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# Development of A Flow Amperometric Enzymatic Method for the Determination of Total Glucosinolates in Real Samples

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**The first amperometric flow analyzer, based on the bio-sensor concept, capable of determining total glucosinolates in real samples, is described. Myrosinase was immobilized on aminopropyl-modified controlled pore glass, which was then used for the construction of a packed-bed reactor. Myrosinase catalyzes the hydrolysis of glucosinolates (sinigrin) to glucose (among the other products), which is then oxidized by the action of glucose oxidase to produce hydrogen peroxide. The glucose enzyme electrode is based on a multimembrane architecture and was mounted on an amperometric flow cell (hydrogen peroxide detection at a platinum anode poised at +0.65 V vs Ag/AgCl/3M KCl). Different membrane types and different activation procedures were tested. The system was optimized to various working parameters, either as a glucose electrode or as a glucosinolate analyzer. The interference effect of various compounds was also investigated. Application of the method to real samples was carried out using glucose/glucose, hydrolyzed sinigrin and glucose/sinigrin solution as calibrators of the glucose electrode and the glucosinolate analyzer. Deviations due to the enantioselectivity of glucose oxidase to the  $\beta$ -glucose anomer were observed, and a data elaboration protocol is proposed. The possibility of the simultaneous determination of glucose and glucosinolates is also demonstrated.**

Glucosinolates ( $\beta$ -thioglucoside-*N*-hydroxysulfates) represent a specific group of natural compounds (secondary plant metabolites) occurring predominantly in vegetables of the family Brassicaceae but also in several other plant families.<sup>1–3</sup> Crucifers is one of the 10 most economically important plant families in the world and includes such vegetables as broccoli, Brussels sprouts, cabbage, cauliflower, kale, radish, rutabaga, and rapeseed. Glucosinolates are found throughout the roots, stems, leaves, and seeds in the cruciferae family<sup>1</sup> and can be hydrolyzed by the enzyme myrosinase ( $\beta$ -thioglucosidase) to glucose, isothiocyanate, nitriles, and sulfate.<sup>4</sup>

Glucosinolates play an extremely important role in the food industry, since their breakdown products produce flavor and aroma constituents of nutritional and commercial importance.<sup>5</sup> Moreover, they have long been known for their fungicidal, bactericidal, nematocidal, and allelopathic properties. The activity of isothiocyanates such as sulforaphane against numerous human pathogens, for example, *Escherichia coli*, *Salmonella typhimurium*, and *Candida* spp. could even contribute to the medicinal properties ascribed to cruciferous vegetables, such as cabbage and mustard.<sup>6,7</sup>

Recently, glucosinolates have attracted intense research interest because of their cancer chemoprotective attributes.<sup>8–10</sup>

Rapeseed, derived from Brassica, is one of the major oilseeds in commerce; its world production amounts to several million metric tons. Removal of the oil from the crushed seeds leaves a residue or meal that contains 40% protein and, hence, is a valued component of animal feed formulations. Rapeseed meal, however, contains glucosinolates, which can undergo chemical or enzymatic hydrolysis, producing a range of products, some of which possess antinutritional or goitrogenic properties.<sup>11–12</sup> The European Community has withdrawn financial support for all batches of seed containing glucosinolates in excess of 20  $\mu\text{mol g}^{-1}$ .<sup>13</sup>

The available methods so far for the determination of total glucosinolates in cruciferous vegetables mainly include chromatographic and enzymatic methods. A detailed presentation of these methods is adduced in the excellent work of Fahey et al.<sup>1</sup> Among the chromatographic methods that have been proposed is the reversed-phase LC method for the quantitative analysis of desulfo-glucosinolates. This method is the most widely used today;

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however, it utilizes an on-column enzymatic desulfation treatment, which is time-consuming.<sup>14</sup> Other chromatographic methods based on liquid chromatography/atmospheric pressure ionization mass spectrometry,<sup>15</sup> supercritical fluid chromatography with light-scattering detection,<sup>16</sup> reversed-phase ion-pair liquid chromatography (IPC),<sup>17</sup> capillary electrophoresis/laser-induced fluorescence via the enzymatically released glucose,<sup>18</sup> hydrophilic interaction chromatography performing along with IPC,<sup>19</sup> and a micellar electrokinetic capillary chromatography<sup>20</sup> have been proposed. A temperature-programmed gas chromatography method<sup>21</sup> and a near-infrared reflectance spectroscopy<sup>22</sup> method have also been published.

A number of enzymatic methods utilizing soluble myrosinase and glucose oxidase in combination with photometric<sup>23–26</sup> or polarographic<sup>27</sup> monitoring of hydrogen peroxidase or oxygen uptake, respectively, have also been proposed. The first biosensor-based approach was proposed by Koshy et al.<sup>28</sup> They described a myrosinase–glucose oxidase bienzyme electrode for the determination of glucosinolates; however, the concentration of the free glucose is co-determined, and the sensor suffers from interference of various electroactive species, mainly ascorbate, which is an activator of myrosinase.

Another approach based on the biosensor concept has been proposed by Leoni et al.<sup>29</sup> and is based on an initial-rate method, using the pH-stat technique. The construction of a myrosinase-modified nylon bioreactor for the production of pure bioactive molecules<sup>30</sup> and the construction of a sulfatase-modified nylon bioreactor for the production of desulfoglucosinolates for the analysis of glucosinolates in combination with an LC<sup>31</sup> have also been proposed.

Evaluating the already existing biosensors,<sup>28–29</sup> it is obvious that there is still a need for improved glucosinolate biosensor-based analyzers, mainly in terms of analytical applicability and performance simplicity.

The present work also deals with an issue of great analytical importance, since a lot of research work refers to the use of some

enzymes, which acting on their specific substrates produce sugars, and the latter are determined by various oxidases or dehydrogenases that are specific to them, for example, glucose oxidase or fructose dehydrogenase. These sugar-specific enzymes are highly enantioselective; however, the standard solutions used as calibrators of the sugar-specific biosensors are (naturally) a mixture of both anomers of the specific sugar. On the other hand, the enzymatically produced sugar, by the action of the first enzyme onto its substrate, is purely one of the anomers. Therefore, the extraction of accurate analytical results by simple subtraction of the results obtained by each enzyme (total sugar minus the free sugar content) is not possible. A possible way to overcome this problem could be the use of an extra enzyme, for example, a mutarotase, to convert the mixture of the anomers in the standard solution of the sugar to a pure solution of one of the two anomers, preferably the one on which the enzyme is enantioselective. This approach, however, introduces some drawbacks, namely, an increase in the cost of analysis, extra incubation steps, more frequent calibrations within runs, and also problems associated with complicated multienzyme systems (cross-selectivity and different working conditions). We proposed here a simple protocol for the elaboration of the received data. The proposed protocol was successfully tested in a series of glucose/sinigrin standard mixtures. The effect of different calibrating systems (glucose/glucose, hydrolyzed sinigrin, and glucose/sinigrin) for the glucose electrode and the glucosinolate analyzer to the validity and the accuracy of the method was investigated. The possibility of the simultaneous determination of glucose and total glucosinolates is demonstrated.

## EXPERIMENTAL SECTION

**Chemicals.** Glucose oxidase Type X-S (GOD, EC 1.1.3.4, from *Aspergillus niger*),  $\beta$ -thioglucosidase (myrosinase) (MYR, EC 3.2.3.1, from *Sinapis alba*),  $\beta$ -D(+)-glucose, cellulose acetate (~40% acetyl), and aminopropyl-controlled-porosity glass (A-CPG) (pore size 500 Å, 200–400 mesh) were obtained from Sigma. Polyvinyl acetate (PVA, MW 167 000 Da), sinigrin monohydrate (SIN), 2-propenyl (allyl) isothiocyanate, 1,3-dicyclo-hexylcarbodiimide (DCC), cyanuric chloride (TsT), dichloromethane (MeCl<sub>2</sub>), and glutaraldehyde 25% grade II were supplied from Aldrich. A Boehringer test kit for D-glucose was purchased from R-Biopharm GmbH (Darmstadt, Germany).

**Membranes.** MF membranes (mixed ester cellulose; 0.45- $\mu$ m porosity; thickness, 120  $\mu$ m) were purchased from Millipore (Poole, U.K.). UltraBind membranes (modified polyethersulfonate membrane possessing aldehyde functional groups; preactivated; of 0.45- $\mu$ m porosity; thickness, 150  $\mu$ m), Biodyne C (negatively charged nylon 66 membrane; 100% carboxyl groups; of 1.2- $\mu$ m porosity; thickness, 150  $\mu$ m), Biodyne A (amphoteric nylon 66 membrane; 50% amino and 50% carboxyl groups; of 0.2- $\mu$ m porosity; thickness, 150  $\mu$ m) were a kind gift of Pall Filtration (Milan, Italy). Polycarbonate membranes (thickness, 10  $\mu$ m; of 0.05- $\mu$ m porosity) were supplied from Nucleopore (MA). For casting cellulose acetate membrane, a wet film applicator (URAI, Milan, Italy) was used.

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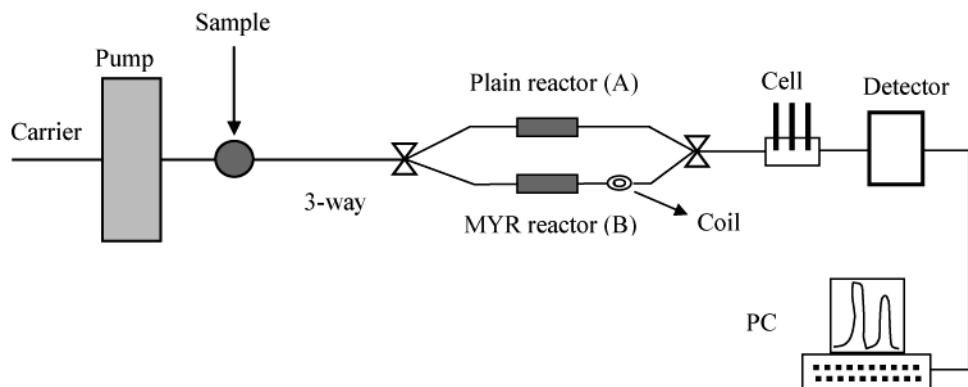


Figure 1. Schematic representation of FI manifold employed for glucosinolates determination

**Apparatus.** The work was carried out using an in-house fully automated FI manifold.<sup>32</sup> Electrochemical experiments were run using a computer-controlled potentiostat (EcoChemie/Autolab). Solutions were pumped through using a four-channel peristaltic pump (Gilson, France), and sample injections were made with a pneumatically actuated injection valve (Rheodyne, Cotati, CA). A three-electrode flow-through detector (Metrohm, Switzerland) was used for the amperometric monitoring of hydrogen peroxide.<sup>32</sup> A three-way valve regulates the performance of the system either as a glucose analyzer (position A) or as a total glucose analyzer (position B), that is, free glucose plus the glucose produced from the hydrolysis of the glucosinolates present in the samples. Kinetic experiments and comparative study of the GOD membranes were performed with steady-state amperometric measurements using a three-electrode amperometric cell (VC-2, BAS). A flow diagram of the system is shown in Figure 1.

**Activation of the Membranes<sup>33</sup>. Cyanuric Chloride Method.** An 860- $\mu$ L portion of TEA and 13 mL of  $\text{MeCl}_2$  were mixed for 2 min; 0.4 g TsT was added, and then 10 pieces of the membrane (0.6-cm diameter) were soaked in the mixture and left to react under stirring for 15 min.

**Carbodiimide Method.** Ten pieces of the membrane were soaked in a 10% (w/v) DCC solution of  $\text{MeCl}_2$  under stirring for 30 min. In both cases, membranes were washed with  $\text{MeCl}_2$  ( $4 \times 10$  mL) for 5 min.

**Glutaraldehyde Method.** Ten pieces of the membrane were soaked in a 5% glutaraldehyde solution of PBS (150 mM NaCl, 40 mM  $\text{Na}_2\text{HPO}_4$ , and 8 mM  $\text{NaH}_2\text{PO}_4$ ) pH 7.4 for 2 h under stirring and finally washed with PBS ( $4 \times 10$  mL) for 5 min. Activated membranes were allowed to air-dry and stored in a desiccator where they were stable for 2 months. A longer lifetime (up to 6 months) can be achieved when they are stored sealed under nitrogen at  $+4^\circ\text{C}$ . **Note:** TsT is a suspected carcinogenic compound and must be handled with extreme caution. Do not inhale vapors!

**Preparation of the Enzymatic Membranes.** GOD was immobilized with covalent bonding or physical adsorption following the spot-wetting method.<sup>34</sup> A 10- $\mu$ L portion GOD (25U) was spotted onto each side of the membrane. Membranes were

allowed to air-dry, and unattached protein was removed by washing them ( $4 \times 3$  mL) with the immobilization buffer (50 mM phosphate, pH 7).

**Preparation of the Myrosinase Reactor.** The PB reactors were prepared by packing 80 mg of A-CPG in glass columns (2.0-mm i.d  $\times$  30-mm length). A-CPG was activated by pumping through the columns a 2.5% v/v solution of glutaraldehyde in 50 mM phosphate buffer, pH 7, for 2 h at room temperature and then washing with double-distilled water (DDW) for another 2 h. The enzymatic solution [6.4 U (20 mg) MYR in 20 mL of 50 mM phosphate buffer, pH 7] was loaded onto the supporting material by circulating it for 12 h at a flow rate of  $0.1 \text{ mL} \cdot \text{min}^{-1}$  at room temperature.<sup>32</sup> (**Note:** myrosinase handling should be carried out wearing a mask and rubber gloves!). The support was washed with DDW (5 min), 50 mM NaCl (10 min), DDW (10 min), and finally, with the buffer of immobilization (10 min). The reactor was stored at  $+4^\circ\text{C}$  filled with the buffer of immobilization.

**Assembly of the Glucose Sensor.** The cellulose acetate membrane (20  $\mu\text{m}$  thick, 100 Da nominal MW cutoff) was first placed on the platinum surface of the electrode to eliminate interference from electroactive species.<sup>34</sup> Then the membrane bearing the GOD was superimposed with an outer polycarbonate membrane in order to prevent microbial attack and leaching of the enzyme. All membranes were tightly fitted over the electrode with the aid of an O-ring.

**Preparation of Glucose Standards from Hydrolyzed Sinigrin.** A 5 mM glucose stock solution was prepared by hydrolyzing a 5 mM solution of SIN by adding to the latter 4 mg of MYR ( $\sim 1.3$  U). Myrosinase was allowed to react for 4 h at room temperature in the working buffer solution and then was deactivated at  $90$ – $100^\circ\text{C}$  for 10 min.

**Sample Preparation.** Broccoli and cauliflower leaves ( $\sim 20$  g) were cut into pieces in a blender with 50 mL of 50 mM phosphate, pH 7 buffer at a temperature near its boiling point. The pulp was further mixed with 50 mL of the same buffer. Seeds of sinapis ( $\sim 10$  g) were preheated at  $120^\circ\text{C}$  for 10 min in order to inactivate the endogenous myrosinase and then were treated as mentioned above. After centrifugation and filtration, the samples were stored at  $-20^\circ\text{C}$ .

**Procedures.** The carrier stream (50 mM phosphate, pH 8) was continuously pumped at a flow rate of  $0.55 \text{ mL} \cdot \text{min}^{-1}$  toward the probe until a stable baseline current was reached ( $1$ – $2$  nA within  $20$ – $30$  min). Standard or sample solutions [ $x$ - $\mu\text{L}$  sample +

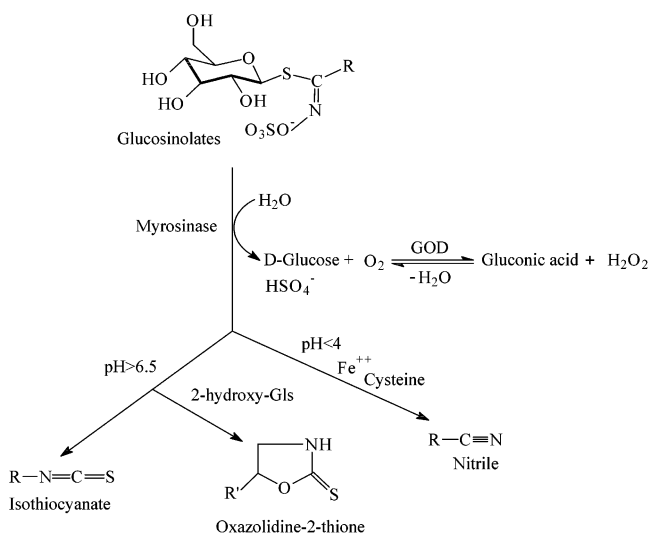
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Scheme 1. Enzymic Pathways of the Hydrolysis of Glucosinolates and Oxidation of Glucose



(5000 -  $x$ )- $\mu$ L carrier] were introduced as short pulses of 120  $\mu$ L via the loop injection valve. The peak height of the current response was taken as a measure of the analyte concentration. Glucose determination was carried out in the absence of the myrosinase reactor (valve position A) using glucose or hydrolyzed sinigrin as standard solutions, whereas glucosinolate determination was carried out by passing the carrier and the samples through the myrosinase reactor (valve position B) and by using glucose or sinigrin or hydrolyzed sinigrin as standard solutions.

## RESULTS AND DISCUSSION

**Enzyme Pathway.** Myrosinase from *Sinapis alba* seeds is a glycoprotein containing various thiol and sulfide groups together with ~18% carbohydrates, mainly hexose. It consists of two identical subunits of 71.1 kDa and shows a pI of 5.1.<sup>35</sup> Myrosinase is present in plants and catalyzes a hydrolytic reaction of glucosinolates, generating compounds such as nitriles, isothiocyanates, or oxazolidinethiones. The formation of these compounds depends strictly on the chemical structure of the glucosinolate side chains and the experimental conditions of the reaction (Scheme 1). According to the literature, pH values close to neutral induce the production of isothiocyanates. Acidic pH, on the other hand, induces the formation of both isothiocyanates and nitriles. In acidic media, the formation of nitriles alone is favored by the presence of reducing agents, such as ferrous ion and cysteine. In isothiocyanates, with a hydroxy group in the b position with respect to isothiocyanate function, a fast cyclization process occurs that produces chiral oxazolidinethiones.<sup>1</sup>

**Enzyme Membranes.** Glucose oxidase is the most widely used enzyme in biosensor research, and experimental results so far have shown that it is an enzyme with excellent behavior in the immobilized form. To find the optimum conditions in our study, we tried a series of different GOD membranes by comparing various activation procedures and methods of immobilization on membranes bearing different functional groups on their

surface. A comparative study of the membrane performance was carried out with steady-state amperometric measurements at an enzyme content of 50 U GOD (25 U/site) at the optimum pH for each membrane (see Table 1). The pH profile of the GOD membrane was also investigated, and optimum values are shown in Table 1. The  $K_m$  constants were calculated by applying the electrochemical Eadie-Hoffstee (E-H) equation [ $I = I_{\max} - K_m(I/C)$ ] and the direct linear (D-L) transformations methodology by applying equation [ $I_{\max} = I + K_m(I/C)$ ].<sup>36</sup> Best results were obtained by using the Biodyne C membrane activated with DCC, and therefore, this membrane was selected for further experiments.

Enzyme loading for GOD was tested in order to define the enzyme loading necessary to obtain diffusional limitation of response, that is, the response maximum. A saturation study of the membrane was made using the Biodyne C membrane activated with DCC. Different enzyme loadings, covering the range 5–100 U GOD (data not shown) were tested, taking as the criterion the sensor sensitivity to a 1 mM glucose concentration. The profile of the system sensitivity reaches a plateau at 40 U GOD, and therefore, further experiments were performed, applying the useful enzyme content (50 U GOD) onto the membrane. The selected membrane shows an excellent operational (>200 injections with no apparent loss of its original activity) and storage (one month at +4 °C) stability.

**Packed-Bed Myrosinase Reactor.** The yield of immobilization of myrosinase onto the glass beads reactor, as a proteinic molecule, is calculated to be >95% by measuring the absorbance of the immobilization solution at 280 nm before and after the immobilization procedure. The remaining activity of the immobilization solution was also tested by steady-state amperometry by applying a conventional fixed-time procedure. Standard solutions of myrosinase, covering the range 0–2 U, were added to a solution of 50 mM phosphate pH 8 containing 5 mM of sinigrin, and the current was calculated 500 s after the addition of enzymatic solution. By constructing a calibration curve  $I$  (nA) =  $f$ (U MYR), the activity of the immobilization solution was found to be 0.5 units. The reactor showed an extraordinary stability, keeping its original activity for >1500 injections within a period of 6 months.

**Optimization of Working Parameters of the Glucosinolates Analyzer.** *Flow Parameters.* All of the experiments were carried out at an overall flow rate of 0.55 mL min<sup>-1</sup>, a rate that reconciles fairly high peaks and satisfactory sample throughput (20 h<sup>-1</sup>) and a sample volume of 120  $\mu$ L, because it prevents peak-broadening and ensures high sensitivity. Under the applied flow parameters, a dispersion coefficient of 1.30–1.36 was calculated.

*pH Profile.* The pH of the working buffer was initially investigated for the glucose sensor using the GOD membranes mentioned before in a 50 mM phosphate buffering system covering the pH range 5.5–8.5 with steady-state amperometric measurements. Optimum pH values (Table 1) range from 5.8 to 8, depending on the nature of the chemical bond and method of activation. The pH profile of the system either as glucose or as sinigrin analyzer was also investigated by coupling the MYR reactor with a Biodyne C membrane activated with DCC in a flow system manifold (FIA). Best results in terms of peak height were

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Table 1.  $K_m$  Values and Analytical Performance of Various GOD Membranes

membrane/activation method	$K_m$ (mM) E-H method	$K_m$ (mM) D-L method	linear range, mM	detection limit, mM	sensitivity, nA/mM	optimum pH
Biodyne A	3.7	4.5	0.03–1.5	0.02	16.9	7.5
Biodyne A/glutaraldehyde	3.8	4.6	0.03–1.1	0.02	18.4	7.0
Biodyne A/DCC	7.5	7.5	0.03–1.1	0.03	19.5	7.5
Biodyne C	3.7	4.6	0.06–1.4	0.04	13.1	8.0
Biodyne C/glutaraldehyde	7.8	6.4	0.04–1.7	0.03	13.9	6.0
Biodyne C/DCC	4.2	3.6	0.005–1.6	0.004	25.1	7.5
Biodyne C/TsT	6.1	6.2	0.04–1.8	0.03	18.1	8.0
MF	9.5	8	0.15–3.4	0.05	3.2	6.2
Ultrabind	3.2	4.6	0.1–1.8	0.05	11.5	5.8
Ultrabind/glutaraldehyde	5.6	5.9	0.01–1.5	0.008	22.3	6.2

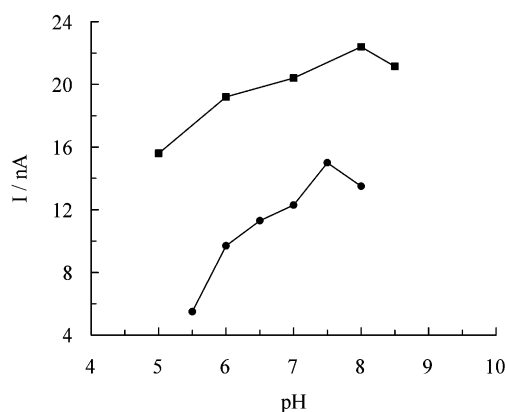


Figure 2. pH profile of the glucose enzyme electrode (●), and the glucosinolate enzyme system (■) under optimum working conditions. Buffer, 50 mM phosphate; glucose concentration, 1 mM; sinigrin concentration, 1 mM.

obtained at 50 mM phosphate buffer pH 7.5 and pH 8, respectively (Figure 2). According to the literature, the optimum pH for soluble or immobilized myrosinase in nylon tubes is near 6.5.<sup>23–27</sup> The shift of the optimum pH value observed in our study can be attributed to the behavior of the GOD membrane, which expresses the hydrolytic efficiency of the myrosinase by means of its sensitivity to the produced glucose.

**Concentration of the Activator (Ascorbic Acid).** According to the literature, myrosinase is activated at various degrees by ascorbic acid, and in some instances, the enzyme is almost inactive in its absence.<sup>37</sup> Activation is not dependent on the redox properties of ascorbate, and according to Ettlinger et al.,<sup>38</sup> it provides a nucleophilic catalytic group. The activation of ascorbate is “uncompetitive”, that is, ascorbate raises both  $V_{max}$  and  $K_m$  for the glucosinolate substrates.<sup>37</sup>

The interference effect of ascorbate on both spectrophotometric and amperometric approaches is well-known.<sup>28,34</sup> The effect of the ascorbic acid was investigated separately for the glucose sensor and the glucosinolate analyzer as an added reagent in the working buffer solution. As expected, ascorbate slightly decreased the sensitivity of the glucose sensor as it reacted with the enzymatically produced hydrogen peroxide in the interface of the enzymatic and the cellulose acetate membranes. On the other hand, it was found to increase the performance of the glucosinolate

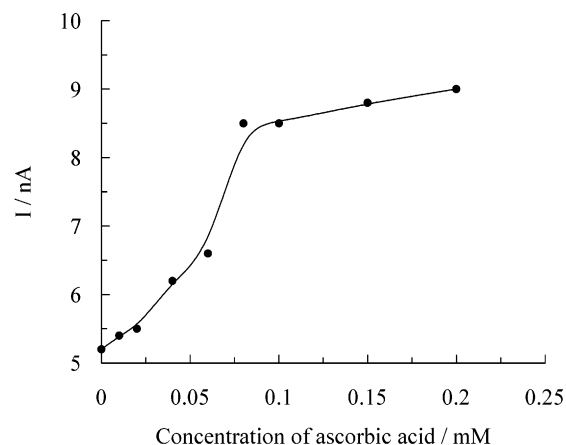


Figure 3. Effect of the concentration of ascorbic acid added in the carrier stream on the performance of the glucosinolate analyzer under optimum working conditions. Sinigrin concentration, 0.4 mM.

analyzer (Figure 3). Optimum concentration of ascorbate was found to be 0.08 mM.

**Interference Effect of Various Compounds.** The interference of some ions and a big variety of organic acids present in real samples was investigated by applying the method of mixed solutions in the presence of 0.2 mM glucose (position A of the valve) and 0.2 mM sinigrin (position B of the valve). The effect of various compounds on the relative responses is shown in Table 2, where the activities of plain 0.2 mM glucose and 0.2 mM sinigrin were taken as 100%. Common organic acids and some ions do not interfere, verifying thus the high selectivity of the used enzymes to their major substrates. The interference effect of allyl isothiocyanate, the major product of the action of myrosinase on sinigrin at pH 8, was found to act as an inhibitor, decreasing the performance of the glucose sensor at a factor of 0.9, whereas it had almost no interference effect on the performance of the sinigrin system. These results refer to the mean value of three runs. The behavior of the glucose sensor is in accordance with the results reported in the literature.<sup>1,28–29</sup> The same behavior in a lower degree, since sinigrin was used as calibrator, was expected for the sinigrin system; however, the bioreactor was found to protect the glucose enzyme electrode by adsorbing to a certain degree the added allyl-isothiocyanate. When allyl-isothiocyanate was added in the carrier stream, its interference effect was reexamined by performing successive glucose and sinigrin injections. After the first five to six runs, the performance of both the enzyme electrode (glucose standards) and the system of the bioreactor and the enzyme electrode (sinigrin standards) started

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Table 2. Interference Effect of Various Compounds on the Assay of Glucose and Total Glucosinolates<sup>a</sup>

interfering species, mM	relative efficiency, %, valve position B	relative efficiency, %, valve position A
sinigrin (0.2)	100	
glucose (0.2)		100
malic acid (1)	99	99
succinic acid (2)	101	103
acetic acid (2)	97	101
oxalic acid (2)	96	97
pyruvic (2)	102	99
glutamic acid (5)	100	97
tartaric acid (10)	103	102
adipic acid (10)	98	96
allyl isothiocyanate (1)	98	89
allyl isothiocyanate as carrier reagent (1)	85	84
Ca <sup>2+</sup> , Cl <sup>-</sup> (2 each)	100	100

<sup>a</sup> The values in parentheses are the concentrations of the compounds in mM.

deteriorating (up to 15% loss of initial activity) as the bioreactor was unable to retain the continuously pumped allyl isothiocyanate. Moreover, allyl isothiocyanate, which is an oily compound, sparingly soluble in the water, accumulated on the polycarbonate membrane. After the exposure of the polycarbonate membrane to the allyl isothiocyanate-spiked carrier stream, the membrane became harder and glossier.

**Application to Real Samples.** Preliminary studies were carried out in broccoli leaves, cauliflower, and sinapis seeds by using glucose standards for measuring the free glucose content (position A of the valve) and sinigrin standards for measuring the total glucose content (position B of the valve). According to that concept, we expected the signal of the total glucose to be greater in order to calculate the glucosinolates content as the difference of the latter with that of free glucose. It is important to point out that most of the proposed approaches that have been reported so far in the literature are based on this philosophy.<sup>18,25–28</sup> However, in a series of experiments and after a detailed control of all of the parameters that may affect the obtained results, the glucosinolates concentration was calculated to be negative or considerably lower as compared to the results obtained with the reference method (Table 3).

The source of the error was then focused on the presence of potentially interfering species produced during the enzymatic conversion of sinigrin to glucose that might act as inhibitors of glucose oxidase. Under the specific experimental conditions, the main product of the enzymatic action of myrosinase is allyl isothiocyanate. Studies of its interference effect (see also Table 2) show an inhibitory action on glucose oxidase.

When the system functions as a glucose analyzer (allyl isothiocyanate is present in the samples in very low, even zero, concentration) the glucose electrode monitors free glucose with a higher sensitivity as compared to that when the system functions as a glucosinolate analyzer, since the latter is calibrated with sinigrin standards up to 1 mM.

To overcome this problem, we used glucose standards for constructing the calibration curves for both free and total glucose. The standards were prepared either by weighting glucose or from enzymatically hydrolyzed sinigrin. Both methods gave positive

values for the total glucosinolates content of the samples (Table 3) with good recoveries to both glucose and sinigrin.

Using glucose standards, two almost identical calibration curves were constructed (Table 4). Geometric characteristics of the plain and the myrosinase reactors are the key factor in order to obtain identical calibration curves when peak height is taken as a measure of the analyte concentration. If analytical results are expressed with respect to peak surface, this factor is less important. Using hydrolyzed sinigrin (HS) standards, two calibration curves were also constructed. As can be seen in Table 4, the glucosinolate system is slightly more sensitive, and this behavior can be attributed to the incomplete hydrolysis of the sinigrin standard solutions. The analytical performance of the glucosinolate analyzer calibrated with sinigrin standard solutions is also presented in Table 4.

Using glucose standards, the recovery of the system to sinigrin, as expected, is high, since the system is more sensitive to sinigrin. This behavior has been also observed in previous studies<sup>28</sup> and has been characterized as not reasonable, since sinigrin is first converted to glucose and then measured as glucose; however, from the results presented in this work this behavior is attributed to the presence of ~36% of the enzymatically inactive  $\alpha$ -anomer of glucose in the glucose standard solution, as compared to the sinigrin solution, which in the presence of myrosinase produces only the enzymatically active  $\beta$ -anomer of the glucose.

Using hydrolyzed sinigrin calibrating solutions (up to 1 mM), the high recoveries of both glucose and sinigrin are attributable to the presence of allyl isothiocyanates, which lowers the sensitivity of the system (Table 2). Consequently, spikes of 0.1 mM glucose (no allyl isothiocyanates present) or sinigrin (the concentration of allyl isothiocyanate produced is up to 0.1 mM) are sensed with higher sensitivity. The partial (hydrolysis lasts 4 h) mutarotation of the enzymatically produced  $\beta$ -glucose to a mixture of both glucose anomers is also a reason which explains the higher recoveries received in the case of sinigrin spikes.

As can be seen in Table 3, the results obtained with the proposed method applying all the proposed calibrating systems are significantly deviated from the results obtained by a reference enzymatic method (application of the Boehringer Mannheim glucose kit to samples that have been prehydrolyzed with a 10 U mL<sup>-1</sup> myrosinase solution at 25 °C for 15 min in the presence of 0.12 mol L<sup>-1</sup> phosphate in 0.34 mmol L<sup>-1</sup> citrate, pH 6, according to a previously reported protocol of the company<sup>4,39</sup>).

After we had tried all of the above-mentioned calibrating systems, we checked a number of parameters that might affect the performance of the system. Examination of any possible activating or inhibiting behavior of sulfate anions (Scheme 1) on myrosinase or glucose oxidase, as well as the catalytic effect of myrosinase onto glucose, was negative.

It was then obvious that the source of the error is originated by the enantioselectivity of glucose oxidase to the  $\beta$ -anomer of glucose.<sup>40</sup> Because of this behavior, the concentration of the free glucose in the samples was measured with a different sensitivity in each case, thus resulting in an incorrect result after the subtraction of the free glucose from the total glucose.

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Table 3. Determination and Recoveries Studies of Total Glucosinolates<sup>a</sup>

sample	sinigrin, mM			recovery, %	
	proposed method <sup>b</sup>	reference method <sup>c</sup>	relative error, %	glucose <sup>d</sup>	sinigrin <sup>d</sup>
Calibration with Glucose Standards					
broccoli	6.80	4.16	63.5	100	116
cauliflower	13.10	7.25	80.7	99	111
sinapis seeds	6.45	3.60	79.2	99	112
Calibration with Hydrolyzed Sinigrin Standards					
broccoli	6.90	4.16	65.9	107	108
cauliflower	13.00	7.25	79.3	109	111
sinapis seeds	5.95	3.60	65.3	107	110
Calibration with Glucose and Sinigrin Standards					
broccoli	2.40	4.16	−42.3	97	97
cauliflower	1.40	7.25	−80.7	97	101
sinapis seeds	3.05	3.60	−15.3	98	97
Standard (Mixed) Solutions Applying the Proposed Data Elaboration Protocol <sup>e,f</sup>					
glu/sin (0.1/0.159)	0.162		1.6		
glu/sin (0.1/0.319)	0.331		3.6		
glu/sin (0.2/0.159)	0.160		0.6		
glu/sin (0.2/0.319)	0.272		−14.7		
glu/sin (0.4/0.159)	0.166		4.4		
glu/sin (0.4/0.319)	0.352		10.3		
glu/sin (0.1/0.637)	0.649		1.8		
glu/sin (0.4/0.637)	0.634		−0.5		
glu/sin (0.2/0.382)	0.365		−4.7		
glu/sin (0.1/0.096)	0.101		5.6		
glu/sin (0.1/0.382)	0.401		4.9		
Real Samples Applying the Proposed Data Elaboration Protocol					
broccoli	3.87	4.16	−6.9	102	96
cauliflower	7.69	7.25	6.1	105	97
sinapis seeds	3.59	3.60	−0.3	99	102

<sup>a</sup> The standard deviation of the means ranges from 0.001 to 0.08 mM. <sup>b</sup> Average of three runs. <sup>c</sup> Modification of the Boehringer test kit method for glucose. <sup>d</sup> Addition of 0.100 mM standard solution in the measuring cell. <sup>e</sup> The concentrations of the glucose and sinigrin in parentheses are in mM. <sup>f</sup> The concentration of sinigrin as calculated after the standardization of the stock solution with the reference method.

Table 4. Analytical Performance of the System Using Different Calibrators

	calibrator			
	glucose	hydrolyzed sinigrin		sinigrin
valve position	A and B	A	B	B
linear range, mM	0.005–1.6	0.01–1.0	0.01–1.0	0.005–1.0
correlation coefficient, $r^2$	0.9992	0.9986	0.9985	0.9982
current (nA) = $f$ ([analyte (mM)])	$y = 0.02 + 25.1$	$y = 0.04 + 13.9$	$y = 0.08 + 14.6$	$y = 0.05 + 41.8$
LOD, mM <sup>a</sup>	0.004	0.009	0.009	0.002
RSD, % <sup>b</sup>	0.88	1.02	1.05	0.76

<sup>a</sup> For signal-to-noise ratio of 3. <sup>b</sup>  $n = 10$ , 0.2mM analyte.

A possible way to overcome this problem could be the transformation of the  $\alpha$ -glucose to the  $\beta$ -glucose by using the enzyme mutarotase (aldose 1-epimerase; EC 5.1.3.3) either in soluble or in immobilized form. Mutarotase has been extensively used in many commercial kits for the determination of glucose in routine analysis.<sup>41</sup> However, its incorporation in the proposed analyzer was not tried in order to avoid a multienzyme structure and complicate the analytical system.

Thus, we attempted to develop a data elaboration protocol that is able to amend the results calculated from the subtraction of the concentration of the free glucose from the concentration of the total glucose, taking into account the different sensitivities of the glucose electrode (calibration with glucose standards) and

the glucosinolate analyzer (sinigrin standards) for measuring the same concentration of pure and enzymatically produced glucose, respectively. A number of protocols were evaluated in a series of standard mixtures of glucose and sinigrin at different ratios. Best results (Table 3) were received by applying the following equation:

$$[\text{sinigrin}] = C_{\text{tg}} - C_{\text{fg}}(S_{\text{g}}/S_{\text{s}})$$

where  $C_{\text{tg}}$  is the total concentration of glucose by using sinigrin standards;  $C_{\text{fg}}$ , the concentration of free glucose;  $S_{\text{g}}$ , the slope of the calibration curve of the glucose electrode by using glucose standards; and  $S_{\text{s}}$ , the slope of the calibration curve of the glucosinolate analyzer by using sinigrin standards. The ratio  $S_{\text{g}}/S_{\text{s}}$  was experimentally calculated to be  $0.62 \pm 3$  ( $n = 10$ ), close to the theoretical value of 0.64 for the specified temperature.

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**Potential Simultaneous Assay of Glucose and Glucosinolates.** The design of the analyzer in a way to provide simultaneous readings for both glucose and glucosinolate concentrations with a single sample injection in a single measuring run was also attempted. To achieve this, we had to manage two parameters: (i) the partition of the sample volume into two equal parts, and (ii) the insertion of a proper delay to one of the substreams in order to manage the successive appearance of the two peaks.

The main stream was divided into two substreams with the use of a three-way valve. The use of the blank reactor is essential in order to obtain the same resistance for both divided streams, to achieve the partition of the sample volume in two equal parts. The effectiveness of this approach was tested by collecting the waste of each substream for a period of 15 min. A delay of 500 s was introduced by inserting a coil of 120 cm ( $\varnothing$  0.8 mm) after the myrosinase reactor (Figure 1).

With this apparatus, we managed the simultaneous determination of glucose and sinigrin with a single injection in a single measuring run. Although the proposed method offers the advantage of the direct determination of two analytes, it suffers in terms of time of analysis, since the simultaneous presence of both reactors within the flow and the insertion of the retarded coil make

it time-consuming. The total time required for the appearance of the two peaks is  $\sim$ 10 min. Moreover, the sensitivity of the system is lower.

## CONCLUSIONS

The proposed method bears all the advantages of biosensors and, moreover, is applicable to real matrixes. The use of the proposed data elaboration protocol seems to overcome some problems raised when the direct subtraction of the free glucose from the total glucose was used. An additional feature worthy of being mentioned is the compatibility of the applied technology (packed-bed bio-reactors and multimembrane architecture) with the most popular biosensor-based commercial analyzers. Further optimization of the data elaboration protocol by incorporating more parameters that may affect the accuracy of the final results, as well as the development of a software able to elaborate the experimental results automatically in the "simultaneous readings" configuration of the analyzer, is under investigation.

Received for review October 10, 2002. Accepted December 11, 2002.

AC026224C