SINIGRIN GLUCOSINOLATE: SPECTRAL AND CHROMATOGRAPHIC CHARACTERISTICS BEFORE AND AFTER ENZYME-ASSISTED SULPHATASE HYDROLYSIS

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Abstract
The main bioactive compounds from *Brassica vegetables*, are glucosinolates, responsable for anticarcinogenic properties, as integral or hydrolysed derivetives. The ussual method to quantify glucosinolates is very laborious but one of the „key" of analysis steps is the enzymatically desulphation of these compounds, leading to stable products. In this study, the activity of sulphatase from *Helix pomatia* (purified by fractionated ethanol) used to hydrolyse sinigrin standard (0.15 mM, pH 5.8) was investigated. Enzymatic hydrolysis of sinigrin determined a specific absorption spectrum and modified its retention time in the HPLC chromatogram (from 4.24 to 11.87 minutes).

Key words: sinigrin, desulphated-sinigrin, sulphatase, HPLC, absorption spectrum

INTRODUCTION

Glucosinolates are a group of secondary metabolites that are present in crops belonging to the family of Brassicaceae.

Regarding to the chemical structure, glucosinolates posses a common basic structure (Figure 1, A) comprising of β-D-glucopyranose residue linked to a sulfur atom (i), or to a (Z)-N-hydroximinosulfate ester (ii) or a variable R group (iii) with different side-chain substituents which give their structural diversity. These compounds are divided into three main groups: aliphatic, indolyl and aromatic chains.

The hydrolysis products of glucosinolates are responsible for the characteristic flavour and odour of many *Brassica* vegetables and also for their anticarcinogenic properties. When the plant tissue is damaged, the glucosinolates are hydrolyzed by endogenous plant enzyme, called myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1.) releasing a breakdown products, like nitriles, isothiocyanates, indoles, amines, epithionitriles, thiocyanates or other products (Figure 1, C).

The analysis of glucosinolates can be divided into the following methods:

- for total glucosinolates: Thymol assay (Tholen et al., 1989), glucose-release enzyme-coupled assay (Heaney et al., 1988), Near

infra-red reflectance (NIR) spectroscopy (Velasco and Becker, 1998)

- for individual intact glucosinolates: HPLC-MS (Schutze et al., 1999), Thermospray LC with tandem MS (Heeremans et al., 1989), GC-MS (Shaw et al., 1989)
- for individual desulfoglucosinolates: HPLC (Griffiths et al., 2000),
- for breakdown products: GC-MS, HPLC.

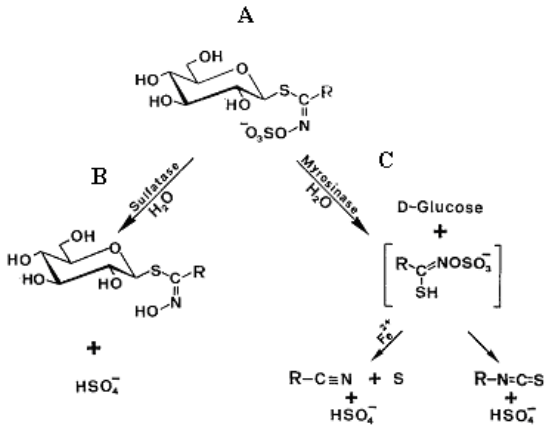

Figure 1. General chemical structure of glucosinolates (A) and enzymatic hydrolysis by sulphatase (B) and myrosinase (C) (Leoni et al., 1988) .

The "gold" method for the analysis of glucosinolates remain HPLC analysis of the enzymatically desulfated glucosinolates. The important step for the analysis of these compounds is the inhibition of myrosinase activity. For this reason, the vegetable materials, before disruption, should be completely dry by freeze-drying, and the aqueous methanol for extraction in combination with high temperatures must be used. Another important step of the analysis is to produce desulfo-glucosinolates by hydrolysis of glucosinolates by sulfatases (aryl sulfohydrolases, EC 3.1.6.1) enzymes (Figure1, B). Desulfo-glucosinolates gives a better separation by HPLC, but are often subject to the difficulties in interpreting results of the individual glucosinolates due to time and enzyme concentration on desulfation products.

The aim of this study was to investigate the sulphatase activity (purify by fractionated ethanol) on sinigrin as substrate, using spectrophotometric and HPLC analysis.

MATERIALS AND METHODS

Preparation of substrate for sulphatase enzyme
To test the activity of sulphatase enzymes, the sinigrin solution 0.15mM, in acetic acid-ethylene diamine buffer (pH5.8) was prepared.

Purification and activation of sulphatase
Helix pomatia (Type H1) sulphatase was a Sigma product. 75 mg of sulphatase was dissolved in 40% ethanol, centrifugation, and the supernatant was treated with ethanol absolute p.a. The precipitate was taken up in 5 ml of distilled water and diluted 10 times before use.

Spectrometric analysis
For the calibration curve, from stock solution of sinigrin, we made dilution between 0.15- 0.07 mM (Figure 2, a). To test the activity of sulphatase, 2 ml of the buffered sinigrin solution was transfer into cell of spectrometer (Shimatzu mini UV-Vis) and 50 µl of purified sulphatase was added and the absorbance at 228 nm was recorded for 210 minutes (Figure 2,b).

HPLC analysis
The chromatographic analyses were carried out on an HPLC system (Shimadzu Corporation, Scientific Instruments, Kyoto, Japan) equipped with a CBM-20A controller, LC-20AD pump, a DGU-20A degaser, a SIL-20AC autosampler, CTO-20AC column oven and a SPD-M20A photodiode array detector. Sinigrin and desulfo-sinigrin were separated on a Platinum (C 18) 100 A column (250 x 4.6 mm, 5 µm), thermostated at 30^0C. The data were processed with the software Labsolution version 5.10.153 (Shimadzu). The flow rate was 0.5 ml/min and the injection volume was 20 µl. The mobile phases consisted of water (eluent A) and acetonitrile (eluent B), using a gradient program as follows: 1 min 1% B; 22 min linear gradient up to 22 % B; 10 min linear gradient down to 1% B. Elution of glucosinolates was monitored at 229 nm. The glucosinolates were identified by retention time, and the UV-Vis spectra.

RESULTS AND DISCUSSION

Flow diagram for the determination of desulfated glucosinolates from vegetables by HPLC is presented in Figure 2. The standard procedure (Official Journal of the European Communities, ECC, 1990) describes the extraction of glucosinolates using methanol, with internal standard added prior the extraction (for the quantification purpose). The next step, is the purification of the extract using ion exchange columns, filled with DEAE

Sephadex A, and sodium acetate buffer (0.02M, pH 4.2) before the enzymatic desulfation of glucosinolates. The desulfoglucosinolate, were eluted from the column with water and then separated using HPLC with UV detection at 229 nm.

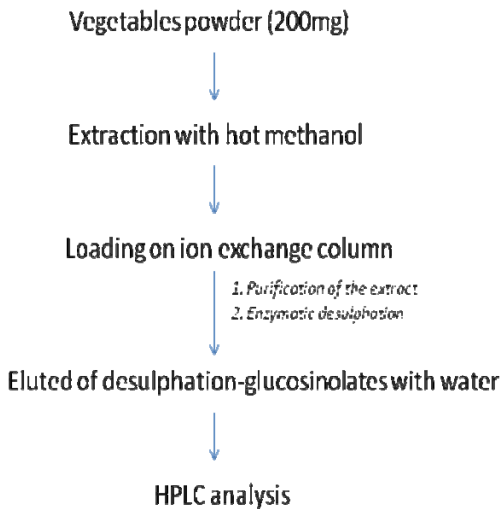

Figure 2. Flow diagram for the determination of desulphation glucosinolates from vegetables sources

The "key" of the best separation of glucosinolates is the enzymatic desulphation by sulphatase from *Helix pomatia*. For this reason, we can try to purify the enzymes by ethanol fraction and test its activity on sinigrin standard (0.15 mM, pH 5.8). The calibration curves of sinigrin with the concentration range between 0.07 and 0.15 mM are presented in the Figure 2 (a), and the kinetic curves of sulphatase purified (but not diluted) are shown in Figure (b). According to the data presented in the Figure 3 (b), after 30 minutes, the desulphatation of sinigrin standard by sulphatase was completed. If, it used the diluted of enzymes (by 10 times), the complete desulphation reaction need 5 hours (data not show). Some study also demonstrates that the time of the hydrolysis depend the nature of the glucosinolates, all of them, the sinigrin are desulphated less rapidly than other glucosinolates (Quinsac and Ribaillier, 1987).

Changes in chemical structure lead to changes in the absorption spectrum of molecules and in the retention time of HPLC chromatography. The UV spectrum and HPLC chromatogram of sinigrin and its desulphated form are shown in Figure 4. In can be notice that enzymatic desulphated of sinigrin produces on its absorption spectrum small different effects. The maxima absorption of sinigrin and desulphated–sinigrin was at 226 nm,

respectively 225 nm. In contrast, the enzymatic removal of sulphate group results in changing the retention time. For example, the retention time of sinigrin was 4.24 minute, while the retention time of desulphated sinigrin was 11.87.

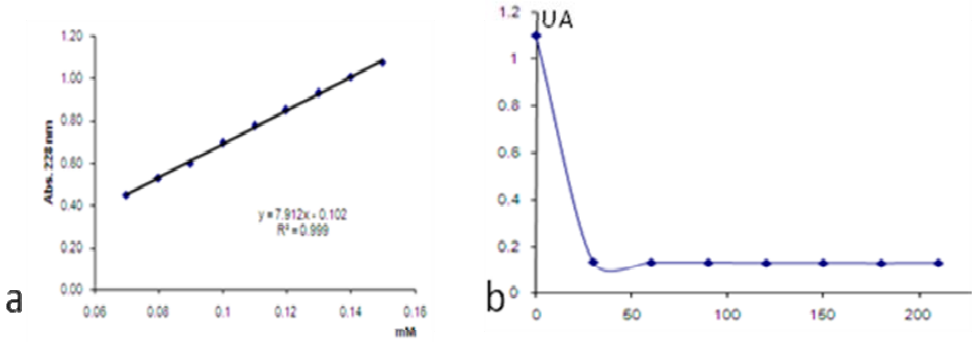

Fig.3 Calibration curve of sinigrin (a) and the kinetic curves for sulphatase activity using sinigrin standard solution (0.15 mM, pH 5.8) as substrate. UA - units of enzyme activity.

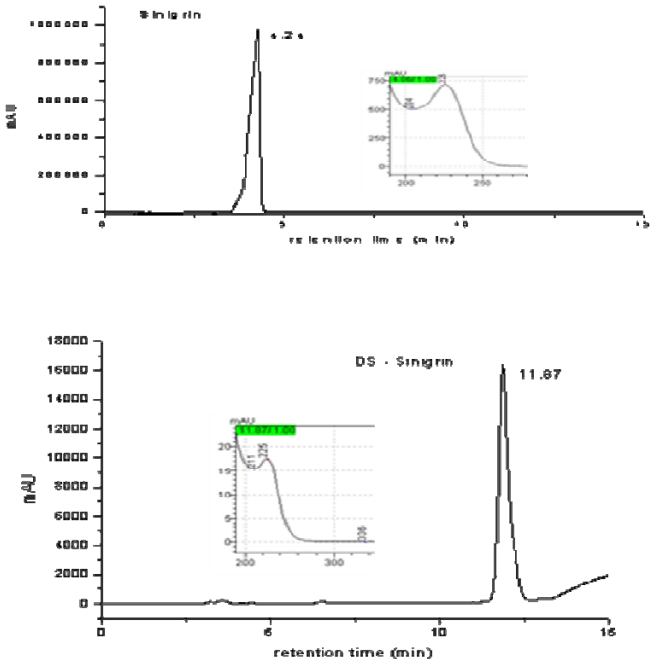

Fig.4. HPLC chromatogram and UV spectra of integral sinigrin and the enzymatically desulphated form.

CONCLUSIONS

The results presented here demonstrated that the enzymatic desulphatation of glucosinolates (in particular, sinigrin) modified the absorption spectrum and the retention time in the HPLC separation. For the analysis of desulfo-glucosinolates, the HPLC analysis remains the best method to demonstrate the effects of the enzymatic hydrolysis of glucosinolates (sinigrin).

Acknowledgements

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REFERENCES

1. Quinsac, A., Ribaillier, D., 1987. Optimization of glucosinolate desulfation before hight performance liquid chromatography. World crops: production, utilization, description. Advances in the production and utilization of cruciferous crops, volum11.
2. Griffiths DW, Birch ANE and Mac Farlane-Smith WH, 1994, Induced changes in the indole glucosinolate content of oil-seed and forage rape (*Brassica napus*) plant in response to either turnip root fly (*Delia floralic*) larval feeding or artificial root damage. *J Sci Food Agric* **65:** 171-178 .
3. Heaney R.K., Spinks E.A., Fenwick G.R., 1988, Improvedmethodfor the determination ofthe total glukosinolate content of rapeseedby determination of enzymically releasedglucose. Analyst 113 (10): 1515–1517
4. Heeremans, C. E. M., van der Hoeven, R. A. M., Niessen,W. M. A., Vuik, J., de Vos, R. H., and van der Greef, J., 1989, Specific screening methods for glucosinolates in sprout extracts using online thermospray liquid chromatography/tandem mass spectrometry. *J. Chromatogr.* **472,** 219–229.
5. Leoni O., Iori R., Haddoum T., Marlier M., Wathelet J-P., Rollin P., Palmieri S., 1998, "Approach to the use of immobilized sulfatase for analytical purposes and for the production of desulfo-glucosinolates" Industrial Crops and Products 7, 335-343.
6. Schutze, W., Mandel, F., Schulz, H., 1999. Identifizierung von Glucosinolaten in Rettich (*Raphanus sativus* L.) und Kreuzungen aus R. sativus L. _Brassica oleraceae L. (Raphanobrassica) mittels LC–MS. Nahrung 43, 245–248.
7. Velasco P, Francisco M, Moreno DA, Ferreres F, García-Viguera C and Cartea ME, 2011, Phytochemical fingerprinting of vegetable *Brassica oleracea* and *Brassica napus* by simultaneous identification of glucosinolates and phenolics. Phytochemical Analysis **22**(2)**:**144-145.