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Myrosinase compatible simultaneous determination of glucosinolates and allyl isothiocyanate by capillary electrophoresis – micellar electrokinetic chromatography (CE-MEKC)

short title:
Simultaneous determination of glucosinolates and AITC by CE-MEKC

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Abstract

Introduction. The functional food Cruciferous vegetables contain glucosinolates which are decomposed by the myrosinase enzyme upon tissue damage. The isothiocyanates are the most frequent decomposition products. Because of their various bioactivities, these compounds and the myrosinase is of high interest to many scientific fields.

Objective. Development of a capillary electrophoresis method capable of myrosinase-compatible, simultaneous quantification of glucosinolates and isothiocyanates.

Methods. Capillary electrochromatography parameters were optimized, followed by optimization of a myrosinase-compatible derivatization procedure for isothiocyanates. Vegetable extracts (Brussels sprouts, horseradish, radish and watercress) were tested for myrosinase activity, glucosinolate content and isothiocyanate conversion rate. Allyl isothiocyanate was quantified in some food products.

Results. The method allows quantification of sinigrin, gluonasturtiin and allyl isothiocyanate after myrosinase compatible derivatization in-vial by mercaptoacetic acid. The chromatograhpic separation takes 2.5 minutes (short end injection) or 15 minutes (long end injection). For the tested vegetables, measured myrosinase activity was between 0.960 – 27.694 and 0.461 – 26.322 μmol min^{-1} mg^{-1} protein, glucosinolate content was between 0 – 2291.8 and 0 – 248.5 μg g^{-1} fresh weight for sinigrin and gluconastrutiin, respectively. The possible specificity of plants to different glucosinolates was also shown. Allyl isothiocyanate release rate was different in different vegetables (73.13 to 102.13 %). The method could also be used for quantification of allyl isothiocyanate from food products.

Conclusions. The presented capillary electrophoresis method requires a minimal amount of sample and contains only a few sample preparation steps, and can be used in several applications (glucosinolate determination, myrosinase activity measurement, isothiocyanate release estimation).

Short abstract

A fast myrosinase-compatible capillary electrophoresis – micellar electrokinetic chromatography method for simultaneous determination of glucosinolates and allyl isothiocyanate was developed. The method was successfully used to determine glucosinolates (sinigrin and gluconasturtiin) from plant matrices (radish, horseradish, Brussels sprouts and watercress), to determine myrosinase activity using either sinigrin or gluconasturtiin as substrates as well as to determine allyl isothiocyanate from food products, and glucosinolate - isothiocyanate conversion rate.

Keywords

myrosinase assay
in-vial derivatization
Brassicaceae vegetables
allyl isothiocyanate
phenethyl isothiocyanate

Abbreviations

CE, capillary electrophoresis
CHES, N-cyclohexyl-ethanesulphonic acid;
FW, fresh weight;
ITC, isothiocyanate;

MEKC, micellar electrokinetic chromatography;

INTRODUCTION

Bioactive natural products are being more and more extensively used for various purposes, including agricultural, nutraceutical and pharmaceutical applications. Consumption of foods containing high amounts of beneficial phytochemicals – the functional foods – is also an important contribution to their use in reducing the risk or severity of diseases (Diplock *et al.,* 1999). The majority of these phytochemicals are biosynthesized and then "simply" accumulated by the plant organs. However, in several plant species, the biologically inactive precursor of the bioactive compound is biosynthesized, with the activating enzyme accumulated in a separate compartment. Upon tissue damage, the two compartments merge, resulting in a reaction that gives rise to the actual bioactive defensive metabolites (Hanschen *et al.,* 2014). A good example for this is the group of the widely cultivated Cruciferous vegetables (and other Brassicaceae species) that biosynthesize precursors called glucosinolates, which are rapidly decomposed by the enzyme myrosinase (EC 3.2.3.1) (Figure 1.a.) (Rask *et al.,* 2000). Myrosinase catalyzes the release of an unstable aglycon (thioglycosidase activity), the fate of which depends on many conditions (Figure 1.a.). In the absence of the so-called specifier proteins, the generated aglycones of most glucosinolates readily undergo Lossen rearrangement to form isothiocyanates (ITCs), making the ITCs the most frequent decomposition products of glucosinolates (Figure 1.a.) (Wittstock and Burow, 2007). The presence of low pH (<5) and/or Fe^{2+} ions also influence the breakdown: in this case nitriles are the main decomposition products, as the Lossen rearrangement of the unstable aglycon is inhibited (Hanschen *et al.,* 2014). Under specific conditions, usually in the presence of specifier proteins like thiocyanate-forming protein or epithiospecifier protein, other volatiles can be the main products (Hanschen *et al.,* 2014) (Figure 1.). The specifier proteins do not inhibit the myrosinase catalyzed breakdown, instead they drive rearrangement of the aglycon into non-ITC products (Wittstock and Burow, 2007). As deterring the aglycon – ITC conversion is a widespread phenomenon in insects feeding on Brassicaceae crops (Wittstock and Burow, 2007), these proteins are of interest for chemical ecologists and crop pest research, among others. Despite that there are many side chain types described, myrosinases are considered to have wide substrate specificity: the myrosinase of a species usually accepts glucosinolates from other species, as shown in (Li and Kushad, 2005), though the hydrolysis rate usually depends on the substrate. The ITCs have high reactivity, and exert various bioactivities, including antifungal, anti-cancer, insect repellent activities, as well as other health benefits for humans, as summarized in our recent review (Nguyen *et al.,* 2013). The ITCs are also key contributors to the sensory characteristics of the foods made from several Brassicaceae crops (horseradish, wasabi and mustard, among others). The different ITCs have characteristic odor that is easily recognized, and several of them are also pungent (Nguyen *et al.,* 2013).

As the generation of beneficial ITCs depends on the active myrosinase, the enzyme is of high interest to the scientific community and the food industry. Myrosinase assays can follow different approaches. Widely used assays like spectrophotometry or pH stat have the advantage of being fast, but lack the specificity that is inherent in different chromatographic separation systems. One can monitor the decrease of the UV absorbance of the substrate, usually sinigrin (Piekarska *et al.,* 2013), but interference from activators (ascorbic acid, dehydroascorbic acid and concentrated plant extracts) may be a serious problem (Kleinwachter and Selmar, 2004) causing significant errors in activity estimation. Other methods measure a product of the reaction, like the released glucose (Wilkinson *et al.,* 1984), sulfate (Nehmé *et al.,* 2014), or H^+ (Piekarska *et al.,* 2013), and are hence prone to less interferences. In

these assays however, it is not possible to assess the rate of conversion of the glucosinolate aglycon to ITC, which is very important from the viewpoint of bioactivity. The most detailed information on enzyme containing extracts decomposing glucosinolates could be obtained by methods simultaneously quantifying the decrease of the substrate and monitoring the increase in the concentration of the products. This is still to be done by sampling the reaction mixture regularly, and subjecting these samples to a complex sample preparation followed by chromatographic separation, usually GC-MS or HPLC. Many HPLC methods available in the literature could theoretically be used for this purpose (Tsao *et al.,* 2002; Song *et al.,* 2005; Herzallah and Holley, 2012; Budnowski *et al.,* 2013). These either detect the isothiocyanates "as is", or the ITCs are derivatized during the sample preparation. A HPLC method used to follow myrosinase activity was reported by (Vastenhout *et al.,* 2014) showing possible application. However, HPLC and GC-MS protocols are not only time-consuming, but the run time per sample is also usually high.

CE can be a very attractive alternative for the analysis of the glucosinolate – myrosinase – isothiocyanate system, because of the small sample demand, and the fact, that proteins and other macromolecules in solution are well tolerated. Despite that CE is quite suitable for determination of the ionic glucosinolates, separation from real matrices is not that straightforward (Karcher and Rassi, 1999). Isothiocyanates usually have poor UV detectability, but still can be determined after derivatization, e.g. hydrolysis to amines followed by labelling with fluorescent probes (Karcher and Rassi, 1999). However, these approaches are not compatible with myrosinase measurement. A study (Bellostas *et al.,* 2006) successfully utilized CE to simultaneously quantify glucosinolates and decomposition products, but used glucosinolates that do not form ITCs at all. The method of (Nehmé *et al.,* 2014) used CE to measure myrosinase activity, but as the released sulfate is quantified, no information on the decomposition products could be obtained. The method uses the much less common conductivity detection.

Our aim was to develop and validate a fast, robust and simple CE-MEKC method for the simultaneous detection of glucosinolates and isothiocyanates, focusing on sinigrin and allyl isothiocyanate, but also attempting its extension to gluconasturtiin and phenethyl isothiocyanate. The sensitivity problem for ITCs was aimed to be addressed by in-vial derivatization not inhibiting the myrosinase enzyme. Then, the method was applied to the quantification of glucosinolate content of the plants, myrosinase enzyme activity, and the release rate of isothiocyanates in different plant extracts. As model plants, Brussels sprouts, radish, watercress and horseradish were used.

EXPERIMENTAL

Chemicals.

For all applications, bidistilled water was used. The used chemicals listed below were all of at least analytical grade. Boric acid, monosodium phosphate monohydrate, ascorbic acid, sodium hydroxide and hydrochloric acid were obtained from Reanal (Budapest, Hungary). Acetic acid, N-cyclohexyl-ethanesulphonic acid (CHES), acetonitrile, mercaptoacetic acid (thioglycolic acid) were bought from VWR Hungary (Budapest, Hungary). Sodium deoxycholate was from Carl-Roth (Karlsruhe, Germany). Sinigrin and gluconasturtiin were from Phytoplan (Heidelberg, Germany). Allyl isothiocyanate, phenethyl isothiocyanate, bovine serum albumine and Bradford reagent were from Sigma-Aldrich (St.Louis, MO, USA).

Plant material and preparation of extracts.

Samples of fresh vegetables (Brussels sprouts, horseradish, radish and watercress) used for the study were obtained from local suppliers. Accurately weighed vegetable plant material (about 15 g fresh weight (FW)) was homogenized in commercial grinders after addition of 15.0 mL of 20 mM phosphate buffer (pH 6.50) at 4 °C. The resulting mush was centrifuged at 20000 rpm for 5 minutes, filtered (0.20 μm pore size), and the supernatants were used for activity measurements directly. Dilutions (if necessary) were done with the extracting buffer. These extracts are referred to as "cold buffer extracts" or "extracts containing active myrosinase" throughout the manuscript. The plant extracts were always made fresh, and stored at 4 °C before being added to the enzyme assay. If the myrosinase had to be inactivated, an aliqout of the extracts containing active myrosinase was put in a test tube and the tube was immersed in boiling water for five minutes. This was used for accuracy measurements, as a "plant matrix".

For glucosinolate determinations, the plant material (about 10 g) was put in a test tube and immersed in boiling water for 10 minutes (watercress) and 30 minutes (other vegetables) to completely inactivate the myrosinase. Thereafter, 10 mL of MeOH was added to the cooked plant material, followed by thorough homogenization, and centrifugation at 13000 rpm for 3 minutes. The supernatant was evaporated to dryness. Prior to analysis, the dried samples were resuspended in water, centrifuged and subjected to analysis by both CE and LC/MS - after dilution with water, if necessary. These extracts are referred to as "methanolic extracts" throughout the manuscript.

Instrumentation

Method development was carried out on a PrinCE-C 700 capillary electrophoresis instrument. A 60 cm fused silica capillary with 50 μm i.d. was used. Effective length was 7.2 cm when injection was accomplished at the capillary end closer to the detector window ("short-end injection"); this mode was used for determination of the myrosinase activity, or isothiocyanate release. Effective length was 52.8 cm when injecting at the other end ("long-end injection"); this mode was used for the determination of different glucosinolates or allyl isothiocyanate quantification from conentrated real matrices. Capillary preconditioning and post-conditioning were previously described (Gonda *et al.,* 2013). Sample injection was hydrodynamic (100 mbar × 0.25 min.). Sinigrin was quantified at 230, gluconasturtiin at 210, ITC derivates at 275 nm.

Optimization of separation

As a first step, a base method had to be developed, which is capable of separating the glucosinolates and the ITCs from the rest of the plant matrix. Solutions of pure sinigrin, gluconasturtiin and methanolic extracts of horseradish resuspended in water (spiked with pure allyl isothiocyanate, where appropriate) were used during this preliminary stage of method development. The latter contains both glucosinolates of interest. As a starting point, the background electrolyte used in our recent study (Gonda *et al.,* 2013) was used, containing N-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS, 20 mM), sodium deoxycholate (250 mM), sodium tetraborate (15 mM), pH 8.50. The development steps aimed to make the method suitable for faster determination, thus reductions of the electrolyte and surfactant concentrations were tested in order to be able to increase the applied voltage, while maintaining sensitivity and stability. The pH value was also readjusted. After achieving sufficient stability, polarity was reversed to use short end injection.

Derivatization studies

The products of the glucosinolate aglycone rearrangement, ITCs were converted to dithiocarbamates in-vial by mercaptoacetic acid according to the scheme in Figure 1.b.

To test the effects of the enzyme activator ascorbic acid on the derivatization reaction, and to find the optimal concentration of the derivatizing agent, 350 μg mL^{-1} allyl isothiocyanate was derivatized in phosphate buffer (10 mM, pH 7.5) using the different concentrations of the derivatization agent mercaptoacetic acid (1, 5, 10 mM), and ascorbic acid (0, 1, 5, 10 mM). All combinations of the above were tested.

To find the working pH range of the derivatization, the amount of product generated by the reaction from allyl isothiocyanate was quantified in reaction mixtures only differing in pH: 350 μg mL^{-1} allyl isothiocyanate was derivatized with mercaptoacetic acid (5 mM) in the presence of ascorbic acid (1 mM) in a pH series of 5.5-9.5 (acetate, phosphate or borate buffers in 10 mM end-concentration). The products were quantified using the proposed CE-MEKC method.

The characterization of derivatization products by LC-MS was run on a Thermo Accela HPLC attached to a Thermo LTQ XL Linear Ion Trap MS (column: Hypersil Gold 50 mm × 2.1 mm × 1.9 μm). ESI ionization parameters were as follows: heater temperature, 300 °C; sheath gas, N_2; flow rate, 20 arbitrary units (arb); aux gas flow rate, 8 arb; spray voltage, 4 kV; capillary temperature, 275 °C; capillary voltage, -28.00 V, negative ion mode. Gradient components were A, water with 0.1% (v/v) formic acid; B, MeCN with 0.1% (v/v) formic acid. The time program was 10% B: 0 – 2 min, 10 – 90% B: 2 – 7 min, 90%B: 7-13 min, 90 – 10% B: 13 – 13.1 min, 10% B: 13.1 – 15 min. Flow rate was 300 μL/min. 1 μL of a derivatized sample (acetate buffer, pH 5.0, mercaptoacetic acid (5 mM), ascorbic acid (1mM)) containing 1 μg mL^{-1} of allyl isothiocyanate and phenethyl isothiocyanate was injected.

Validation

The short end injection version of the CE method was subjected to validation. Five-point calibration curves for the allyl isothiocyanate were measured. Derivatized allyl isothiocyanate solutions (4.5, 9, 45, 90, 450 μg mL^{-1}) were obtained as follows: the serial dilutions of the allyl isothiocyanate were mixed with buffered derivatization solution (NaH_2PO_4 (100 mM), mercaptoacetic acid (50 mM), ascorbic acid (10 mM), pH 7.50) in a 9:1 ratio. During preparation of the buffered derivatization solution, NaOH for pH adjustment had to be added before the addition of mercaptoacetic acid. Stock solutions of allyl isothiocyanate (25 mg mL^{-1}) were prepared in MeCN, the serial dilutions were performed with water. Seven-point calibration curves (5, 10, 50, 100, 500, 1000, 5000 μg mL^{-1}) were prepared from sinigrin and gluconasturtiin in water. From the calibration curves, LOD, LOQ, R^2 and regression equations were calculated.

For interday and intraday reproducibility studies, 5 injections of 100 μg mL^{-1} derivatized solutions of allyl isothiocyanate, and 100 μg mL^{-1} solutions of the glucosinolates were done, on three days of measurement (n=5x3). These concentrations are realistic during myrosinase studies. The RSD between the area under curve values and retention times were obtained by DAx 8.1 software.

For accuracy measurements, samples containing 10 μL of the previously inactivated cold buffer extracts of the plants (radish, horseradish) / 100 μL volume were spiked with 100 μg mL^{-1} standards (separately), and the recoveries were calculated. The absence of residual glucosinolates in these plant extracts was confirmed by injecting samples without adding glucosinolate standards.

Quantification of glucosinolates and allyl isothiocyanate from real matrices

The methanolic extracts of the four tested vegetables (Brussels sprouts, horseradish, radish and watercress) were assayed for glucosinolate (sinigrin and gluconasturtiin) content by the proposed method in long end injection (CE). The same extracts were assayed for sinigrin and gluconasturtiin by LC-ESI-MS, and the obtained values were compared.

Glucosinolate determination by LC-ESI-MS was done on a Thermo Accela HPLC attached to a Thermo LTQ XL Linear Ion Trap MS, column: Kinetex XB-C_{18} (100 × 2.10mm × 2.6 µm, Phenomenex). Five-point calibration curves of sinigrin and gluconasturtiin in water ranging from 0.5 to 40 µg mL^{-1} were used as calibration curves. Gradient components were A, water with 0.1% (v/v) formic acid; B, MeCN with 0.1% (v/v) formic acid. The time program was 5% B: 0 – 1 min, 5 – 25% B: 1 – 4 min, 25 – 60% B: 4-5 min, 60 – 5% B: 5 – 6 min, 5% B: 6-8 min. Flow rate was 250 µL/min. 1 µL of the diluted methanolic extract was injected, typically 5-100-fold dilutions with water were appropriate. The instrument was tuned automatically for sinigrin to obtain the optimal ESI parameters. ESI ionization parameters were as follows: capillary temperature, 275 °C; source heater temperature, 300 °C; sheath gas, N_2; sheath gas flow, 30 arbitrary units (arb); aux gas flow, 5 arb; source voltage, 3 kV; capillary voltage, -1.00 V, negative ion mode.

Four food products, mustard (condiment), two types of horseradish sauce, and a wasabi cream were assayed for allyl isothiocyanate as follows: 100 mg of food product was diluted with a mixture of 100 µL of buffered derivatization solution (NaH_2PO_4 (100 mM), ascorbic acid (10 mM), mercaptoacetic acid (50 mM), pH 7.5) and 800 µL of water. The resulting sample was thoroughly mixed, centrifuged and the supernatant directly analyzed by CE in long-end injection mode. The products containing significant amounts of vinegar required readjustment of pH to 7.5.

Study of myrosinase activity and allyl isothiocyanate release

After the preparation of cold buffer extracts from the vegetables, their myrosinase activity was determined. The reaction mixture was obtained as follows: The myrosinase containing plant extracts were diluted with a 9:1 mixture of buffer (NaH_2PO_4 (100 mM), ascorbic acid (10 mM), pH 6.50) and water. Thereafter, to 195 µL of this mixture, 5 µL of glucosinolate stock (10 mg mL^{-1}) was added. Addition of the substrate was the reaction start point, negative controls were obtained by using plant extracts that were previously boiled to inactivate the myrosinase. The reaction was run at 25 °C for 5 minutes, then terminated by heating the test tubes at 100 °C for 5 minutes, followed by the sinigrin concentration determination by CE after centrifugation at 13000 rpm (1 minute). Substrate concentration decrease was kept below 10%. The plant extracts were also checked for the presence of residual substrates (no sinigrin added). Protein determination from these extracts was done using Bradford's reagent with bovine serum albumine as standard.

Under the same conditions, a series of different initial sinigrin concentrations were tested to obtain the K_m constant for myrosinase. Tested initial concentrations were 20, 35, 50, 65, 100, 150, 225, 300 µg mL^{-1}, the determination was run in three replicates. The added myrosinase containing 50-fold diluted horseradish extract was allowed to decompose sinigrin for 5, 8.75, 12.5, 16.25, 25, 37.5, 56.25, 75 minutes, respectively, keeping decomposed substrate below 10%, allowing the estimation of the initial reaction rate (v_0). The K_m value was calculated by fitting the Michaelis Menten equation ($v_0 = v_{max}[S] / K_m+[S]$) to the obtained data. For non-linear curve-fitting, the nls package in R was used (n=3).

The same vegetable extracts were also assayed for myrosinase activity by the widely used pH stat assay (Piekarska *et al.,* 2013). The reaction mixture was the same as that for CE, except that it was

not buffered: to 7.66 mL of water 80 µL of ascorbic acid solution (100 mM, pH adjusted to 6.50 with NaOH), 80 µL of plant extract (diluted if necessary) was added. After the pH drift stopped after a few minutes, the reaction was initialized by addition of the substrate (final concentration: 250 µg mL^{-1}). Thereafter, freshly prepared 1 µM NaOH was added under slow constant stirring to keep the pH at 6.50. The amount of NaOH consumed by the released H^+ during glucosinolate decomposition was registered for 5 minutes.

Extracts of the four vegetables (Brussels sprouts, horseradish, radish, watercress) were compared for the sinigrin aglycon – allyl isothiocyanate conversion rate. The reaction mixture was: 10 µL of buffered derivatization solution (NaH_2PO_4 (100 mM), ascorbic acid (10 mM), mercaptoacetic acid (50 mM), pH 7.5), 60 µL bidistilled water, 10 µL enzyme containing extract (not diluted) and 20 µL of sinigrin stock solution (10 mM). The experiment was designed to result in 2 mM allyl isothiocyanate if the conversion ratio is 100%.

Software

ChemAxon MarvinSketch was used for drawing chemical structures and reactions, while Calculator Plugins were used for structure property prediction and calculation. Version 6.2.3_b915, 2014 was used (http://www.chemaxon.com). Figures were generated using scripts in R 3.1.1. (R Development Core Team, 2009) using ggplot 0.9.3.1.

RESULTS AND DISCUSSION

CE method development

The background electrolyte of our recent study (Gonda *et al.,* 2013) served as a starting point for the method development, as it gave good resolution for glucosinolates (sinigrin / gluconasturtiin) in the methanolic extract of horseradish. The later parts of the method optimization served two purposes: 1., to decrease analysis time; 2., to increase the sensitivity for ITCs.

In the first part of the method development, we applied long end injection. Borate could be completely omitted from the background electrolyte, as it showed no contribution to the resolution, but it increased retention times for the analytes. Sodium deoxycholate concentration could be reduced from 250 mM to 175 mM without losing resolution between sinigrin and gluconasturtiin, giving us the ability to increase the voltage, and reduce the analysis time. The pH was modified to 9.0 to increase EOF strength, which also resulted in lower analysis time and higher stability. At pH 9.0, CHES (*N*-Cyclohexyl-2-aminoethanesulfonic acid) was chosen to be used as a buffering agent (significant buffering capacity, low UV absorption).

Other tested approaches, like addition of organic solvents, employing different surfactants (also as mixtures) all resulted in loss of resolution and/or sensitivity for some analyte of interest. The final background electrolyte composition was CHES (20 mM), sodium deoxycholate (175 mM), pH 9.0, applied voltage was 20 kV. Good resolution for sinigrin / gluconasturtiin was observed in long end injection, and no major interfering peak was found in the methanolic extract of horseradish (Figure 2.a.). The method could also well separate allyl isothiocyanate from the other analytes of interest (not shown). Unfortunately, the low specific absorbance of allyl isothiocyanate has made its LOD so high, that it could not have been used for the study of allyl isothiocyanate released on-line. Therefore, to increase the sensitivity of the method for ITCs, they were converted to dithiocarbamates in-vial, with mercaptoacetic acid, as detailed below.

Derivatization studies

The reaction scheme (Wilson *et al.,* 2011) used in some determination studies of ITC is plotted in Figure 1.b. This approach is similar to that of (Bjergegaard *et al.,* 1999) who successfully derivatized allyl isothiocyanate by amino acids, enabling more sensitive detection of ITCs by CE. However, their method is unsuitable for myrosinase-compatible determination of ITCs because: 1., optimized reaction time is 15 minutes; 2., the reaction between the isothiocyanates and the amino acids resulted in different products, 3., the optimal reaction conditions require strongly alkaline, organic reaction media (50% triethylamine, 25% pyridine), and is followed by liquid-liquid extraction. Therefore, derivatization is applicable after the complete decomposition of glucosinolates. The authors also note that cysteine gave much better results, but the applicability for the determination of on-line generated ITCs was not addressed (Bjergegaard *et al.,* 1999). We therefore tested several thiols to react with ITCs in aqueous solutions. We found that the reaction is much faster than that reported for amines; it was actually immediate at a rather wide pH range, in accordance with (Hanschen *et al.,* 2014). Mercaptoacetic acid has the following advantages (in contrast to the tested other thiols containing more chromophores): it is inexpensive, miscible with water at any pH, the dithiocarbamate products (Figure 1.b.) carry charge at the background electrolyte pH 9.0, as shown by calculations in ChemAxon MarvinSketch v6.2.3 (Supplementary Figure 1.a.). The presence of the dithiocarbamate product and the absence of side-products was also confirmed by subjecting an ITC reaction mixture to an LC-ESI-MS/MS study in negative ion mode. The ions with m/z 190 and 254 were abundant peaks in the reaction mixture, and corresponded to the $[M-H]^-$ ions of the dithiocarbamates of allyl isothiocyanate, and phenethyl isothiocyanate, respectively, with fragmentations supporting the proposed structure. The products are especially polar at pH $\geq$ 7, as shown by the logD values predicted by ChemAxon MarvinSketch v6.2.3 (Supplementary Figure 1.b.) resulting in much better water solubility and lower retention time in CE-MEKC than that of the underivatized ITC.

The myrosinase enzymes can operate at a wide pH range (Li and Kushad, 2005), so, pH dependence of the derivatization was examined. As the formation of dithiocarbamate products from ITCs was found to be influenced by pH, as in (Hanschen *et al.,* 2014), different calibration curves should be used for ITC products at different pH if the method is to be applied for some application that requires usage of a wide range of pH values. It is important to state however, that this sensitivity does not cause any inaccuracy in determination: interday RSD was 4.45% when the pH of the derivatization solutions was 7.5 ± 0.1. The reaction gives higher sensitivity between pH 6.5-9.5, and significantly less, but still detectable amounts of products at pH 4.5-5.5. The final derivatization solution composition, subjected to validation was: mercaptoacetic acid (5 mM) and ascorbic acid (1 mM), pH 7.5, because this pH is a good compromise between the myrosinase pH optimum (Li and Kushad, 2005) and the derivatization pH optimum.

The derivatization reaction was not affected significantly by ascorbic acid concentration in the range 0-10 mM tested ($p > 0.05$). Thus, the ascorbic acid concentration range usually used in assays of myrosinase (Kleinwachter and Selmar, 2004; Bellostas *et al.,* 2006; Nehmé *et al.,* 2014) worked without any experienced problems. Inhibiton by higher concentrations (Travers-Martin *et al.,* 2008) could also be tested without major interferences.

The concentration of mercaptoacetic acid influences the product amount to a minor extent, but using two-fold concentrations of mercaptoacetic acid (10 mM) only caused a 19.7% increase in the average peak area of the product, compared to the proposed 5 mM, thus, this phenomenon did not

result in inaccuracy. As soon as the mercaptoacetic acid was in excess to ITC, no problems of low reproducibility were experienced.

Validation results

The method with short end injection was validated. As our instrument is capable of applying pressure on both capillary ends, the area under curve values for the same samples were the same in case of long-end and short-end injections. The RSDs of the determination were acceptable, the method showed sufficient stability during the validation. The main method characteristics are shown in **Table 1**. The presence or absence of mercaptoacetic acid in the solution did not influence the characteristics of the glucosinolates, their detection was sensitive and reproducible. Gluconasturtiin behaved very similar to sinigrin during the analysis, it can be reproducibly analyzed with the proposed method.

The derivatization increased sensitivity for allyl isothiocyanate by about an order of magnitude, opening the way to the determination of the minute amounts of on-line generated allyl isothiocyanate (Table 1.). On the other hand, phenethyl isothiocyanate calibration curves showed worse interday reproducibility – perhaps because the product formed from phenethyl isothiocyanate still had poor solubility in water (Supplementary Table 1.). In this case, usage of fresh reference standards was found to be of extreme importance: deteriorated ITC resulted in much less solution stability. The method can be still used for quantification of phenethyl isothiocyanate, if a considerable amount of organic solvent (e.g. 20% MeCN) is added to the solution, but this is not fully compatible with enzymatic studies. Apart from that, the useful linear range, sensitivity, and intraday RSD for the gluconasturtiin and phenethyl isothiocyanate pair was comparable to that of sinigrin, and allyl isothiocyanate, respectively (Table 1, Supplementary Table 1.).

Recoveries of sinigrin, gluconasturtiin and allyl isothiocyanate were found to be good even from samples containing high amounts of inactivated cold water extracts: extracts equivalent to 5 mg FW in 100 μL sample. The cold water extracts did not contain any detectable residual glucosinolates in any sample tested (not shown). When spiking with allyl isothiocyanate, allyl isothiocyanate solutions in water (500 μg mL^{-1}) had to be used.

The resolution was excellent between the substrate, the product, ascorbic acid and the excess derivatization reagent even in short-end injection mode for both glucosinolate – isothiocyanate pairs (Figure 2.b. and Supplementary Figure 3. b.). If desired, an enzyme containing sample subjected to study could be injected about every 4 minutes, as shown in a sequential injection in Supplementary Figures 2-3.

Applications

Determination of glucosinolates and isothiocyanates from plant and food matrices. The proposed methodology can be simply used to quantify sinigrin and gluconasturtiin from different matrices, perhaps also other glucosinolates in long end injection mode (Figure 2.a.).

The tested four vegetables' sinigrin and gluconasturtiin content is summarized in Table 2. It can be seen that the values closely match those obtained by a different analytical methodology, LC-ESI-MS, the difference between the two methods ranged from 82.3-108.6 %, with an average of 99.6%.

The tested commercial condiments were shown to contain 369 – 418 μg / g allyl isothiocyanate. The method provided a good resolution despite the simple sample preparation procedure, as plotted in Figure 2.c-d.

The presented long end injection method separates the analytes of interest within 15 minutes

from real matrices (Figure 2.a,c,d.). This falls in the range of the fastest HPLC methods available for determination of sinigrin and allyl isothiocyanate (Tsao *et al.,* 2002). Typical methods last 20-25 minutes or more (Herzallah and Holley, 2012; Budnowski *et al.,* 2013), consume 1 mL min^{-1} solvent and require the removal of protein and fat before analysis. Direct, simultaneous quantifications from complex matrices can be even longer (Song *et al.,* 2005). Isothiocyanate determination – even when derivatized – can also be time demanding (Wilson *et al.,* 2011).

The presented method is similar in speed to the fastest available CE methods for glucosinolates (Karcher and Rassi, 1999; Bellostas *et al.,* 2006), an analysis time of 15-25 minutes is typical. However, the separation of isothiocyanate adducts usually requires more time (Bjergegaard *et al.,* 1999). In our case, separation of allyl isothiocyanate dithiocarbamate was also done within 15 minutes.

The CE screening is also faster as compared to GC-MS methods. The usual time of a GC-MS for different isothiocyanates measurement is 30-35 mintues (Zhao *et al.,* 2007; Khoobchandani *et al.,* 2010).

Study of myrosinase activity and allyl isothiocyanate release of vegetable extracts. The average K_m values obtained by non-linear regression analysis of reaction velocity versus sinigrin concentration (at pH 6.50, 25 °C, 0.1 mM ascorbic acid) fell in the range of 0.129 ± 0.025 μM. This is the same order of magnitude found for sinigrin in several papers (e.g. (Li and Kushad, 2005; Nehmé *et al.,* 2014)) for different myrosinases, thus we can state that the presented activity is truly that of myrosinase.

The tested vegetables showed very different activities in the assay (**Table 3**). Expressing the activity in μmol sinigrin decomposed per minute (U) yielded the following results: Activity of sinigrin decomposition ranged from 4.42 U g^{-1} FW (watercress) to 208.26 U g^{-1} FW (horseradish) in 10 mM phosphate, 1 mM ascorbic acid, pH 6.50, 25 °C, initial substrate concentration: 250 μg mL^{-1}. The method was shown to be suitable to measure myrosinase activity from low-activity mixtures without major interferences. The obtained myrosinase activities with sinigrin as the substrate were compared to those found in the widely used pH-stat assay. Under the same conditions (1 mM ascorbic acid, pH 6.50, at 25 °C, initial substrate concentration: 250 μg mL^{-1}), the myrosinase-containing extracts of the vegetables had very similar activity (93.7% – 116.9%, average: 107.1%, Table 3).

If one compares the activity ratio of the same extracts with sinigrin and gluconasturtiin as the substrate, some level of specificity can be found: Brussels sprouts that contains sinigrin but no gluconasturtiin (Table 2) had 2.08-fold activity against sinigrin as compared to gluconasturtiin. Watercress containing gluconasturtiin but no sinigrin (Table 2) also showed some specificity towards its own glucosinolate. Horseradish, which contains both glucosinolates (Table 2), decomposed both with similar efficacy (Table 3). Though the measured specificity is not that striking as the one described for *Crambe abyssinica* by (Finiguerra *et al.,* 2001), it suggests that using sinigrin as the sole substrate for myrosinase activity determination may sometimes result in serious under- or overestimation of the biologically relevant myrosinase activity of plant extracts. Therefore, if possible, methods capable of using substrates other than sinigrin should be integrated into myrosinase activity tests. The phenomenon clearly requires more in-depth study, for which the current method can be used.

There are popular methods for myrosinase measurement, most of which are based on spectrophotometry instead of chromatographic separation. These include the indirect measurement of the glucose released (Wilkinson *et al.,* 1984) or measuring the breakdown kinetics of the substrate (decrease of absorbance maximum) (Piekarska *et al.,* 2013). It is also possible to use the released H^+ for

quantification in a pH-stat assay (Piekarska *et al.,* 2013). As glucosinolate absorbance maxima are usually around 210-230 nm, many compounds can interfere with quantification in UV-Vis, especially when using more concentrated raw extracts. Higher specificity can only be achieved by subjecting the reaction mixture to chromatographic separation. For this purpose, the CE methods of both (Bellostas *et al.,* 2006; Nehmé *et al.,* 2014) can be used. They operate with a minimal amount of sample and are able to study decomposition of different glucosinolates, but neither can give information on the ITC release rate that is of primal biological significance. HPLC methods have excellent reproducibility and sensitivity (Vastenhout *et al.,* 2014), but are frequently time consuming and require much more reagents than CE determinations. The reaction mixtures used in this study are simple enough to use short-end injection, which results in less, but sufficient resolution, and less analysis time. In this case, the presented method is capable of separating a glucosinolate – isothiocyanate pair from the reagents in 2.5 minutes. With a capillary reconditioning applied after every sixth injection, a number of about twelve injections per hour can be reached. This is comparable to the widely used assays' time demand (Travers-Martin *et al.,* 2008; Piekarska *et al.,* 2013), yet, chromatographic separation takes place, which gives the least interferences from for example ascorbic acid (Kleinwachter and Selmar, 2004).

For many vegetables, conversion of sinigrin to allyl isothiocyanate was not found to be 100% in the literature. The isothiocyanate yield from the glucosinolate aglyca can range from a few percent (Piekarska *et al.,* 2014) to near 100%. This is usually attributed to the presence of specifier proteins that cause the glucosinolate aglycon to rearrange into different volatile products such as nitriles, epithionitriles, thiocyanates among others (Figure 1.b.) (Piekarska *et al.,* 2014).

In the current study, four vegetable extracts (Brussels sprouts, horseradish, radish, watercress) were successfully compared for ITC conversion rate, with minimal amount of reagents. The procedure also does not require laborious sample preparation (e.g. liquid-liquid extraction) needed to study the ITC content by GC-MS, as in (Piekarska *et al.,* 2014). The amount of allyl isothiocyanate generated from the same amount of added sinigrin (1 mM) significantly differed among the vegetable extracts ($p<0.05$, n=3, ANOVA). Conversion rate was found to range between 73.13±0.27% and 102.13±0.94% (Table 3). The HPLC method of (Vastenhout *et al.,* 2014) successfully separates allyl isothiocyanate and sinigrin from model matrices with active myrosinase within 6 minutes, and was successfully used to measure ITC release. However, it was not tested for the ability to measure myrosinase activity or ITC release from real matrices, and operates with a 1 mL min^{-1} solvent flow. Testing of real matrices would also require the removal of protein and fat as sample preparation steps.

The advantages of the proposed CE method include saving time and solvents as compared to many HPLC methods. Hence, it is suitable as a screening method for glucosinolates and allyl isothiocyanate. The method was used as a higher specificity myrosinase assay that also allows quantification of on-line generated isothiocyanates, the main bioactive products. Only 25-50 µg glucosinolate per sample is sufficient for a myrosinase study, which is especially important in the case of glucosinolates other than sinigrin. Analysis of factors affecting the glucosinolate – isothiocyanate conversion rate is also possible.

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FIGURES

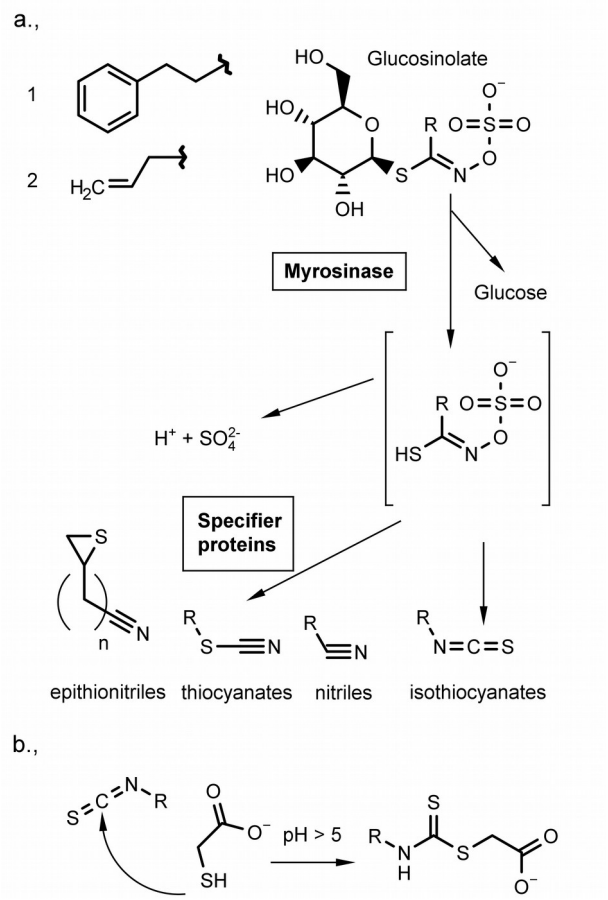


Figure 1. a. Scheme of myrosinase catalyzed breakdown of glucosinolates and main pathways of the rearrangement of the glucosinolate aglycon to various volatile natural products. Glucosinolates: R=1., gluconasturtiin (phenethyl glucosinolate); R=2., sinigrin (allyl glucosinolate). Isothiocyanates: R=1., phenethyl isothiocyanate (phenethyl isothiocyanate); R=2., allyl isothiocyanate (allyl isothiocyanate).
Figure 1. b. Derivatization scheme of myrosinase generated isothiocyanates by mercaptoacetic acid.

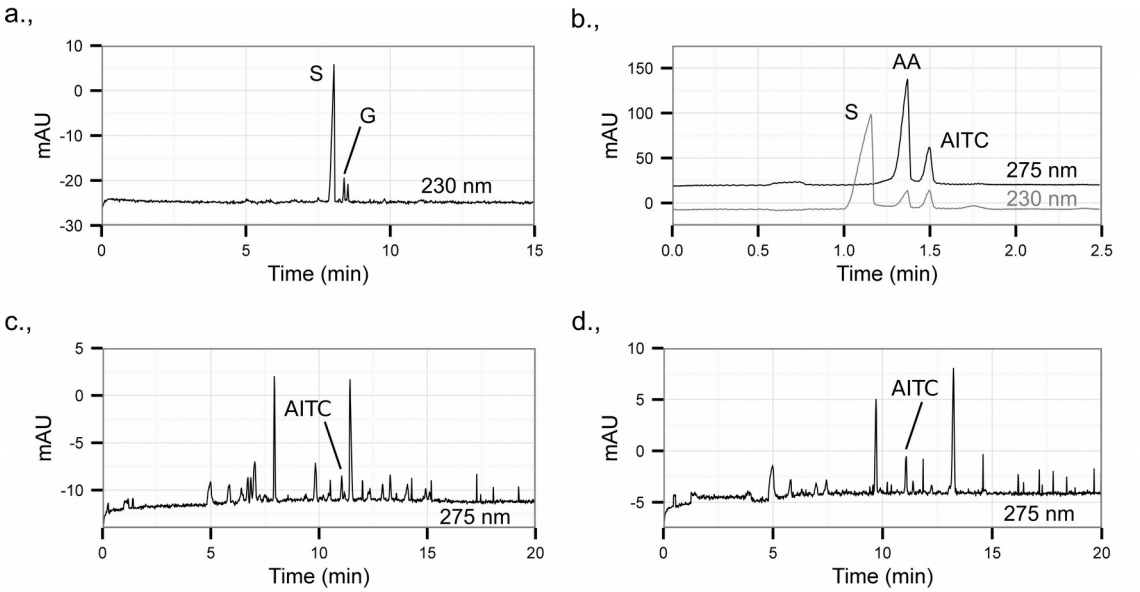


Figure 2. Electropherograms obtained using the proposed capillary electrophoresis – micellar electrokinetic chromatography (CE-MEKC) method showing possible applications. Background electrolyte: CHES (20 mM), sodium deoxycholate (175 mM), pH 9.0. Isothiocyanates are present as dithiocarbamates during separation. Sample matrices: phosphate (10 mM, pH 7.5), ascorbic acid (1 mM), mercaptoacetic acid (5 mM). Subplots: **a.**, Methanolic extract of horseradish root redissolved in water, measured in long-end injection mode (see **Instrumentation**). **b.**, Standard mixture (sinigrin, allyl isothiocyanate) in derivatization buffer, analyzed in short-end injection mode (see **Instrumentation**). **c.-d.** Detection of allyl isothiocyanate from food products in long-end injection mode. c., mustard (condiment); d., horseradish sauce with wasabi. Abbreviations: AA, ascorbic acid; AITC, allyl isothiocyanate (as its dithiocarbamate); G, gluconasturtiin; S, sinigrin.

TABLES

Table 1. Characteristics of the proposed CE-MEKC method.

	Sinigrin	**Allyl isothiocyanate** [a]	**Gluconasturtiin**
Detection wavelength (nm)	230	275	210
Linear Regression Equation (µg mL^{-1})	f(x) = 0.0085 x + 0.0263	f(x) = 0.0289 x - 0.1534	f(x) = 0.0061 x + 0.0142
Linear Regression R^2	0.9999	0.9999	0.9998
Upper limit of quantification (µg mL^{-1} / mM) [b]	5000 / 12.58	450 / 4.54	5000 / 10.95
LOD (µg mL^{-1} / mM) [b]	11.93 / 0.030	13.50 / 0.136	9.99 / 0.022
Retention time RSD% (interday)	3.07 %	2.01 %	1.55 %
Retention time RSD% (intraday)	2.02 %	1.36 %	1.93 %
Area under curve RSD% (interday)	5.51 %	4.45 %	6.42 %
Area under curve RSD% (intraday)	4.03 %	4.64 %	5.07 %
Accuracy (recovery at 100 µg mL^{-1}, n=3)	98.14 %	105.13 %	100.39 %

[a]: Values for allyl isothiocyanate are given as allyl isothiocyanate equivalent, but the compounds are actually quantified after derivatization with mercaptoacetic acid in-vial, in phosphate buffer (10 mM, pH 7.5).

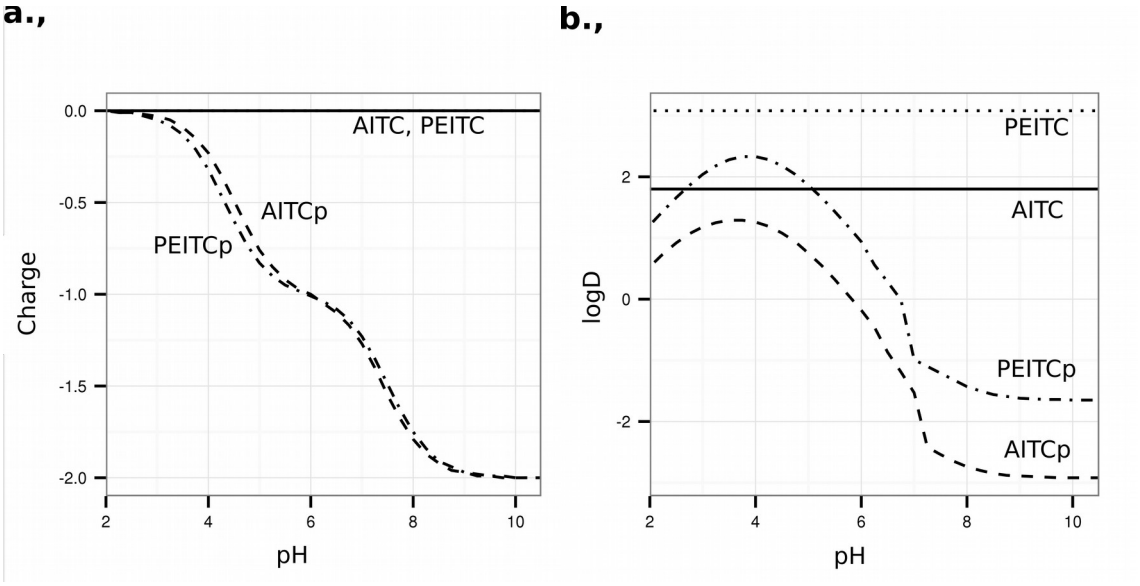
[b]: Values are given for injection with 100 mbar × 0.25 minutes.

Table 2. Glucosinolate content of the four tested vegetables, as measured by the proposed CE method, or by LC-ESI-MS. Abbreviations: CE, capillary electrophoresis; FW, fresh weight.

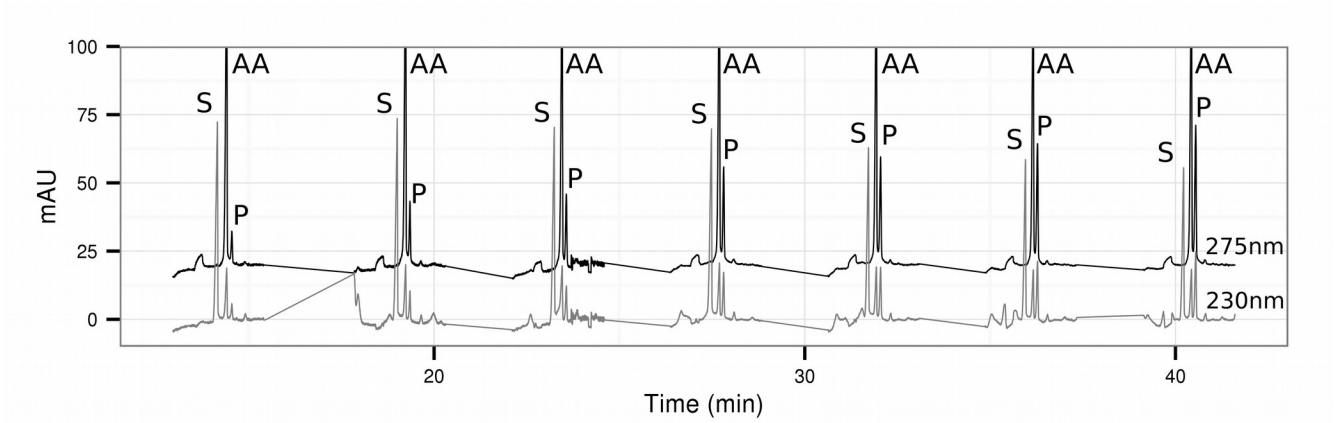
Vegetable	**CE**	**LC-ESI-MS**	**CE**	**LC-ESI-MS**
	Sinigrin (µg g^{-1} (FW))		**Gluconasturtiin (µg g^{-1} (FW))**	
Radish	n.d.	n.d.	n.d.	n.d.
Brussels sprouts	161.0	148.2	n.d.	n.d.
Watercress	n.d.	n.d.	162.9	153.7
Horseradish	2291.8	2784.4	248.5	244.3

Table 3. Myrosinase activtity of the four tested vegetables using sinigrin or gluconasturtiin as substrate, measured by the proposed CE method and pH stat assay. One unit (U) of activity is defined as 1 µmol per minute. Abbreviations: CE, capillary electrophoresis; FW, fresh weight; S/G AR: Ratio of myrosinase activity with sinigrin as the substrate / gluconasturtiin as the substrate. The presented values are mean ± SD of three measurements.

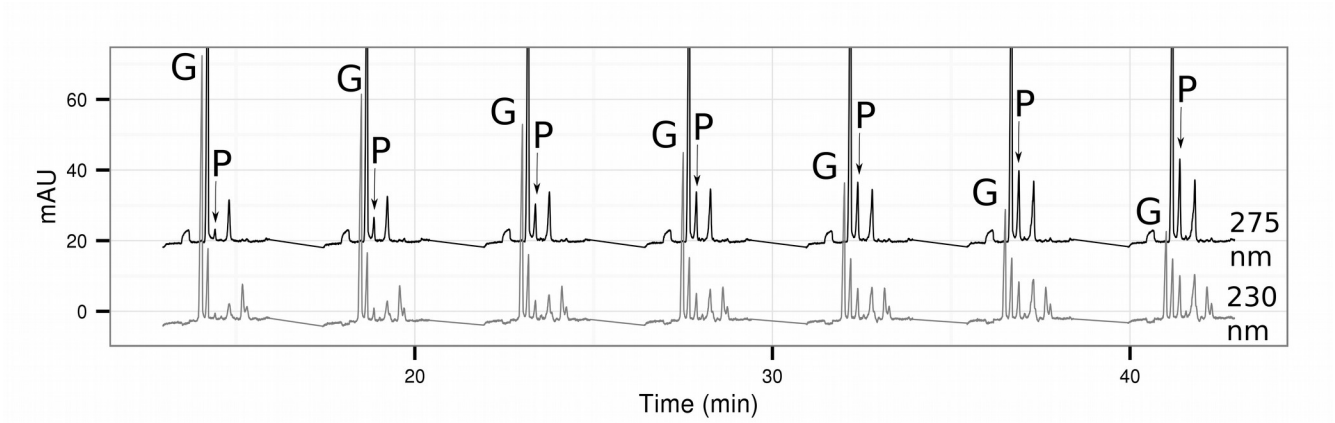
Vegetable	**sinigrin, CE**	**sinigrin, CE**	**sinigrin, pHstat**	**sinigrin, pHstat**
	U g^{-1} FW	**U mg^{-1} protein**	**U g^{-1} FW**	**U mg^{-1} protein**
Radish	10.31±1.31	3.41±0.43	11.00±0.94	3.64±0.31
Brussels sprouts	7.72±0.6	0.96±0.07	6.83±0.24	0.85±0.03
Watercress	4.42±0.5	0.76±0.09	4.22±0.94	0.73±0.16
Horseradish	208.26±42.94	27.69±5.71	178.13±4.42	23.69±0.59
Vegetable	**gluconasturtiin, CE**	**gluconasturtiin, CE**	**S/G AR**	**ITC release (%)**
	U g^{-1} FW	**U mg^{-1} protein**		
Radish	5.35±1.02	1.77±0.34	1.93	102.13±0.94%
Brussels sprouts	3.71±0.09	0.46±0.01	2.08	92±4.39%
Watercress	6.01±0.12	1.04±0.02	0.73	98.25±3.02%
Horseradish	197.94±33.98	26.32±4.52	1.05	73.13±0.27%



Supplementary Fig. 1. Predicted chemical parameters of the dithiocarbamates formed in-vial by the reaction between mercaptoacetic acid and the myrosinase generated isothiocyanates. Calculations were done by ChemAxon MarvinSketch v6.2.3., using default method parameters. Abbreviations: AITC: allyl isothiocyanate; PEITC: phenethyl isothiocyanate; AITCp allyl isothiocyanate dithiocarbamate product; PEITCp: phenethyl isothiocyanate dithiocarbamate product. Subplots: a., Charge of isothiocyanates and their mercaptoacetic acid dithiocarbamate products in the pH range usually used for capillary electrophoresis. Note, that above pH 6, -1 or -2 charge chemical species are dominant, increasing the solubility compated to the isothiocyanates. b., logD values of isothiocyanates and their mercaptoacetic acid dithiocarbamate products in the pH range usually used for capillary electrophoresis. Note the polar logD of the products at pH 7 and 9, the pH of the sample, and background electrolyte, respectively.

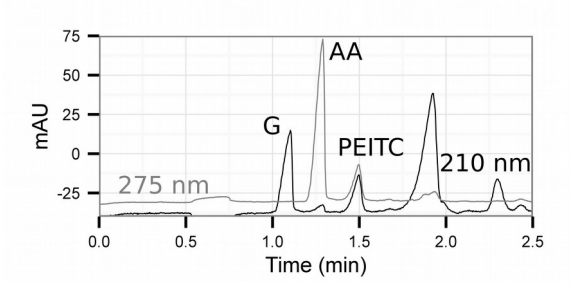


Supplementary Fig. 2. Electropherogram series from seven sequential injections of an active myrosinase-containing mixture (radish extract) using the proposed capillary electrophoresis-micellar electrokinetic chromatography (CE-MEKC) method. Conditions: effective capillary length 7.2 cm (short end injection), voltage: -20.0 kV, background electrolyte: CHES (20 mM), sodium deoxycholate (175 mM), pH 9.0; sample matrix: phosphate (10 mM, pH 7.5), ascorbic acid (1 mM), mercaptoacetic acid (5 mM). Note the decomposition of sinigrin (substrate), the increase in the product isothiocyanate derivate concentration, and the lack of interfering peaks from the unpurified plant extract. Abbreviations: AA, ascorbic acid; S, substrate (sinirgin); P, product (allyl isothiocyanate derivatized to dithiocarbamate). Excapillary electrophoresisss derivatization reagent (mercaptoacetic acid) is detectable, but is not shown, as it passes the detector at the time of the injection of the next sample.



Supplementary Fig. 3. a. Electropherogram series from seven sequential injections of an active myrosinase-containing mixture mixture (250-fold diluted horseradish extract) using the proposed capillary electrophoresis-micellar electrokinetic chromatography (CE-MEKC) method (Conditions: effective capillary length 7.2 cm (short end injection), -20.0 kV, background electrolyte: 20 CHES, 175 sodium deoxycholate, pH 9.0; sample matrix: 10 mM phosphate, pH 7.0, 1 mM ascorbic acid, 5 mM mercaptoacetic acid).

Note the decomposition of gluconasturtiin (substrate), the increase in the product isothiocyanate derivate concentration. Abbreviations: G, substrate (gluconasturtiin); P, product (phenethyl isothiocyanate derivatized to dithiocarbamate).



Supplementary Fig. 3. b. Close-up of a gluconasturtiin to phenylethyl isothiocyanate decomposition study in short-end injection mode. Conditions are as in Fig. 3.a. Abbreviations: AA, ascorbic acid; G, substrate (gluconasturtiin); PEITC, phenethyl isothiocyanate derivatized to dithiocarbamate.

Supplementary Table 1. Validation data for phenethyl isothiocyanate.

	Phenethyl isothiocyanate [a]
Detection wavelength (nm)	275
Linear Regression Equation (µg mL^{-1})	f(x) = 0.0093 x + 0.0018
Linear Regression R^2	1.0000
Upper limit of quantification (µg mL^{-1} / mM) [b]	450 / 2.78
LOD (µg mL^{-1} / mM) [b]	13.75 / 0.084
Retention time RSD% (interday)	0.71 %
Retention time RSD% (intraday)	1.64 %
Area under curve RSD% (interday)	14.9 %
Area under curve RSD% (intraday)	1.87 %

[a]: Values for phenethyl isothiocyanate are given as phenethyl isothiocyanate equivalents, but the compounds are actually quantified after derivatization with mercaptoacetic acid in-vial, in phosphate buffer (10 mM, pH 7.5). [b]: Values are given for injection with 100 mbar x 0.25 minutes.