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Optimization of glucosinolate separation by micellar electrokinetic capillary chromatography using a Doehlert's experimental design

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Abstract

The aim of this study was to optimize by micellar electrokinetic chromatography the separation of four glucosinolates, i.e. sinigrin, glucobrassicin and methoxyglucobrassicin involved in Cruciferae resistance mechanisms and glucotropaeolin used as an internal standard. The separation borate buffer which contained sodium dodecyl sulphate, tetramethylammonium hydroxide and methanol was firstly optimized by using a three variable Doehlert experimental design. The optimum concentrations found enabled, for the first time, to obtain an acceptable resolution between the two indole glucosinolates, glucobrassicin and methoxyglucobrassicin. Modifications of the method such as a capillary pre-rinse with pure borate buffer and a step change in voltage during experiment were performed to improve the resolutions between glucosinolates and to reduce the analysis time. This method was validated by a statistical analysis and showed good linearity, repeatability and reproducibility. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Doehlert's experimental design; Mobile phase composition; Glucosinolates; Sinigrin; Glucobrassicin; Methoxyglucobrassicin; Glucotropaeolin

1. Introduction

Glucosinolates are secondary sulfur-containing metabolites found in every organ of cruciferous plants (cauliflower, rape, radish, Brussels sprouts, . . .). All have the common structure presented in Table 1 and can be divided into three classes (aliphatic, aromatic or indole compounds) according to the chemical nature of the side-chain, R group.

Glucosinolates and their breakdown products have various physiological effects; they are suspected of participating in the pests- and diseases-resistance mechanisms. Among aliphatic glucosinolates, sinigrin and/or its major breakdown product are known as powerful antifungal compounds [1,2]. Moreover, several papers have dealt with the effects of either indole glucosinolates, i.e. glucobrassicin and methoxyglucobrassicin, or their derived products known to accumulate further to wounding or pathogen attack [3,4].

In a previous paper, we showed that sinigrin, glucobrassicin and methoxyglucobrassicin contents depend on cauliflower varieties according to their

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Table 1
Structures of the four investigated glucosinolates

General glucosinolate structure	
R—C(=N—O—SO_3^-)—S—D- glucose	
Structure of R group	
CH_2=CH—CH_2—	C_6H_5—CH_2—
Sinigrin	Glucotropaeolin
(indol-3-yl)—CH_2—	(4-OMe-indol-3-yl)—CH_2—
Glucobrassicin	Methoxyglucobrassicin

susceptibility or resistance to infection by the downy mildew agent, *Peronospora parasitica* [5]. We also highlighted that glucobrassicin and methoxyglucobrassicin could be considered as resistance markers to this disease. These results have shown the requirement of a fast and reliable method to quantify both sinigrin and the two indole glucosinolates.

Glucosinolates in Cruciferae samples are usually extracted and purified according to the reference method of the European Community [6] prior to quantification by high-performance liquid chromatography (HPLC). Unfortunately, this method has a major drawback: it requires a long glucosinolate desulfatation step. In order to save an appreciable amount of time, Michaelsen et al. [7] and Feldl et al. [8] have developed capillary electrophoresis-based separation and quantification methods to analyze intact glucosinolates (without a desulfatation step). Even though these techniques have proved to be efficient in the separation of the main glucosinolates, they have led to an incomplete separation of glucobrassicin and methoxyglucobrassicin.

This present paper reports on a new method for the separation of the four following glucosinolates, i.e. sinigrin (aliphatic compound), glucobrassicin and methoxyglucobrassicin (indole compounds) all involved in cauliflower resistance to *Peronospora parasitica* and glucotropaeolin (aromatic compound) usually employed as internal standard (Table 1). This separation was achieved by using a micellar electrokinetic capillary chromatography (MECC) technique which had the peculiarity of operating with sodium dodecyl sulphate (SDS) in association with an ion-pair reagent [tetramethylammonium hydroxide (TMAH)].

The first step was to determine the concentrations of the three constituents of the separation buffer, i.e. SDS, TMAH and methanol (MeOH) allowing the best separation of the four glucosinolates. The classical approach consists in varying each experimental parameter independently of the others; however, this method has several drawbacks such as possible interactions between solutes which can not be taken into account, an uncertain identification of optimum conditions due to a lack of information on the behavior of solutes. So, the application of a factorial design such as a Doehlert matrix was well-suited to our study to optimize with a reduced number of experiments the separation of the four glucosinolates. Unusual elution and step change in voltage were then tested to improve the method. The results obtained were finally subjected to a statistical analysis to assess the method linearity, repeatability and reproducibility.

2. Experimental

2.1. Reagents and reference substances

TMAH and SDS were purchased from Sigma (St. Louis, MO, USA); anhydrous sodium tetraborate was obtained from Merck (Darmstadt, Germany). MeOH was of high-performance liquid chromatography (HPLC) grade and was purchased from Carlo Erba (Val de Reuil, France). Water was obtained from a Millipore MilliQ system (Saint-Quentin, France).

Glucobrassicin, methoxyglucobrassicin and glucotropaeolin were isolated and purified from natural sources [9]. Sinigrin was purchased from Sigma.

2.2. Instrumentation

Experiments were performed using a Beckman P/ACE 5500 capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) connected to a P/ACE Diode Array detector module (Beckman). A 57 cm×75 μm I.D. fused-silica capillary tube (Supelco, Bellefonte, PA, USA) was used at a temperature of 40°C. Detection was carried out at 224 nm. Data were processed by System Gold software.

2.3. Procedure

2.3.1. Optimization of separation buffer composition

The separation buffer was composed of SDS, TMAH, MeOH and sodium tetraborate. The concentrations of the three components (SDS, TMAH and MeOH) investigated in the Doehlert experimental design were described in Section 2.4. The sodium tetraborate concentration was fixed at 24 m*M* further to preliminary experiments. The pH of the mixture was adjusted to 10.

The various glucosinolates were dissolved in water to get the following concentrations: sinigrin: 1.9 μmol/ml; glucotropaeolin: 1.7 μmol/ml; glucobrassicin: 0.5 μmol/ml and methoxyglucobrassicin: 0.3 μmol/ml. Solutions were filtered through a 0.45 μm porosity size membrane prior to use.

Before each analysis the capillary was first washed with 1.0 *M* NaOH for 2 min, then with water for 1 min, and lastly with separation buffer for 2 min. The samples were introduced into the positive end of the capillary under pressure for 5 s. The separation was performed at a constant voltage of 15 kV.

2.3.2. Method improvement

The method applied was then modified as follows: the separation buffer used in the pre-rinse step before injection was replaced by sodium tetraborate (95 m*M*). A step change in voltage which consisted of a first 6 min sequence at 15 kV followed by a 21 min sequence at 20 kV was applied during the separation process. The buffer-component concentrations used were the optimum ones obtained further to the optimization of the separation buffer.

2.4. Experimental design

The 'Doehlert uniform shell design' [10] used in this study consists of a set of points uniformly distributed in a cubo-octahedron. The whole experimental domain is explored through a minimum number of experiments which depends on the number of factors studied: indeed, k factors will require a minimum of k^2+k+1 experiments. In the present study, the three-factor design used (SDS, TMAH and MeOH) required at least 13 different experiments (Table 2). Each of them was repeated twice or three times to perform a statistical analysis.

The ranges of the three concentrations (SDS, TMAH and MeOH) under optimization were selected as follows: (i) SDS concentrations ranged between 100 and 200 mM; the lower limit ensured a minimum separation of glucosinolates while the upper one prevented a too high ionic strength, and consequently avoided a too high current during separation process. (ii) TMAH concentrations ranged between 10 and 50 m*M*. (iii) MeOH percentage in separation buffer varied from 5% to 25% to respectively expand the migration time-window and avoid disrupting micellar structure.

The analytical data obtained from the 13 experiments were processed with the specific multi-function software NEMROD® [11] using a second-degree mathematical model. The coefficients of each polynomial function representing the relationship between two glucosinolates were calculated. As we

Table 2
Buffer-component concentrations used according to the Doehlert experimental design

Experiment	TMAH (m*M*)	SDS (m*M*)	MeOH (%)
1	50	150	15
2	10	150	15
3	40	200	15
4	20	100	15
5	40	100	15
6	20	200	15
7	40	167	25
8	20	133	5
9	40	133	5
10	30	183	5
11	20	167	25
12	30	117	25
13	30	150	15

investigated four different glucosinolates (six 2-by-2 couples), we obtained six different models whose accuracy was statistically tested by the software. The response surfaces showing the resolution between two glucosinolates versus buffer-component concentrations were drawn by the software. A specified function of the software, named 'Desirability', based on the search for a global optimum of the variables tested [12] was also used; it gave a unique quantitative measure representing the global quality of the compromise.

2.5. Resolution calculations

The efficiency of the MECC separation between two glucosinolates was analyzed in terms of resolution (Rs) between their peaks with:

$$Rs = 2(MT_2 - MT_1)/(W_2 + W_1)$$

where MT_2 and MT_1 are the migration times measured at the maximum peak height, W_1 and W_2 are the peak widths measured at the peak base.

2.6. Statistical validation

Statistical data were validated in compliance with the criteria from Caporal-Gautier et al. [13].

In order to assess the linearity of the method, measurements were carried out for five different concentrations of sinigrin, glucobrassicin and methoxyglucobrassicin; each measurement was made in triplicate. Statistical tests were then performed on the whole set of data at a significance level of 1%.

In order to test the repeatability and reproducibility of relative migration times and of relative areas six consecutive experiments with the glucosinolate concentrations previously described in Section 2.3.1. were performed and repeated at three different days.

The relative migration time (RMT) is defined as follows: $RMT = MT_1/MT_2$ where MT_1 is the migration time of a given glucosinolate and MT_2 is the migration time of the internal standard (glucotropaeolin in our experiments) whose RMT is equal to one.

The relative area (RA) is defined as follows: $RA = A_1/A_2$ where A_1 is the area of the peak of a given glucosinolate and A_2 is the area of glucotropaeolin peak whose RA is equal to one.

Relative standard deviations (RSD) were calculated to estimate repeatability and reproducibility for RMT and RA.

The limit of detection (LOD) and the limit of quantification (LOQ) are defined as the sample concentration producing a peak whose height is respectively three and ten times the baseline noise.

3. Results

3.1. Optimization of separation buffer composition

As previously mentioned in Section 2.4, 13 runs were performed with SDS–TMAH–MeOH buffers at various concentrations (Table 2) to apply the Doehlert factorial design. Three of the resulting electropherograms are presented in Fig. 1 where remarkable changes in the separation quality are quite noticeable. For instance, methoxyglucobrassicin is not separated from glucobrassicin in the run no. 5 (Fig. 1A) whereas it is fused with sinigrin in the run no. 6 (Fig. 1B). On the contrary, Fig. 1C (run no. 12) illustrates quite a good separation of the four glucosinolates.

The optimum concentrations of SDS, TMAH and MeOH were then determined in order to separate the four glucosinolates with the best resolution. The response surfaces calculated by NEMROD® showed that the glucobrassicin–methoxyglucobrassicin resolution (gb–megb Rs) was the highest when the glucotropaeolin–methoxyglucobrassicin resolution (gtl–megb Rs) was the lowest (unpublished data). Consequently, as the corresponding optimum concentrations were not in the same range, they were optimized by the software 'Desirability' function, which established a global optimum corresponding to 26.3 mM for TMAH concentration, 158.6 mM for SDS and 24.4% for MeOH. The resulting electropherogram (Fig. 2A) evidences the best separation of the four glucosinolates with Rs in the 1.41–4.47 range (Table 3a) validating thus the theoretical concentrations predicted by the software.

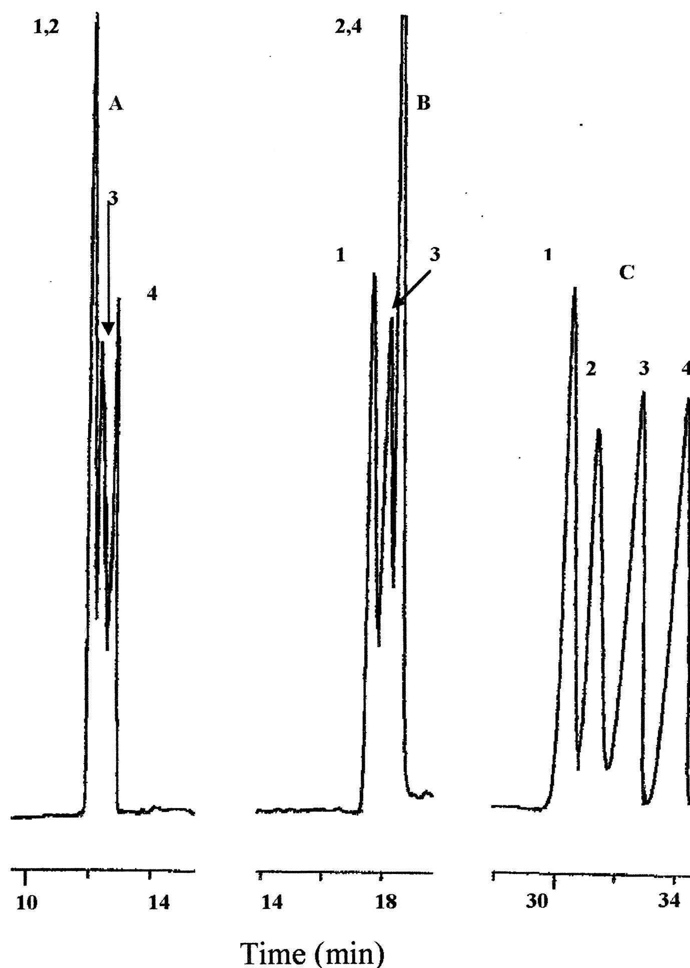

Fig. 1. Electropherograms of runs no. 5 (A), no. 6 (B) and no. 11 (C) obtained from the experiments noticed in Table 2. [(1) Glucobrassicin; (2) methoxyglucobrassicin; (3) glucotropaeolin; (4) sinigrin.]

3.2. Method improvement

The separation method was modified to both improve the resolution of glucosinolate separation and reduce the analysis time. The separation buffer first used in the pre-rinse step was replaced by a solution of pure borate (95 m*M*), which shortened the retention times by 5 min for sinigrin, and 7 min for indole glucosinolates (Fig. 2B); this change also improved the resolution of glucosinolate separation except for gb–megb *Rs* ($Rs = 1.12$), which, nevertheless, remained satisfactory (Table 3b). The capillary pre-rinse with borate instead of separation buffer modified the current intensity. When separation buffer was used in pre-rinse, a constant current of 120 μA was observed over the whole experiment whereas its replacement by borate induced change in current intensity. In the first 10 min of experiment this intensity decreased from 170 μA (characteristic of the borate concentration) to 120 μA and then remained stable (unpublished data). In order to get an optimum current close to 200 μA all over the experiment, a gradient voltage of 15 kV in the 6 first min enhanced to 20 kV in the next 21 min was applied. These voltage modifications associated to the pre-rinse step with a borate solution shortened

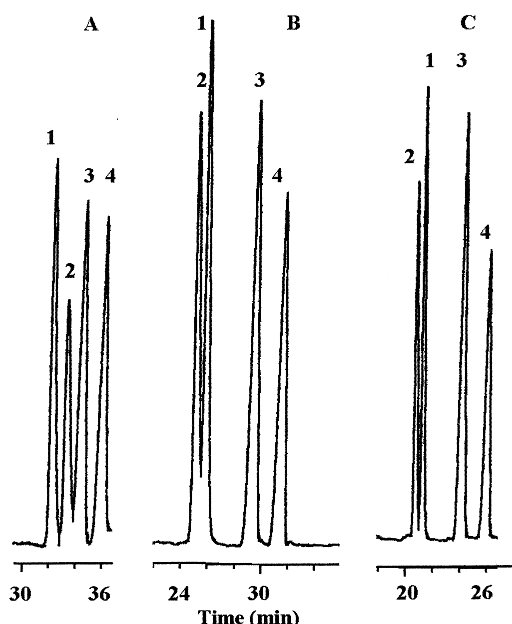

Fig. 2. Electropherograms obtained further to the optimization of the separation buffer composition; (A), changes in the pre-rinse solution composition; (B) and in the electric voltage (with the same pre-rinse solution as in B), (C) [(1) glucobrassicin; (2) methoxyglucobrassicin; (3) glucotropaeolin; (4) sinigrin].

the analysis time from 32 to 26 min (Fig. 2C) and improved resolution. For example, the gtl–megb *Rs* enhanced from 4.95 to 5.70 whereas the gb–megb *Rs* increased slightly from 1.12 to 1.20 (Table 3c).

3.3. Statistical validation

The concentrations used to test the linearity of the method ranged from 0.055 to 1.503 m*M*. The different statistical tests carried out both validated the linearity of the method and showed correlation coefficients higher than 0.998 (Table 4).

The relative standard deviations (RSD) for RMT and RA repeatability and reproducibility were calculated (Table 5). They indicate a good repeatability, below 0.3% for RMT and 1.3% for RA, and a satisfying reproducibility, below 0.5% for RMT and 2.2% for RA.

The limits of detection (LOD) and quantification (LOQ) were estimated (Table 4); they ranged between 4 and 27 nmol/ml and between 14 and 90 nmol/ml respectively.

4. Discussion

Even though cetyltrimethylammonium bromide (CTAB) has been commonly used as surfactant by Michaelsen et al. [7] and Feldl et al. [8] to quantify glucosinolates in MECC, it was not included in the separation buffer used in the present study. Indeed, preliminary experiments carried out with CTAB and various conditions of temperature, pH, 2-propanol or

Table 3
Resolutions (*Rs*) between glucosinolates obtained further to the optimization of the separation buffer composition (a), with borate used in pre-rinse (b) and step change in voltage (c) (sin: sinigrin; gtl: glucotropaeolin; gb: glucobrassicin and megb: methoxyglucobrassicin)

	Rs					
	sin–gtl	sin–gb	sin–megb	gtl–gb	gtl–megb	gb–megb
(a)	1.46	4.47	3.19	2.6	1.40	1.41
(b)	1.86	6.33	7.25	4.10	4.95	1.12
(c)	2.35	8.33	8.85	5.00	5.70	1.20

Table 4
Concentration range, correlation coefficients, limits of detection (LOD) and limits of quantification (LOQ) for the three glucosinolates tested

	Concentration range (m*M*)	Correlation coefficients (R^2)	LOD (nmol/ml)	LOQ (nmol/ml)
Sinigrin	0.376–1.503	0.999	27	90
Glucobrassicin	0.094–0.378	0.999	5	17
Methoxyglucobrassicin	0.055–0.220	0.998	4	14

Table 5
Relative standard deviation (RSD) on repeatability and reproducibility of the relative migration times (RMT) and relative areas (RA) for the three glucosinolates tested

		Sinigrin	Glucobrassicin	Methoxyglucobrassicin
RSD on	RMT (%)	0.2	0.3	0.3
repeatability	RA (%)	1.3	1.3	1.1
RSD on	RMT (%)	0.2	0.4	0.5
reproducibility	RA (%)	1.3	1.8	2.2

methanol did not result in a sufficient separation of glucobrassicin and methoxyglucobrassicin. On the contrary, the use of a separation buffer containing the SDS surfactant in association with an ion-pair reagent (TMAH) and an organic modifier (MeOH) enabled separation of the four glucosinolates with, for the first time, an acceptable resolution between glucobrassicin and methoxyglucobrassicin.

The addition of tetraalkylammonium salts to SDS solution to separate anionic substances was first described by Nishi et al. [14] who succeeded in separating two compounds with structural similarities. According to the study of Nishi, the chemical mechanism occurring inside the capillary is as follows: anionic analytes such as glucosinolates form paired-ions with the ammonium ion of TMAH reducing thus the electrostatic repulsion between the anionic SDS micelle and glucosinolates. In this study, the use of such reagents enables us to separate glucobrassicin and methoxyglucobrassicin which only differ in a methoxy group and thus have low different hydrophobicity.

MeOH was added to the separation buffer because it contributes to reduce the electro-osmotic velocity and expand the migration time window [15].

The use of a Doehlert experimental design presents several advantages. First, it reduces the number of runs required to find the optimum component concentrations because its specific properties consist in an easy displacement of the design into an unexplored portion of the experimental domain. Indeed, it provided us with the optimum concentrations of the separation buffer (SDS = 158.6 m*M*, TMAH = 26.3 m*M* and MeOH = 24.4%) although these values had not been selected as experimental concentrations in the 13 runs. Moreover, the addition of new variables like pH or temperature not scheduled at first in the experimental design is possible: only further experiments are necessary to complete the results already obtained from the experimental design.

The optimized method described in this paper included changes in voltage. Usually, operating at a constant voltage is advisable to avoid possible slight inter-day variations in electrolyte composition, which modify conductivity and may significantly alter the current intensity over the run [16]. The step change in voltage used in this present study did not cause such variability as indicated by the good reproducibility of retention times and areas. These unusual modifications in the composition of pre-rinse solution and/or step change in voltage could be thus applied to other methods in order to reduce analysis time and improve resolution.

The method reported here is thus of interest because it allows the quantification of glucobrassicin and methoxyglucobrassicin that are usually separated with great difficulty. In addition, as the four glucosinolates were separated in 26 min and no desulfatation step was required, the total procedure was quite fast. It is also a reliable method in terms of linearity, repeatability, reproducibility, LOD and LOQ as shown by its validation and can be then applied to the analysis of cauliflower extracts.

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