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Micellar electrokinetic capillary chromatography—Synchronous monitoring of substrate and products in the myrosinase catalysed hydrolysis of glucosinolates

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Abstract

A micellar electrokinetic capillary chromatography (MECC) method has been developed for monitoring the myrosinase catalysed hydrolysis of 2-hydroxy substituted glucosinolates and the simultaneous formation of the corresponding degradation products (oxazolidine-2-thiones (OZTs) and nitriles). Glucosibarin ((2*R*)-2-hydroxy-2-phenylethylglucosinolate) was chosen as the model glucosinolate owing to the difficulties in determining hydrolysis rates of this type of substrates in traditional UV-assays. The method was afterwards validated with glucobarbarin ((2*S*)-2-hydroxy-2-phenylethylglucosinolate) and progoitrin ((2*R*)-2-hydroxybut-3-enylglucosinolate). Aromatic glucosinolates without a 2-hydroxy group in their side chains, such as glucotropaeolin (benzylglucosinolate) and gluconasturtiin (phenethylglucosinolate) were also tested. Formation of the glucosinolate hydrolysis products was monitored simultaneously at 206 nm and 230 nm. This allowed estimation of the extinction coefficient of the OZT derived from glucosibarin, which was found to be 18,000 M^{-1} cm^{-1} and 12,000 M^{-1} cm^{-1} at 206 nm and 230 nm, respectively. The developed method has limit of detection of 0.04 mM and 0.06 mM and limit of quantification of 0.2 mM and 0.3 mM for the glucosibarin derived OZT and nitrile, respectively. Linearity of the glucosinolate concentration was examined at six concentration levels from 2.5 mM to 100 mM and at 206 nm a straight line ($R^2 = 0.9996$) was obtained. The number of theoretical plates (N) at the optimal system conditions was 245,000 for the intact glucosibarin, 264,000 for the OZT and 252,000 for the nitrile.

Keywords: Glucosinolates; Myrosinase; Nitriles; OZTs; MECC; Synchronous method

1. Introduction

Glucosinolates are alkyl-*N*-hydroximine esters with a β-D-thioglucopyranoside group attached to the hydroximine carbon in *Z* configuration to the sulphate group [1–3]. These allelochemicals are present in all plants of the order Capparales [4] and have as well been reported in some few other species [1,5]. Myrosinase isoenzymes (EC 3.2.1.147) co-exist with glucosinolates and catalyze the hydrolysis of the β-D-thioglucopyranoside bond releasing an aglucone that further rearranges to a variety of products depending on the parent glucosinolate and the environmental conditions [6–10]. At neutral pH, aliphatic glucosinolates generally yield isothiocyanates, while at acidic pH or in the presence of ferrous ions the formation of nitriles is favoured (Fig. 1) [11–16].

Glucosinolate-containing plants have traditionally attracted much attention due to the physiological effects of the various glucosinolate degradation compounds. Oxazolidine-2-thiones (OZTs), especially goitrin from progoitrin (Fig. 2), have appreciable antinutritional or negative effects on the quality of food and feed used for monogastric animals, whereas other OZTs do not have so strong physiological effects [17,18]. Other glucosinolate and isothiocyanate derived products, especially sulphoraphane from glucoraphanin and its dithiocarbamates, and the degradation compounds of the indol-3-ylmethylglucosinolates have been reported to have antioxidant and anticarcinogenic properties [6,19–21]. Isothiocyanates have also been found to be fungicidal [22,23], nematocidal [24] and bactericidal [25]. The great variety of physiological effects of the different glucosinolate derived compounds calls for further attention, especially with respect to the factors controlling glucosinolate hydrolysis and the conditions under which the different transformation products are produced. The development of analytical methods able to

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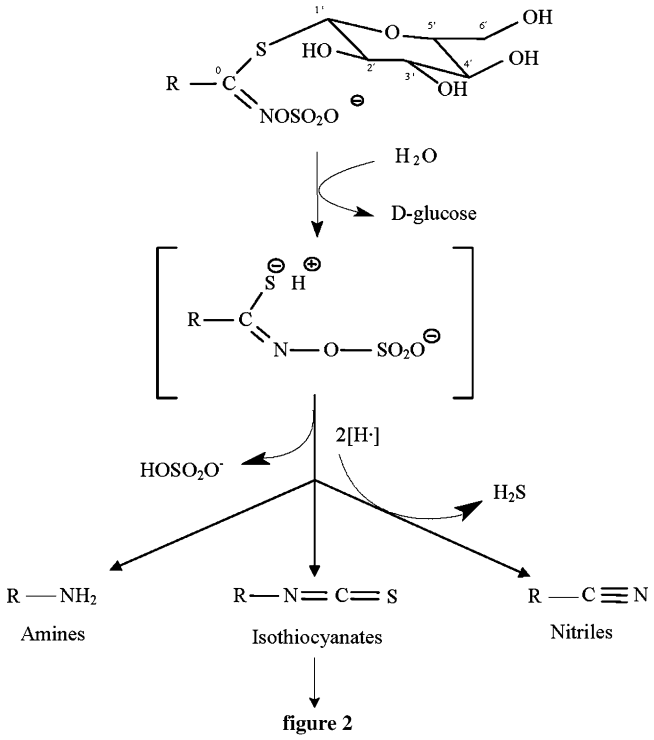

Fig. 1. Glucosinolate degradation by myrosinase and products obtained from aliphatic glucosinolates [6].

determine the compounds produced under different conditions and capable of studying the role that the type of myrosinase, cofactors and additional proteins play in the hydrolysis of glucosinolates is therefore of great importance in this respect.

Numerous methods have been developed for the measurement of the activity of myrosinase. Indirect methods involve the measurement of the released products, such as glucose [26] isothiocyanates and nitriles [1,2,14]. GC, LC and HPLC techniques for determination of glucosinolates and glucosinolate degradation products require in most cases more or less time consuming sample preparation steps prior to the chromatographic separation [3,5,14,27–29]. Micellar electrokinetic capillary chromatography (MECC) has also been developed as a method of analysis for determination of glucosinolates, desulphoglucosinolates [30,31] and some of the glucosinolate degradation products, such as OZTs and nitriles [32–34]. There is however, no GC, HPLC or HPCE method described up to date for the simultaneous determination of glucosinolates and their degradation products. Direct methods have been used both for determination of myrosinase activity and kinetic parameters, such as the determination of liberated acid, which is titrated with alkali using pH-stat apparatus (pHSA) [35], direct spectrophotometric assay (DSA) of glucosinolate degradation [12,36], the spectrophotometric coupled enzyme assay (SCEA) of liberated glucose [37,38] and the polarographic coupled assay (PCA) [39]. The pHSA, the DSA and the SCEA are used in routine analysis of myrosinase activity of *Brassica* extracts due to their being simple, fast and inexpensive. However, despite their accuracy in the quantitative determination of the amount of glucosinolate degraded in assays based on only one glucosinolate, they are neither able to determine differences in the speed of degradation of glucosinolate mixtures nor the lack of degradation of thioglucoside substituted glucosinolates [3,5]. Furthermore, these methods do not allow determining the types of products formed and in which proportions they are produced.

In recent years, capillary electrophoresis has been increasingly used for monitoring of enzymatic reactions [40–42], both for determination of the total amount of reaction products [43] as well as for the determination of the enzyme kinetic parameters and the efficiency of the reaction [44,45]. In the present study, we have developed a method for the simultaneous monitoring of the hydrolysis of glucosinolates by myrosinase and the formation of the degradation compounds. The method is based on the separation of glucosinolates in crude extracts by MECC [34]. Glucosibarin ((2*R*)-2-hydroxy-2-phenylethylglucosinolate) was chosen as the model glucosinolate and the method was afterwards validated with other glucosinolates, such as glucobarbarin ((2*S*)-2-hydroxy-2-phenylethylglucosinolate), progoitrin ((2*R*)-2-hydroxybut-3-enylglucosinolate), glucotropaeolin (benzylglucosinolate) and gluconasturtiin (phenethylglucosinolate). Formation of the corresponding degradation products was mon-

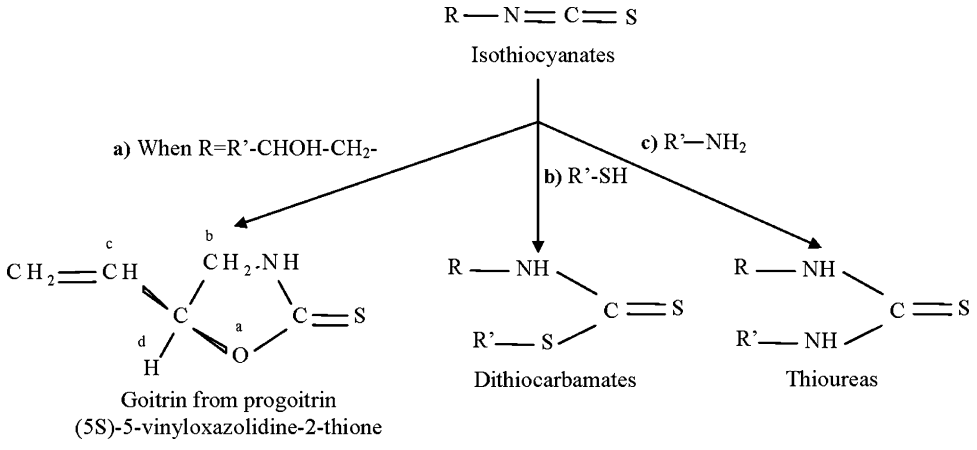

Fig. 2. Products formed from isothiocyanates: (a) the isothiocyanates derived from glucosinolates with a 2-OH group cyclise spontaneously to form the corresponding OZT; (b) dithiocarbamates are formed by the reaction of isothiocyanates with thiol groups; (c) thioureas are formed by the reaction of isothiocyanates with free amino groups.

itored at a neutral pH and this allowed estimating the response factor of the OZT derived from glucosibarin on the basis of the parent glucosinolate. The further implications of the use of this method for the study of the enzyme kinetics as well as the myrosinase–glucosinolate system in general are also discussed.

2. Materials and methods

2.1. Chemicals and supplies

Sodium cholate, taurin and trigonellinamide (TNA) were purchased from Sigma–Aldrich (Steinheim, Germany). Disodium hydrogenphosphate was purchased from Riedel-de Häen (Seelze, Germany) and ascorbic acid from Bie & Berntsen (Rødovre, Denmark). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). The intact glucosinolates and myrosinase isoenzymes (*B. carinata* cv. BRK-147-A) were from the laboratory collection [5,34,46–48].

2.2. CE instrumentation

Analyses were performed using a Hewlett-Packard HP^{3D} CE capillary electrophoresis system (Agilent, Waldbronn, Germany) equipped with diode array detector. All separations were performed on fused silica capillaries (Agilent, USA), 50 μm i.d. × 64.5 cm total length (56 cm effective length). Samples were injected from the anodic end of the capillary (vacuum injection 40 mbar, 1 s) and detection was performed on-column at 230 nm and 206 nm. The electrophoreses were run at 30 °C for 15 min and the voltage applied was 17 kV. Between each run the capillary was rinsed with 1.0 M NaOH (2 min), water (2 min) and run buffer (5 min). Data processing was carried out by use of a HP Vectra 5/100 mHz Pentium with HP ChemStation V. 6.01.

2.3. Buffer

The run buffer was: 35 mM Sodium cholate, 100 mM disodium hydrogen phosphate, 50 mM taurine, 2% 1-propanol. The pH was kept at 8.2. The buffer was filtered through a 0.20 μm membrane filter before use.

2.4. Procedure for the in-vial enzyme reaction

The reaction media was adapted from the standard reaction assay for myrosinase activity used in our laboratory [47]. A stock solution of the glucosinolate (364 μL 0.05 M), buffer (63 μL, disodium hydrogen phosphate 0.1 M pH 6.5), purified water (119 μL) and TNA as an internal standard (364 μL, 0.1 M) was prepared. Aliquots (91 μL) of the stock solution described above, ascorbic acid (4 μL, 0.01 M) and myrosinase (5 μL of a 2.6–3 U/mL solution = 0.013–0.015 U in vial) were run three times. Ascorbic acid and myrosinase were added 1 min before injection and the reaction was allowed to run for 325 min (a total of 15 injections).

2.5. Linearity

The quantification linearity of glucosibarin as a model glucosinolate and TNA as the internal standard was examined at six concentration levels in the range from 2.5 mM to 100 mM.

3. Results and discussion

3.1. Use of an internal standard—the choice of TNA

An internal standard (TNA) was included in the experiments in order to be able to determine the relative changes in the concentrations of the glucosinolate and the degradation product/s as well as to be able to control the variations in the concentration of the compounds due to changes in the injected volume. TNA was considered as the optimal internal standard as it appears as a sharp peak before the electroosmotic flow (EOF), therefore far from the area where glucosinolates and degradation products are expected. Furthermore, and according to test results, it does not react with glucosinolates or their degradation products and it is neither degraded at the conditions used nor hydrolysed by myrosinase.

3.2. Validation of the method—linearity, LOD, LOQ and number of theoretical plates

The tested concentration-normalized area (NA) relation for glucosibarin and TNA was shown to be linear (Table 1). When different concentrations of glucosibarin were combined with a fixed concentration of TNA (100 mM), a linear relationship between the concentration of glucosibarin and its relative normalized area (NA (glucosibarin)/NA (TNA)) was also obtained (Table 1). The NA of an analyte is obtained by dividing the integrated peak area of the analyte by its migration time. The use of NA is required for on-column detection as it allows correcting for the speed of the analytes through the detection window.

At the optimal system conditions, the developed method was found to have limit of detection (LOD), limit of quantification (LOQ) and number of theoretical plates (N) for glucosibarin and its corresponding OZT and nitrile as presented in Table 2.

Table 1
Linear relation between concentration and NA for glucosibarin (e-18), TNA and e-18 in relation to 100 mM TNA at 206 nm and 230 nm (in parenthesis)

Parameter	Compound		
	e-18	TNA	e-18/TNA
Equation	$y = 2090x + 0.06$ ($y = 1070x + 0.33$)	$y = 2190x + 5.09$ ($y = 718x + 1.47$)	$y = 41.9x - 0.06$ ($y = 23.9x - 0.01$)
R^2	0.999 (0.999)	0.995 (0.996)	0.998 (0.999)

Table 2
LOD, LOQ (concentrations in mM and amounts in pmol) together with theoretical plates of the method for glucosibarin (e-18) and its corresponding OZT and nitrile at the optimal system conditions

Parameter	Compound		
	e-18	OZT	nitrile
LOD (mM/pmol)	0.1/0.2	0.04/0.08	0.06/0.12
LOQ (mM/pmol)	0.5/1	0.2/0.4	0.3/0.6
Theoretical plates (*N*)	245000	264000	252000

All values are from the runs with detection at 206 nm, except the theoretical plates for e-18 which is based on detection at 230 nm.

3.3. Synchronous on-line measurement of the degradation of the glucosinolates (progoitrin and glucosibarin) and the formation of the products—oxazolidine-2-thiones and nitriles

Glucosibarin belongs to the group of glucosinolates bearing a 2-hydroxy group; therefore upon myrosinase catalyzed hydrolysis the major degradation product is an isothiocyanate that rearranges to form an OZT (Fig. 2). At room temperature, water and alcohol groups do not react with isothiocyanates to an appreciable extent (vide infra); however, due to the stereochemistry of the isothiocyanate molecule formed in the myrosinase catalysed hydrolysis of 2-hydroxy substituted glucosinolates, the alcohol group is placed at the right position to give a fast reaction with the highly electrophilic C atom in the isothiocyanate group, hence forming the OZT [3]. At the pH used and with this particular type of myrosinase, a nitrile was also formed, which was clearly observed at 206 nm due to the high extinction coefficient of the phenyl ring (Fig. 3).

The present MECC method allowed for the synchronous monitoring of the degradation of glucosibarin and the simultaneous formation of the correspondent OZT and nitrile with no other compound detected at the used wavelengths (Fig. 3). When progoitrin was used as the initial glucosinolate, the corresponding OZT (goitrin ((5*S*)-5-vinyloxazolidine-2-thione) was also detected by the present method (Fig. 4). No nitrile was detected as a product from the myrosinase catalysed hydrolysis of this glucosinolate.

The spectrophotometric measurement of the degradation of 2-hydroxy substituted glucosinolates at a fixed wavelength is normally troublesome, as both the glucosinolate and the OZT contribute to UV absorption at 230 nm, which is the wavelength used for the DSA, pHSA and SCEA methods [36]. The present MECC method allows for the simultaneous monitoring of product and substrate, which appears as a main advantage with respect to the traditional spectrophotometric assay, since overlapping of maximum wavelengths of substrate and product is then avoided. The ability of the HPCE system to simultaneously register different wavelengths as well as record the full UV-spectra of the peaks in the electropherogram allows detecting the presence of artefacts and/or other products than the ones expected from the reaction, which is also an advantage with respect to the traditional spectrophotometric assay.

Despite the reaction was conducted at neutral pH and there were no Fe(II) ions added to the sample, the hydrolysis of glucosibarin by myrosinase also produced the corresponding nitrile, which could easily be observed due to the presence of the phenyl group (Fig. 3). Nitriles were also observed when the two aromatic glucosinolates, glucotropaeolin and gluconasturtiin, were used as substrates (data not shown). Upon myrosinase catalysed hydrolysis at neutral pH, glucotropaeolin and gluconasturtiin produce mainly isothiocyanates (Fig. 1). Water and alcohol groups have no significant reactivity against isothiocyanates at room temperature [34], hence, as expected, no isothiocyanate–alcohol reaction product was observed. The thiol and free amino groups in the myrosinase isoenzymes were only

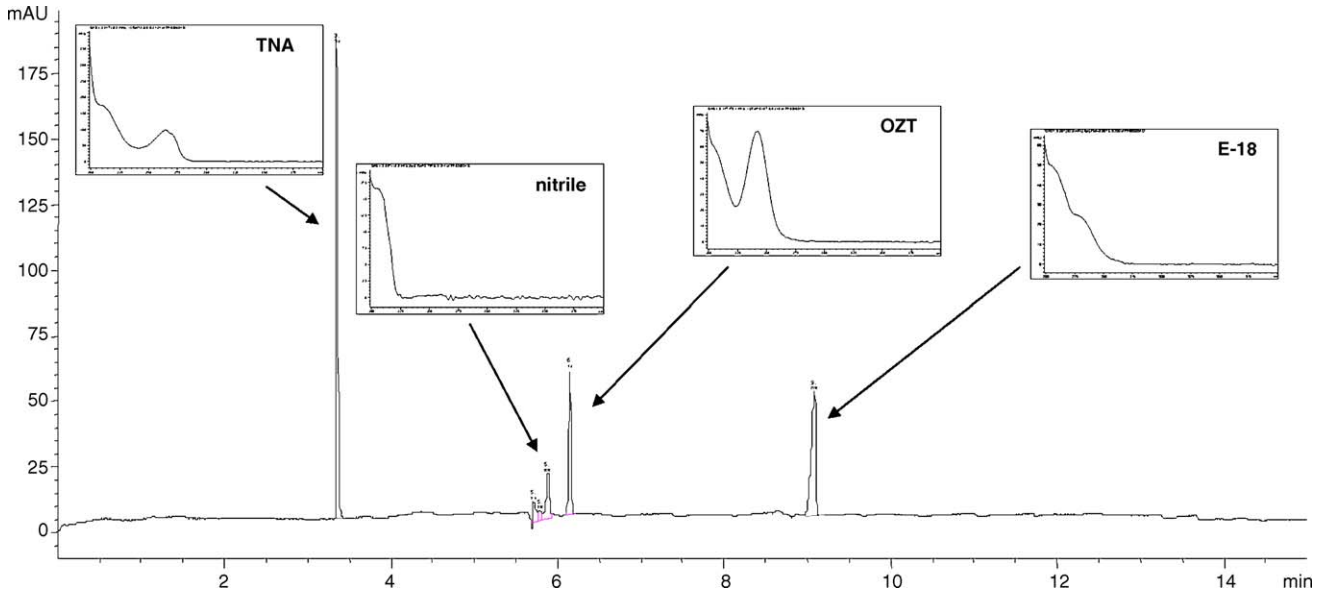

Fig. 3. Electropherogram showing the simultaneous appearance of glucosibarin (e-18) and its degradation products at 206 nm. The spectrum of the different compounds is also shown.

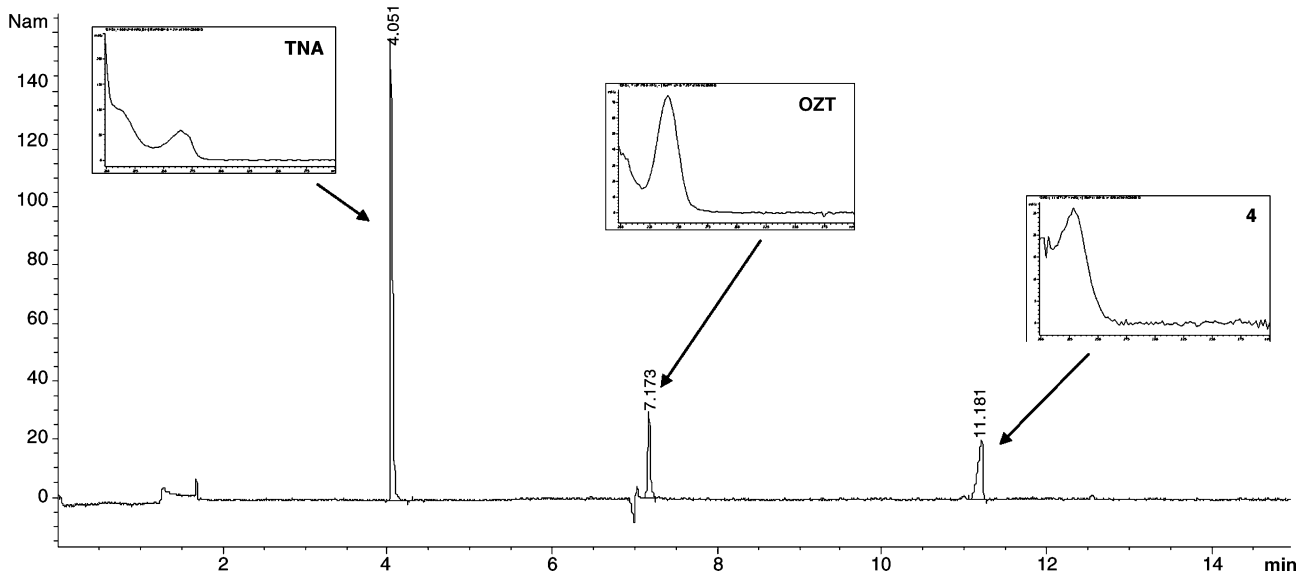

Fig. 4. Electropherogram showing the simultaneous appearance of progoitrin (4) and its degradation products at 206 nm. The spectrum of the different compounds is also shown.

present in trace amounts in the reaction mixture and therefore no dithiocarbamates and thioureas were detected. The present MECC method was not optimised with respect to the direct detection of the isothiocyanates produced from these glucosinolates; however, it was still capable of determining the disappearance of the substrates and the formation of the nitriles. The use of initial low concentrations of these glucosinolates allowed for the monitoring of the reaction, despite the baseline of the electropherogram was slightly disturbed due to the interaction of the isothiocyanates with the micelles. In-vial derivatization with amines or thiol containing compounds such as GSH, results in the production of the corresponding hydrophilic thioureas or dithiocarbamates (Fig. 2) [49], which were also found to be detectable by the developed procedure (data not shown).

The low wavelength (206 nm) used in the present method allows detecting compounds with phenyl groups, S atoms and double bonds in their molecules, although some specific compounds with low extinction coefficients may require the use of Z-shaped or bubble flow cells [48]. Hence, the present method can potentially be used to detect the products of hydrolysis from most of the glucosinolates expected in *Brassica* species [34].

3.4. Degradation of glucosibarin and formation of the products as function of time—calculation of extinction coefficients (ε-value)

The simultaneous appearance of glucosibarin and its corresponding degradation products was determined in the same electropherogram by detection at 206 nm and 230 nm (Fig. 3). This allowed calculating the extinction coefficient for OZT at both wavelengths. The calculation was based on an extinction coefficient value for sinigrin of 8000 M^{-1} cm^{-1} at 227 nm and a relative response factor of glucosibarin to sinigrin of 0.95 at 230 nm [50], which gave extinction coefficient values for glucosibarin of 8400 M^{-1} cm^{-1} (230 nm) and 16,400 M^{-1} cm^{-1} (206 nm). The choice of the extinction coefficient for sinigrin (8000 M^{-1} cm^{-1} at 227 nm) was based on previous literature values [31,36,51]. The relation between the normalised areas of glucosibarin (Table 1) and OZT at 230 nm and 206 nm allowed for the calculation of the extinction coefficients of the OZT at both wavelengths, which resulted in values of 12,000 M^{-1} cm^{-1} (230 nm) and 18,000 M^{-1} cm^{-1} (206 nm). The extinction coefficient for the glucosibarin derived OZT has been previously determined to be 19,000 M^{-1} cm^{-1} at 245 nm in 96% ethanol [52]. According to the spectrum (Fig. 3), the extinction coefficient of OZT at 245 nm was estimated to be 19,600 M^{-1} cm^{-1}, although the maximum value (20,600 M^{-1} cm^{-1}) was observed at 242 nm. These values fit well with the literature, despite the low volume of sample used and the fact that the OZT was present in a mixture with the parent glucosinolate and the nitrile. The use of the present method may therefore result in an advantage with respect to traditional spectroscopic methods,

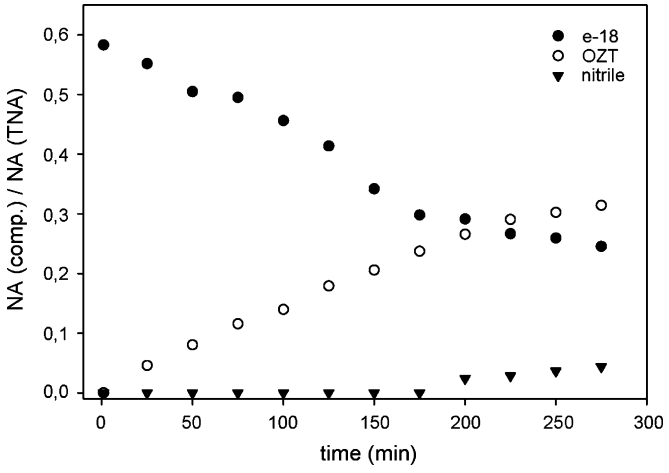

Fig. 5. Degradation of glucosibarin and formation of the two products at pH 6.5.

which are limited in their estimations by the purity of the compound and the interference from the solvent.

At neutral pH, OZT was formed from the beginning of the hydrolysis of the parent glucosibarin, whereas the corresponding nitrile started to appear later in the reaction (Fig. 5). The present method allows determining the pattern and timing of the production of the degradation compounds, since it allows following the reaction over a period of time at different wavelengths.

4. Conclusion

The present MECC method has proven to have a series of advantages with respect to the traditional spectrophotometric myrosinase assays. It is based on the use of small volumes of glucosinolates and myrosinases therefore it is a cheap method that does not need high quantities of reactants. The reaction could be followed over a period of time of over 24 h (data not shown) and therefore it can also be used in studies with myrosinase isoenzymes of very low activity. The present method is an automated process that makes it easy to use and does not need constant operation as it is based on subsequent injections from the same sample vial. The system worked at different pHs from 3 to 8 (data not shown) and the micelles proved to be stable with the different buffers used in the samples. Despite the myrosinase was also injected with the rest of the sample, the same capillary was used over a high number of runs, which proved its resistance to being worn out by the constant injection of the protein.

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