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RESEARCHES REGARDING THE ISOLATION, PURIFICATION AND ANALYSIS OF SINIGRIN GLUCOSINOLATE FROM *BRASSICA NIGRA* AND *ARMORACIA RUSTICANA*

Stoin D., F. Radu, D. Dogaru

Banat's University of Agricultural Sciences and Veterinary Medicine, 119 Calea Aradului, 300645, Timişoara, România, Faculty of Food Processing Technology,
119 Calea Aradului, 300645, Timişoara, România
*email: mucetedaniela@yahoo.com

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Abstracts. The objectives of research activities aimed natural substances with antibiotic activity, come from spontaneous flora (horseradish and black mustard), which used in the shape of standardized extracts as food additives, to replace in a near future, the compounds obtained through chemical synthesis. Such compound is glugosinolate sinigrin, an exceptional source of enzymatic inductors with active microbiologic role.

In various temperature conditions (65-100°C),various solvents extraction (distillated water, methanol, ethanol, mixture: ethanol:water (1:1), methanol:water (1:1), purification by ionic exchange chromathography (DEAE Sephadex A-50), extraction time: 3-4 hours, a sinigrin purity of 98.01-99.11% and an efficiency of 82.85-83.62%, respectively has been obtained.

INTRODUCTION

Glucosinolates are un group of plant secondary metabolites found exclusively in dicotyledonous plants. The highest concentrations are found in the *Brassicaceae* family.

The *Brassicaceae* family comprises many commonly consumed vegetables, condiments, forages and oil containing plants, such as cabbage, broccoli, horseradish, mustard, cauliflower, Brussels sprouts and rape *(Fahey et. al., 2001; Oerlemans et al, 2006).*

Glucosinolates and their degradation products are responsible for the characteristic taste and odor of crops such as horseradish, cabbage, mustard and broccoli. The GLS content is higher in black mustard seeds and horseradish roots (over 10% by dry weight), than in the other constituent parts of the *Cruciferae (Fahey et. al., 2001; Moreno et. al., 2006).*

These sulfur-containing compounds seams to play an important role in resistance to fungi, nematodes and other plant pathogens *(Bonnes and Rossiter, 1996).* On the other hand glucosinolates and/or their breakdown products have recently attracted intense research interest because of their possible cancer chemo protective attributes *(Fahey et. al., 2001).*

The glucosinolate known as sinigrin (*thio-β-glucopyranosyl-1-N-sulphate-2-propenylimidate*) are in significant content in horseradish *(Armoracia rusticana*) roots *(Thies, 1988).*

Glucosinolates in *Cruciferae* samples are usually extracted and purified according to the method of the European Union prior to HPLC determination *(Helboe, 1980).*

MATERIAL AND METHODS

Vegetable material

The horseradish roots have been harvested from Recas an the black mustard seeds from Didactic Experiment Station of Banat's University of Agricultural Sciences and Veterinary Medicine Timisoara's lot. After harvest, the samples were separated from impurities and then stored in propper conditions.

In order to attain one's object reguarding isolation, purification and analysis of sinigrin glucosinolate from *Brassica nigra* and *Armoracia rusticana*, running throug more experimental stages was necessary, each one representing a symbiothical whole of analysis methods with characteristic feature, that is:

The isolation of sinigrin from vegetable samples by solid-liquid extraction at warm conditions

The extraction protocol presented in the following represents an adaptation of the methods elaborated by Thies (1988) and Chunchang and Brain (2003) respectively.

100 g of vegetable material was weighed. The samples were stored at 120°C for 10 minutes in order to inactivate myrosinase. After being cooled at 20°C, the samples were homogeneised for 10 minutes in a blender, untill obtaining a homogeneous paste in the case of horseradish roots and a fine powder in the case of mustard seeds, respectively. The samples so obtained were quantitatively placed with 500mL phosphate buffer solution (20 mM, pH=7) in an Erlenmayer. The obtained mixture was heated untill boiling in a basin of hot water for 20 minutes. After the expireing extraction time, the suspension obtained was cooled on ice, and then centrifugated to 6000 rpm for 10 minutes at 4°C from the obtained supernatant, 20mL were taken with a dropper, volume that was placed into another tube of centrifuge, where 5mL solution were previously introduced in order to defecation. After centrifugation at 4°C temperature, a volume of 2mL supernatant was quantitatively placed in a glass of 25mL and then 15mL distilated water were added. The obtained solution was filtered and stored for following analyses.

The separation of sinigrin by ionic exchange chromatography on DEAE Sephadex A-50 column

The filling of the column of which help the separation of sinigrin was accomplished, was composed of *DEAE Sephadex A-50* brought to acetate form with piridin-acetate 0,5M solution. The samples prepared according to the protocol previously described were added in the column. The column was then washed twice with 20mL water volumes, each time the eluate being removed. Afterwards, the sinigrin was eluated with K_2SO_4 0.3M solution. About 20mL of the eluated sinigrin solution from the column, was placed into a reaction glass, and then concentrated under vacuum at 55°C until close to dry. The obtained white precipitate, was filtered under vacuum. The glass content was washed with every 5ml alcoholic solution 50%. The white crystalline precipitate was dried out with draught under vacuum at 40-45°C. Recrystallization was made from ethylic alcohol 96% .

The obtained quantity of sinigrin was 296.691mg in the case of horseradish extract and 2.358g in mustard extract, respectively.

The determination of sinigrin content from purified fraction by RP-HPLC – UV/VIS

The purity of sinigrin from horseradish and black mustard was determined through HPLC analysis using a JASCO MD 1510 apparatus coupled with a DAD JASCO MD 1510

detector. The analysis protocol was the following one *(Jen et. al., 2001; Wathelet et. al., 2001, 2004)*: it was used a NUCLEOSIL C18 column (25cm x 4,6mm, 5µm particles enlargement); volume 20µL; sample flow 1mL /min; mobile phase: methanol: phosphate buffer solution pH=7, (95:5); column temperature:35°C; wavelength: 227 nm. Data processing was made with a BORWIN 1.5 soft.

RESULTS AND DISCUSIONS

From the literature data and preliminary experimental studies , it has been concluded that isolation of sinigrin from the horseradish roots and black mustard seeds , respectively is dependent of various factors that influence the obtaining efficience and the product purity, too. The most important parameters of the process are:

The influence of inactivation treatment

In order to emphasize the influence of inactivation treatment on the process of sinigrin isolation from the horseradish roots and black mustard seeds,respectively, it has been worked in parallel with two by two samples mentaining invariably the extraction solvent (500mL phosphate buffer solution pH=7), extraction temperature (100°C), the ratio extraction vegetable material: extraction solvent and extraction time (20minutes). The calculation of the efficience was made reporting the quantity of raw crystals practical obtained to the quantity of sinigrin existent in the horseradish roots, and mustard seeds, respectively (0.65% d.w. in horseradish and 2.81% in mustard seeds, respectively).

The study's results are shown in table1. from the experimental obtained data, it can be seen that the inactivation treatment applied to horeseradish and black mustard samples, has a significant influence on the sinigrin separation in a positive sense with the extraction efficience as it can be seen from the data showed in table 1. This regarding the samples that were submitted to inactivation of myrosinase enzyme, the extraction efficiency was almost double in comparison to samples to which this treatment was not applied .

In exchange the inactivation treatment hasn't determined the decreasing of sinigrin purity, which confirms Thies (1988) appreciations. The variation in a positive sense of the extraction efficiency in the case of active samples of vegetable material can be explained considering the conditions in which the isolation process was made, too, that is : the extraction solvent was an aquous solution and the medium pH was neutral, both conditions being favourable for the process of sinigrin enzymatic hydrolisis *(Shahidi and Gabon, 1990)*.

Table 1. The influence of inactivation treatment on the separation efficiency and on the sinigrin purity from vegetable material

No. sample	Obtained raw crystals mass	Efficiency of sinigrin extraction , (%)	Melting point (°C)	Molar coefficient of extinction ε^{227nm} ($L*mol^{-1}*cm^{-1}$)	Sinigrin purity (%)
P_1	2.11	75.08	129 - 132	7450±10.1	98.6
P_2	1.05	37.36	126 - 129	7320±9.5	98.5
P_3	0.21	60.00	127 - 130	7600±22.1	98.8
P_4	0.13	32.14	125 - 127	7150±15.2	98.3

Note: P1- mustard seeds inactivated sample; P2 - mustard seeds activated sample; P3-horseradish inactivated sample; P4-horseradish activated sample

The influence of extraction temperature of sinigrin from analysed vegetable material

This parameter influences directly the isolation proces through the solvent used for the separation process. According to speciality literature there are known various conditions of sinigrin isolation, where there are mentioned various solvents used *(Thies, 1988)*.

Taking into consideration that sinigrin solubilises itself only in polar solvents, we have chosen for the study of the influence of the low tide temperature the following solvents: (1) ethilic alcohol 96% (v/v) (b.p. 75°C); (2) water (b.p. 100°C); (3) methilic alcohol (b.p. 65°C); and also the mixture 1:1 (volume) ethilic alcohol:water (b.p. 86-88°C) (4) and methilic alcohol:water (b.p. 72-74°C) (5) respectively. All horeseradish and mustard samples, respectively, were previously inactivated. The results of the study are shown in table 2 and 3.

Table 2. The influence of extraction temperature on the separation effieciency of sinigrin from black mustard seeds and horseradish roots,respectively

No. sample	Extraction solvent	Extraction temperature (°C)	Black mustard seeds		Horseradish roots	
			Obtained raw crystals mass (g)	Sinigrin extraction efficiency (%)	Obtained raw crystals mass (g)	Sinigrin extraction efficiency (%)
P_1	Ethanol (96%)	78	2.15	76.51	0.22	62.85
P_2	Methanol	65	1.61	57.29	0.12	34.28
P_3	Distilled water	100	2.35	83.62	0.29	82.85
P_4	Ethanol:Water (1:1)	86	2.20	78.29	0.28	80.00
P_5	Methanol:Water (1:1)	74	1.90	67.61	0.18	51.42

Table 3. The influence of extraction temperature on the purity of sinigrin separated from black mustard seeds and horseradish roots , respectively

No. Samp	Extraction solvent	Extraction temperature (°C)	Black mustard seeds			Horseradish roots		
			Melting point (°C)	Molar coefficient extinction ε^{227nm} ($L*mol^{-1}*cm^{-1}$)	Sinigrin purity (%)	Melting point (°C)	Molar extinction coefficient ε^{227nm} ($L*mol^{1}*cm^{-1}$)	Sinigrin purity (%)
P_1	Ethanol (96%)	78	128 - 132	7551±25.4	98.22	129 - 132	7541±25.4	98.20
P_2	Methanol	65	126 - 130	7330±10.1	98.02	126 -129	7335±10.1	98.05
P_3	Distilled water	100	127 - 131	7652±8.9	99.10	127 - 130	7662±8.9	99.11
P_4	Ethanol: Water (1:1)	86	124 - 127	7150±16.2	98.15	124 - 128	7140±16.2	98.13
P_5	Methanol: Water (1:1)	74	125-126	7224±11.5	98.25	125-127	7214±11.5	98.21

The experimental values shown in table 2 and 3, suggest that the extraction temperature and the solvent used in the isolation process implicit represent a major influence factor that confirms the previous research of Shahidi and Gabon (1990). However, this parameter can not exceed a certain limit because at termic values higher than 95°C may interfere ciclocondensation , thermic degradation processes *(Thies, 1988; Wathelet, 2001)*, which can explain the situation occured to samples P_3, P_4, too, when although the extraction temperature significantly increased (from 86°C to 100°C), the efficiency showed close values (78.29 and 83.62% in the case of mustard samples; or 80, 82, 85%, respectively in the case of houseradish samples). The polarity of the solvent influences the efficiency of sinigrin isolation, too. Thus, according to the experimental data from the table 2 and 3, it can be seen, that, although the boiling points of methanol and methanol: water mixture, respectively, in ratio 1:1 are relative close, the extraction efficiencies of sinigrin from samples P_5 (67.61% and 51.42%, respectively) increased with over 40% in comparison to the values showed at samples P_2 (57.29%; 34.28%). This aspect can be explained if we take into account the hypothesis of Abramski and Chmielewski (1996), which according to, the sinigrin is found in plants mostly as monohidrated potasium salt.$C_{10}H_{16}NO_9S_2K$ and in order to extract it as much as possible, it is necesarly to add at the organic solvent a certain quantity of water, too.

The influence of column filling on sinigrin purity degree

In order to purify the sinigrin extract from *Armoracia rusticana* and *Brassica nigra*, ionic exchange chromatography was used *(Jen et. al., 2001)*. The concentrated extracts of horseradish and black mustard (15mL) were passed through the column, eluated with 0,3M K_2SO_4 solution. It has been collected a number of 20 fractions of 5 mL eluate, each one. As a result of aqueous extracts purification, it was ascertained that sinigrin began to be detected from the fraction number 5, reached a maximum in fraction 10, and after that a slow decreasing was noticed at the beginning (fraction 11) and then a sudden decreasing until fraction 13, and starting with fraction 14, sinigrin was no longer present in eluate.

The sinigrin detection was made through HPLC analysis at a wavelength = 227 nm, using the external standard method *(Wathelet, 2001)*. The chromatrograms of the respective standard of the isolated and purified sinigrin from horseradish roots and mustard seeds are shown in figure 1-3.

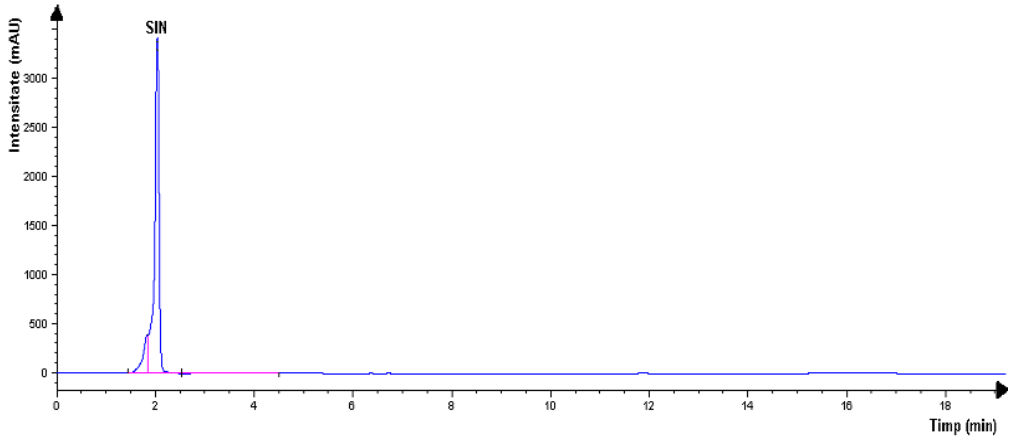

Figure 1. Chromatogram HPLC of sinigrin (standard solution)

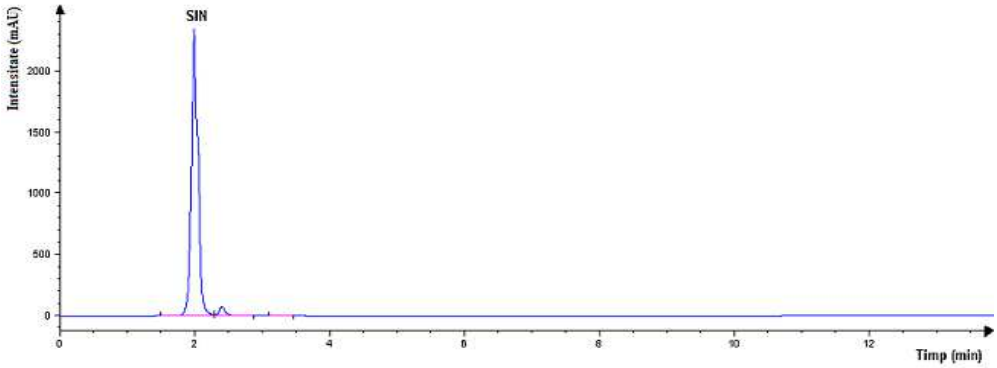

Figure 2. Chromatogram HPLC of sinigrin from mustard

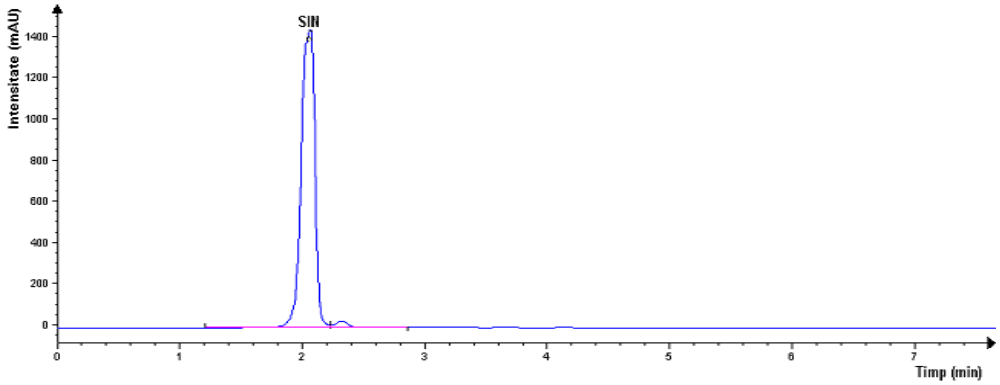

Figure 3. Chromatogram HPLC of sinigrin from horseradish

CONCLUSIONS

The method proposed by our research staff concerning isolation, purification and analysis of sinigrin, an exceptional source of enzymatic inductors with active microbiologic role, can be successfully used in order to obtain standardized extracts of *Armoracia ruticana* and *Brassica nigra*. Thus, the optimum popper conditions of obtaining the standardized extracts are: extraction solvent: water or phosphate buffer solution (pH=7); extraction time: 20 minutes; crystalization period: 3-4 hours; purification: eluation on column with DEAE Sephadex A-50; inactivation temperature:100°C. Using the optimum parameters specific to sinigrin extraction, a purity of 98.01-99.11% and an extraction efficiency of 82.85-83.62%, respectively, was obtained. The method which we presented here, is exactly, rapidly, and may be used in the majority of the laboratories.

BIBLIOGRAPHY

Abramski W., M. Chmielewski, 1996, Practical synthesis of sinigrin, Journal of Carbohydrate Chemistry 15, 109-113

Bones, A.M., J.T. Rossiter, 1996, The myrosinase-glucosinolate system, its organisation and biochemistry, Physiologia Plantarum, 97, 194-208

Chunchang T., H.E. Brian, 2003, Preliminary Investigation on Gls Extraction from Yellow Mustard Meal, American Society of Agricultural and Biological Engineers, PNW-03-118

Fahey, J.W., A.T. Zalcmann, P. Talalay, 2001, The chemical diversity and distribution of glucosinolates and isothiocyanates among plants, Phytochemistry, 56, 5-51

Helboe P., 1980, Separation of glucosinolates by high-performance liquid chromatography, Journal of Chromatography, 197, 199-205

Jen J., T. Lin, J. Huang, W. Chung, 2001, Direct determination of sinigrin in mustard seed without desulfation by reversed-phase ion-pair liquid chromatography, Journal of Chromatography A, 912, 363-368

Moreno D.A., M. Carvajal, C. L´opez-Berenguer, Garcia Viguera, 2006, Chemical and biological characterisation of nutraceutical compounds of broccoli, Journal of Pharmaceutical and Biomedical Analysis, article in press

Oerlemans K., Diane Barrett, Carme Bosch Suades, R. Verkerk, M. Dekker, 2006, Thermal degradation of glucosinolates in red cabbage, Food Chemistry, 95, 19-29

Shahidi F., J.E. Gabon, 1990, Fate sinigrin in methanol/ammonia/water-hexane extraction of B. juncea mustard seed, J. Food Sci., 55, 3, 793-799

Thies W., 1988, Isolation of sinigrin and glucotropaeolin from cruciferous seeds, Fat Science and Technology, 90, 311-314

Wathelet J.P., Iori R., Mabon N., Palmieri S., Leoni O., Rollin P., Marlier M., 2001, Determination of the response factors of several desulfo-glucosinolates used for quantitative analysis of Brassicaceae, Réunions techniques du GCIRC, Poznan, Pologne