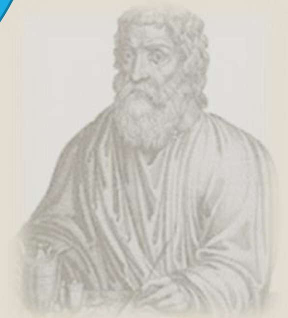
- *In vitro* testing of the antiproliferative activity was performed against two human cancer cell lines: breast cancer cell line MDA-MB-231 and bladder cancer cell line TCCSUP
- Cell viability and proliferation was determined by measuring cellular metabolism *via* MTT assay



- Antimicrobial activity (bacteria, fungi, yeasts)

The End…

Hippocrates recommends white mustard for internal use against gastrointestinal problems and for externally, mixed with vinegar to extract inflammation!



Thank you for your attention!