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ARTICLE

Semiquantitative Analysis of 3-Butenyl Isothiocyanate To Monitor an Off-flavor in Mustard Seeds and Glycosinolates Screening for Origin Identification

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The present paper describes the development of an analytical method for the semiquantitative analysis of 3-butenyl isothiocyanate in mustard seeds, this compound being linked to an undesirable (at least for the European palate) off-flavor. 3-Butenyl isothiocyanate is one of the enzymatic degradation products of gluconapin, a member of the glucosinolate family of compounds. A headspace−gas chromatography−mass spectrometry (HS-GC-MS) method has been developed for the rapid analysis of 3-butenyl isothiocyanate in mustard seeds. The cross-check of this HS-GC-MS method has been made on the basis of the analysis of the native gluconapin using liquid chromatography coupled to time-of-flight mass spectrometry (LC-TOF-MS). Both techniques gave comparable results. The HS-GC-MS method was kept as the method of choice as it is rapid and solvent-free. Because yellow mustard seeds do not normally contain gluconapin, its presence in such seeds above the limit of detection was already considered as a criterion for potentially problematic mustard batches. However, "organoleptically" acceptable brown mustard seeds already contained measurable amounts of gluconapin and had to be differentiated from mustard seeds containing nonacceptable levels of gluconapin, as it is typically the case for brown mustard originating from the Indian subcontinent. Thus, a 3-butenyl isothiocyanate content "cut point" has been established to discriminate between batches. This limit could then be applied for the acceptance or rejection of mustard seed batches. In addition, LC-TOF-MS screening of mustard seeds from different geographic origins showed the heterogeneity of the glucosinolate profile and the difficulty to find good origin markers.

KEYWORDS: Mustard seeds; glucosinolate; gluconapin; isothiocyanate; 3-butenyl isothiocyanate; HS-GC-MS; LC-TOF-MS; off-flavor; quality control

INTRODUCTION

Mustard seeds have been known since ancient times and today represent the largest volume in the international spice trade (*1*). The main countries producing mustard seeds are Canada, Nepal, Russia, Ukraine, Czech Republic, Myanmar, and the United States. The worldwide production was 703 738 t in 2004 (*2*).

Mainly two different mustard seed types are used for culinary applications originating from two different species of the Brassicacea family: yellow mustard (*Sinapis alba*) and brown mustard (*Brassica juncea*). Differences are based not only on their anatomy but also on their composition, and especially on the composition of their glucosinolates, which affects the organoleptic properties of the mustard. Yellow mustard contains glucosinalbin as the main glucosinolate, whereas the major brown mustard glucosinolate is sinigrin (*3*). These glucosinolates are present in the intact cells and are enzymatically degraded upon cell rupture. Different degradation products may be produced depending on pH, temperature, and other cofactors. The major hydrolysis products are often isothiocyanates, but thiocyanates, nitriles, and cyano-epithioalkanes have also been identified (*4*). The reaction is driven by the endogenous enzyme myrosinase (thioglucosidase, EC 3.2.3.1). In the case of glucosinalbin, a nearly odorless but strong-tasting 4-hydroxybenzene-1-isothiocyanate is produced. From sinigrin, the pungent volatile allyl isothiocyanate is formed.

In the context of this work, another glucosinolate named gluconapin may play an important role. It is known that certain strains of *B. juncea*, mainly originating from the Indian subcontinent, contain gluconapin, which is converted into 3-butenyl isothiocyanate (see **Figure 1**) and may lead to an undesirable (at least for the Western palate (*1*)) off-flavor. The obtained off-flavor can be described as cabbage-like, aromatic pungent, and sulfur-like.

Consequently, because yellow as well as brown mustard seeds are being widely used by industry in various applications for European consumers, the quality of the seeds in terms of

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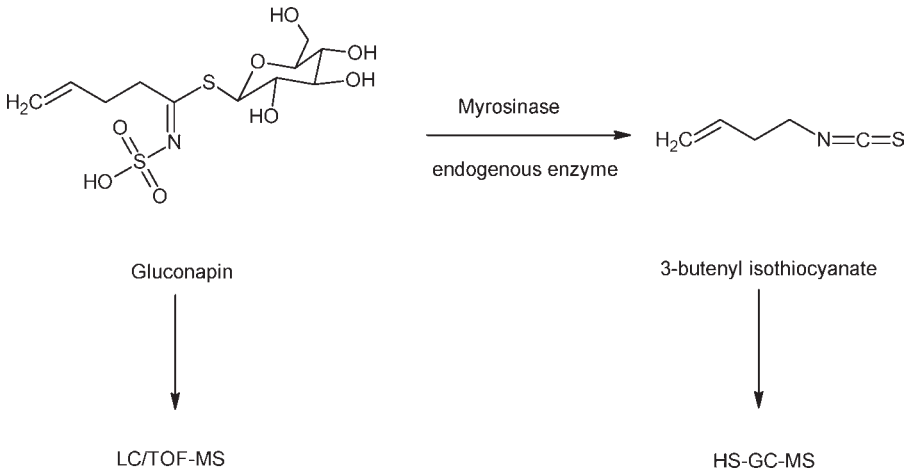


Figure 1. Conversion of gluconapin to volatile 3-butenyl isothiocyanate catalyzed by means of the enzyme myrosinase and the associated technologies used for their analysis.

glucosinolate content is a critical point for ensuring the desired organoleptic properties of the final products. More specifically, the analysis of gluconapin and/or 3-butenyl isothiocyanate may consequently allow monitoring off-flavors in mustard seeds.

The present paper focuses on two aspects to ensure quality control of mustard seeds. The first one targeted the development of an analytical method for semiquantification of volatile compounds responsible for the off-flavor. We report on the development of 3-butenyl isothiocyanate analysis by direct headspace−gas chromatography−mass spectrometry (HS-GC-MS) in mustard seeds. The innovative approach presented here is based on a statistical analysis of different batches to build a model that can then be applied for the rejection or acceptance of the seed batches.

The second aspect of mustard seed quality control addressed the question of variability of glucosinolate content depending of production area. To better understand and to investigate the possible origin of the high gluconapin content in some batches responsible for quality outlier products, the influence of the geographic origin of seeds on glucosinolate content has been tested through a large-scale glucosinolate screening by LC-TOF-MS.

EXPERIMENTAL PROCEDURES

Samples. Different mustard seed batches were provided by several mustard seed suppliers for analysis (37 yellow mustard seeds and 18 brown mustard seeds). Among these, two samples per mustard seed type (yellow and brown) were included as acceptable and nonacceptable reference samples, based on the sensory evaluation of seeds (see Sample Preparation for "Sniffing" Sensory Test). All four reference samples were analyzed during each analytical sequence to control intersequence variability. Furthermore, other mustard seeds with a certified geographic origin have been provided to better understand the possible origin of gluconapin and to highlight glucosinolate-based heterogeneity of seeds. This panel of batches was made up three yellow, two brown, and one black mustard seed batches from India, one yellow mustard seed batch from Ukraine, one yellow mustard seed batch from Moldavia, and two kinds of Oriental mustard (unknown origin).

Chemicals. Acetonitrile (LC grade), buffer solution at pH 4 (citric acid/sodium hydroxide/hydrogen chloride), methanol (Lichrosolv grade), distilled water, and formic acid (98%) were from Merck KGaA (Darmstadt, Germany). 3-Butenyl isothiocyanate (C_5H_7NS) was from TCI Europe (Tokyo, Japan, 95% purity).

Technical Equipment. The different kinds of mustard seed were finely ground with a Perten laboratory mill 3303 from Seedboro (Chicago, IL).

Sample Preparation for "Sniffing" Sensory Test. The sensory test was used to identify acceptable and nonacceptable reference samples. Furthermore, acceptable yellow mustard seeds were spiked with different concentrations of 3-butenyl isothiocyanate standard diluted in acetonitrile to roughly estimate the concentration of 3-butenyl isothiocyanate in nonacceptable samples. The sensory test was performed as follow: 1 g of ground yellow mustard seed powder was put into a 100 mL Erlenmeyer, 3 mL of distilled water was added, and the Erlenmeyer was immediately closed with a glass stopper. The Erlenmeyer was gently swirled to ensure total hydration of the powder and left for 2 min. The addition of standard solution or acetonitrile (blank) was made directly on the wet mustard powder (maximum volume of acetonitrile of 30 μL), the Erlenmeyer being immediately closed and left for another 2 min before sensory testing. This sensory test was performed for the reference samples acceptable and nonacceptable as well as with 10, 1, 0.3, and 0.1 mg of spiked reference acceptable samples.

Sample Preparation for Headspace−Gas Chromatography−Mass Spectrometry (HS-GC-MS). Test portions of milled sample (200 mg of mustard, except for linearity test for which 600 mg of yellow mustard was taken) were weighed into 20 mL headspace vials, 500 μL of a pH 4 buffer solution (citric acid/sodium hydroxide/hydrogen chloride) was added (1.5 mL for the yellow mustard linearity check), and the vials were closed immediately. The closed vials were incubated for 1 h at 60 °C in an oven (Heraeus Kelvitron T, Langenfeld, Germany). Each sample was analyzed in duplicate and on three different days to evaluate the variability of the method.

Headspace Gas Chromatography−Mass Spectrometry. GC-MS analyses were performed using an HP 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA) coupled to an HP 5973 series mass spectrometer (Agilent Technologies) and equipped with a Gerstel MPS2 autosampler (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany). A fused silica capillary column (ZB-Wax) was used, 30 m × 0.25 mm i.d. and film thickness = 0.25 μm (Phenomenex, Torrance, CA). The sample was equilibrated at 50 °C under stirring (250 rpm) for 10 min. A 2.5 mL syringe was used to inject 500 μL of headspace with a syringe temperature at 60 °C (injection speed of 125 μL/s) using the pulsed split mode (+20 psi for 3.5 min) with a split ratio of 1:100 and with an injector temperature set at 280 °C. The flow rate of the helium carrier gas was 1.2 mL/min (constant flow). For the separation, the following oven temperature program was used: 40 °C hold for 4 min, to 80 °C at 5 °C/min (8 min), afterward to 240 °C at 20 °C/min (12 min), and hold for 10 min.

The mass spectrometer was operated in electron ionization mode at 70 eV using full-scan mode from m/z 35 to 350. The transfer line temperature was set at 250 °C and the source temperature at 230 °C.

For the extraction of the chromatogram of 3-butenyl isothiocyanate the following ions were selected: m/z 72, 113, 55, and 85. Typical retention time was 14 min. The semiquantification of all samples was performed by using ion m/z 113.

Test of Linearity. "Artificial" samples were produced to check the linearity of the developed GC method. To cover a relevant range of potential gluconapin contents, different mixes of the two reference samples

Table 1. Composition of Each Sample Mix Used for the Linearity Test

percentage of acceptable mustard seeds per sample	mustard acceptable (g)	mustard nonacceptable (g)
0	0	5
20	1	4
40	2	3
60	3	2
80	4	1
100	5	0

(acceptable and nonacceptable) were prepared for both kinds of mustard seed. The composition of each sample mix is presented in **Table 1**.

Setup of Cut-Point Value for the Discrimination of Acceptable and Nonacceptable Samples. In the particular case of brown mustard seeds, identification of nonacceptable sample batches was done with a statistical model described elsewhere by Mire-Sluis et al. (*5*). It considers that the content of an acceptable sample class is normally distributed around a mean value. This approach is based on the determination of an assay cut point, that is, the level of response above which a sample is defined as nonacceptable. This was obtained statistically using 22 samples and a normalization approach based on an interday analysis of variability, performed in three steps. The first one identifies a population normally distributed within these 22 samples thanks to a test of normality (Shapiro Francia test, 5% confidence). The second step consists of calculating the cut-point value above which samples would be considered as statistically different from the acceptable population. A 99.99% confidence level has been chosen here. The analyses were repeated on three different days, which led to three cut-point values. Finally, to correct the inherent interday drift, the cut point of each day is normalized by dividing it by the value of a reference sample and then averaged on the three days values to obtain a normalization factor K to be used for assessment of future samples.

Sample Preparation for Liquid Chromatography−Time of Flight Mass Spectrometry (LC-TOF-MS). The seed powders used for LC-TOF-MS analysis were the same as those prepared for the analysis of the 3-butenyl isothiocyanate by HS-GC-MS. The extraction of glucosinolate performed here followed the same principles of methods already described elsewhere (*4*, *6*, *7*): 1 g of mustard powder was weighed into a 50 mL Teflon tube, 10 mL of a methanol/water solution (70:30, v/v) heated at 70 °C was added, and the Teflon tube was incubated for 30 min at 70 °C in a water bath. Every 10 min the samples were vortexed and sonicated for approximately 2 min. After incubation, samples were centrifuged. To avoid saturation of the detector and to decrease the amount of injected organic solvent, 50 μL of the clear supernatant was transferred into a glass tube containing 20 mL of acidified water (0.1% formic acid). Finally, 5 μL of this solution was injected into the LC-TOF-MS system.

Liquid Chromatography−Time of Flight Mass Spectrometry. Analyses by LC-TOF-MS were performed using a 1200 SL Agilent HPLC (Agilent Technologies) coupled to a 6220 Accurate-Mass TOF MS analyzer (Agilent Technologies). Among the tested columns, the Atlantis dC18 (Waters, Milford, MA), 2.1 × 150 mm with a particle size of 3 μm, gave the most efficient chromatographic separation of polar compounds such as glucosinolates. A gradient elution was performed, with mobile phase A consisting of water with 0.1% formic acid and mobile phase B consisting of acetonitrile with 0.1% formic acid. The gradient program with 5 μL injection volume was applied at a flow rate of 300 μL/min as follows: 1% of B for 3 min, increased to 25% B from 3 to 7 min, then to 95% B from 7 to 10 min, kept at 95% B for 2 min, and re-equilibrated from 12 to 16 min at 1% B.

The ionization and detection of the glucosinolates were performed with an electrospray ionization source operated in the negative mode, using the following operation parameters: capillary voltage, 3500 V; nebulizer pressure, 50 psig; drying gas flow rate, 9 L/min; gas temperature, 350 °C; skimmer voltage, 60 V; octapole DC 1, 37.5 V; octapole RF current, 250 V; fragmentor voltage (in-source collision induced dissociation (CID)), 190 V, except during glucosinolate identity confirmation when the fragmentor voltage was increased to 230 V to enhance in-source fragmentation. The high-resolution mode (4 GHz, $R > 12000$) with an acquisition range of m/z 100−1600 (2 scan/s) was used. Accurate mass measurement was achieved thanks to an automated calibrant delivery system for mass spectra correction. A dual-nebulizer electrospray source was introduced at the outlet of the chromatograph at the same time as the calibrant solution containing purine ($C_5H_4N_4$, m/z 121.050873) and HP-0921 ([hexakis-(1*H*,1*H*,3*H*-tetrafluoropentoxy)phosphazene], $C_{18}H_{18}O_6N_3P_3F_{24}$, m/z 922.009798). The full-scan spectral data were processed with Agilent Mass Hunter software (version B.02.00, Build 2.0.197.0).

Building of Glucosinolates Database with Accurate Mass and Characteristic Fragments. Because TOF-MS analyses provide exact mass measurement with < 2 ppm error, the identification of glucosinolates was performed on the basis of exact mass measurement and their retention time. Check of identity of glucosinolates was done by injection of extracts at a higher fragmentor voltage (230 V) to produce characteristic fragments of glucosinolate (see Screening of Glucosinolates in Various Geographic Origin Seeds for observed fragments). Once identity was confirmed through fragmentation, the retention time of the considered glucosinolate was recorded as another matching criterion. As this technique is dedicated to screening approaches, the opportunity was taken to search for other known glucosinolates in the different samples provided. Combining results already published by Lee et al. (*7*), Cataldi et al. (*6*), and Bennett et al. (*4*), the theoretical exact monoisotopic masses of some glucosinolates searched has been calculated and gathered in a database as illustrated in **Table 2** (see also **Table 3**). This database was subsequently used by Agilent Mass Hunter software for data treatment. Data were processed in a targeted way. The glucosinolates present in the database were searched in the samples. The matching criteria were accurate mass tolerance (< 5 ppm error in measured mass) and retention time tolerance (±0.2 min).

RESULTS AND DISCUSSION

The degradation product 3-butenyl isothiocyanate from gluconapin has earlier been reported to produce an undesirable off-flavor in mustard (*7*) . Starting from mustard seeds presenting this off-flavor, we tried to mimic it by diluting different volumes of pure 3-butenyl isothiocyanate in a freshly milled yellow mustard seed matrix free of gluconapin (acceptable reference). A non-acceptable reference sample was also processed without the addition of 3-butenyl isothiocyanate as a positive control. A panel of three people could not differentiate between the non-acceptable reference sample and the acceptable sample spiked with 3-butyenyl isothiocyanate. This confirmed 3-butenyl isothiocyanate to be responsible for the off-flavor in accordance with the literature (*7*). In addition to this, the nonacceptable reference sample was always easily positioned at the same place on a scale generated by acceptable reference samples spiked with different amounts of 3-butenyl isothiocyanate.

Several papers described the analysis of glucosinolates (*4*, *7*) as well as that of isothiocyanates (*8*, *9*). All glucosinolates are amenable to analysis by reversed phase liquid chromatography coupled to mass spectrometry (LC-MS), but not to analysis by GC because they are not volatile. On the other hand, the isothiocyanates they generate may be either volatile or not. The volatile ones are usually analyzed by GC-MS. For the purpose of this study, HS-GC-MS was used for the analysis of the volatile 3-butenyl isothiocyanate (**Figure 1**) and LC-TOF-MS was used for the analysis of gluconapin due to its high selectivity and robustness.

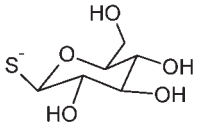
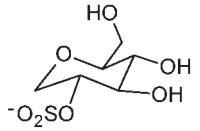
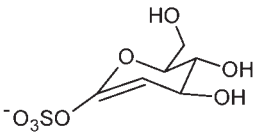
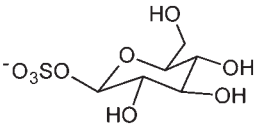
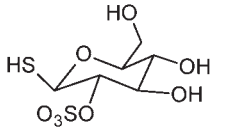
Establishment and Selection of the Analytical Method. To avoid any bias induced by the conversion of gluconapin into 3-butenyl isothiocyanate during the LC-TOF-MS analysis, the inactivation of the myrosinase during extraction of glucosinolates was ensured by the addition of a hot methanol/water solution. The extraction protocol was a minor modification of methods that have been described elsewhere (*4*, *7*, *10*) . The obtained extracts were injected into the LC-TOF-MS system and subjected only to a semiquantitative approach as no gluconapin standard was used.

For the analyses of the volatile 3-butenyl isothiocyanate, GC-MS analyses using either Solid Phase Micro Extraction (SPME) or HS as sampling system were evaluated. As these GC-MS

Table 2. Theoretical Exact Monoisotopic Masses of Some Glucosinolates

glucosinolate	formula	theor mass
(*R*)-*p*-hydroxy-2-hydroxy-2-phenylethyl glucosinolate	$C_{15}H_{21}NO_{11}S_2$	455.0556
(*R*)-*p*-methoxy-2-hydroxy-2-phenylethyl glucosinolate	$C_{16}H_{23}NO_{11}S_2$	469.0713
10-methylsulfonyldecyl glucosinolate	$C_{18}H_{35}NO_{11}S_3$	537.1372
10-methylthiodecyl glucosinolate	$C_{18}H_{35}NO_9S_3$	505.1474
2-(*R*-L-rhamnopyranosyloxy)benzyl glucosinolate	$C_{20}H_{29}NO_{14}S_2$	571.103
3,4,5-trimethoxybenzyl glucosinolate	$C_{17}H_{25}NO_{12}S_2$	499.0818
3-hydroxy, 4-(*R*-L-rhamnopyranosyloxy)-benzyl glucosinolate	$C_{20}H_{29}NO_{15}S_2$	587.0979
4-GDB-GLSd	$C_{17}H_{31}NO_{14}S_4$	601.0627
4-hydroxyglucobrassicin	$C_{16}H_{20}N_2O_{10}S_2$	464.0559
4-methoxyglucobrassicin	$C_{17}H_{22}N_2O_{10}S_2$	478.0716
5-hydroxyglucobrassicin	$C_{16}H_{20}N_2O_{10}S_2$	464.0559
5-methylsulfonylpentyl glucosinolate	$C_{13}H_{25}NO_{11}S_3$	467.059
7-methylthioheptyl glucosinolate	$C_{15}H_{29}NO_9S_3$	463.1005
8-methylsulfonyloctyl glucosinolate	$C_{16}H_{31}NO_{11}S_3$	509.1059
8-methylthiooctyl glucosinolate	$C_{16}H_{31}NO_9S_3$	477.1161
9-methylsulfonylnonyl glucosinolate	$C_{17}H_{33}NO_{11}S_3$	523.1216
9-methylthiononyl glucosinolate	$C_{17}H_{33}NO_9S_3$	491.1318
dehydoerucin	$C_{12}H_{21}NO_9S_3$	419.0379
DMB-GLS	$C_{22}H_{40}N_2O_{18}S_6$	812.0601
glucoalyssin	$C_{13}H_{25}NO_{10}S_3$	451.0641
glucoarabin	$C_{17}H_{33}NO_{10}S_3$	507.1267
glucoberteroin	$C_{13}H_{25}NO_9S_3$	435.0692
glucobrassicanapin	$C_{12}H_{21}NO_9S_2$	387.0658
glucobrassicin	$C_{16}H_{20}N_2O_9S_2$	448.061
glucocamelinin	$C_{18}H_{35}NO_{10}S_3$	521.1423
glucocapparin	$C_8H_{15}NO_9S_2$	333.0188
glucocheirolin	$C_{11}H_{21}NO_{11}S_3$	439.0277
glucoconringiin	$C_{11}H_{21}NO_{10}S_2$	391.0607
glucoerucin	$C_{12}H_{23}NO_9S_3$	421.0535
glucoerysolin	$C_{12}H_{23}NO_{11}S_3$	453.0433
glucohesperin	$C_{14}H_{27}NO_{10}S_3$	465.0797
glucohirsutin	$C_{16}H_{31}NO_{10}S_3$	493.111
glucoiberin	$C_{11}H_{21}NO_{10}S_3$	423.0328
glucoiberverin	$C_{11}H_{21}NO_9S_3$	407.0379
glucolesquerellin	$C_{14}H_{27}NO_9S_3$	449.0848
glucolimnanthin	$C_{15}H_{21}NO_{10}S_2$	439.0607
glucomalcomiin	$C_{16}H_{23}NO_{10}S_2$	453.0763
glucomatronalin	$C_{14}H_{19}NO_{11}S_2$	441.04
gluconapoleiferin	$C_{12}H_{21}NO_{10}S_2$	403.0607
gluconasturtiin	$C_{15}H_{21}NO_9S_2$	423.0658
gluconapin	$C_{11}H_{19}NO_9S_2$	373.0501
glucoputranjivin	$C_{10}H_{19}NO_9S_2$	361.0501
glucoraphanin	$C_{12}H_{23}NO_{10}S_3$	437.0484
glucoraphenin	$C_{12}H_{21}NO_{10}S_3$	435.0328
glucosibarin	$C_{15}H_{21}NO_{10}S_2$	439.0607
glucosiberin	$C_{15}H_{29}NO_{10}S_3$	479.0954
glucosinalbin	$C_{14}H_{19}NO_{10}S_2$	425.045
glucotropaeolin	$C_{14}H_{19}NO_9S_2$	409.0501
isobutyl glucosinolate	$C_{11}H_{21}NO_9S_2$	375.0658
MB-GLS	$C_{11}H_{21}NO_9S_3$	407.0379
methylpentyl-GLSc	$C_{13}H_{25}NO_9S_2$	403.0971
n-butyl glucosinolate	$C_{11}H_{21}NO_9S_2$	375.0658
neoglucobrassicin	$C_{17}H_{22}N_2O_{10}S_2$	478.0716
n-hexyl-GLSc	$C_{13}H_{25}NO_9S_2$	403.0971
n-pentyl glucosinolate	$C_{12}H_{23}NO_9S_2$	389.0814
n-propyl glucosinolate	$C_{10}H_{19}NO_9S_2$	361.0501
n-sulfoglucobrassicin	$C_{16}H_{20}N_2O_{12}S_3$	528.0178
p-methoxy-2-phenylethyl glucosinolate	$C_{16}H_{23}NO_{10}S_2$	453.0763
progoitrin/epiprogoitrin	$C_{11}H_{19}NO_{10}S_2$	389.045
sinigrin	$C_{10}H_{17}NO_9S_2$	359.0345

Table 3. Glucosinolate Fragments and Their Theoretical Exact *m*/*z*, Combined from References *7* and *6*

FRAGMENT	FORMULA	THEORETICAL m/z
	$C2H3OS^-$	74.9910
	$SO3^-$	79.9574
	$SO4^-$	95.9523
	$HSO4^-$	96.9601
	$C6H11O5S^-$	195.0333
	$C6H11O7S^-$	227.0231
	$C6H9O8S^-$	241.0024
	$C6H11O9S^-$	259.0129
	$C6H11O8S2^-$	274.9901

methods require the conversion of gluconapin into 3-butenyl isothiocyanate by myrosinase, it was important to find experimental conditions erasing any kinetic differences between sample preparations and ultimately allowing comparison of results obtained by LC-TOF-MS and GC-MS. For this purpose a standardized incubation at 60 °C for 1 h as described under Experimental Procedures was used prior to analysis.

Although SPME and HS techniques appeared to be sensitive and reproducible, the SPME approach suffers from saturation of the fiber due to the potentially high isothiocyanate concentrations that may be generated (glucosinolates can represent up to 3% of the seed content (*1*)), thus preventing an unambiguous differentiation from samples highly contaminated with 3-butenyl isothiocyanate. This saturation problem did not appear when using direct headspace injection with a 1:100 split as illustrated by the linearity study applied to the brown mustard shown in **Figure 2**. The static headspace approach was chosen for the following experiments.

As no gluconapin standard was used for LC-TOF-MS and no labeled standard was commercially available for the GC analysis, semiquantitative results were normalized with the unambiguous nonacceptable reference sample identified during sensory trials. Gluconapin and 3-butenyl isothiocyanate content were expressed as the peak area ratio between the analyte in the sample and the analyte in the nonacceptable reference.

The nonacceptable reference sample was analyzed within each sample series. This normalization allowed the comparison between LC-TOF-MS and GC-MS results. The obtained values summarized in **Table 4** show a good correlation (r^2 = 0.99) between both analytical techniques. Such a good positive

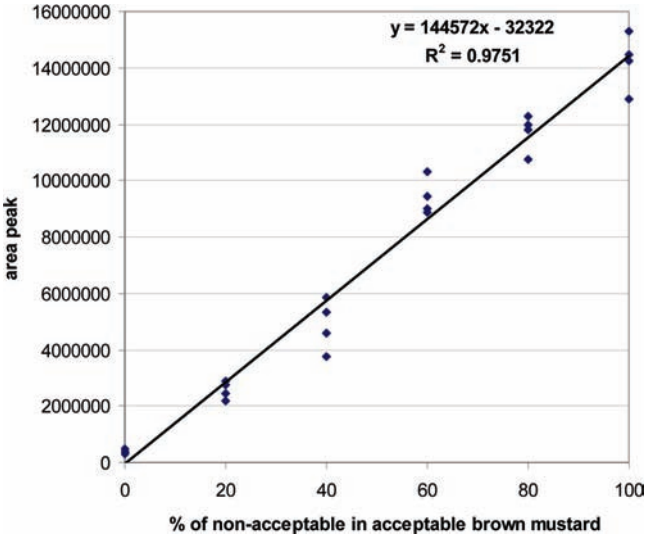


Figure 2. Regression line for the analysis of yellow mustard by HS-GC-MS.

Table 4. Comparison between LC-TOF-MS (Gluconapin) and GC-MS (3-Butenyl Isothiocyanate) Results for Some Analyzed Mustard Seed Samples

sample	gluconapin ratio (sample/reference) (%)	3-butenyl isothiocyanate ratio (sample/reference) (%)
brown mustard 5	0.6	0.6
brown mustard 3	0.5	0.5
brown mustard 9	0.5	0.5
brown mustard 7	0.5	0.5
brown mustard 18	0.5	0.5
brown mustard 4	0.7	0.7
yellow mustard 37	26.2	32.3
mustard Indian origin 4 (brown mustard seed)	1.2	1.6
brown mustard 16	0.6	0.4
mustard Indian origin 3 (yellow mustard seed)	116.4	102.4

correlation between the two techniques suggests that either method should be applicable for the analysis. The HS-GC-MS method was preferred because it was faster and required no hot solvent extraction. The HS-GC-MS method was used for the development of the statistical model and to define the cut point for acceptance or rejection of samples.

By mixing the acceptable and nonacceptable reference samples it was possible to generate samples containing different concentrations of 3-butenyl isothiocyanate (**Table 1**). These samples were used to probe the linearity of the HS-GC-MS method. The regression line for brown mustard seeds is shown in **Figure 2**. The data were analyzed using an in-house statistical tool. The obtained regression line having a high coefficient of determination (R^2 = 0.949 for yellow mustard and R^2 = 0.975 for brown mustard) as well as a normal distribution of residuals indicated that the proposed method is linear for concentrations ranging from our acceptable to our nonacceptable reference samples.

Determination of a Cut Point for the Discrimination of Acceptable and Nonacceptable Samples by HS-GC-MS. In the case of the yellow mustard seeds, discrimination between acceptable and nonacceptable samples was clear as either no 3-butenyl isothiocyanate or large amounts of 3-butenyl isothiocyanate were observed in the samples. The 3-butenyl isothiocyanate chromatographic peak presenting a signal-to-noise ratio higher than 10 was thus considered as a cut point. If the 3-butenyl isothiocyanate peak exceeded this ratio, the sample was considered to be

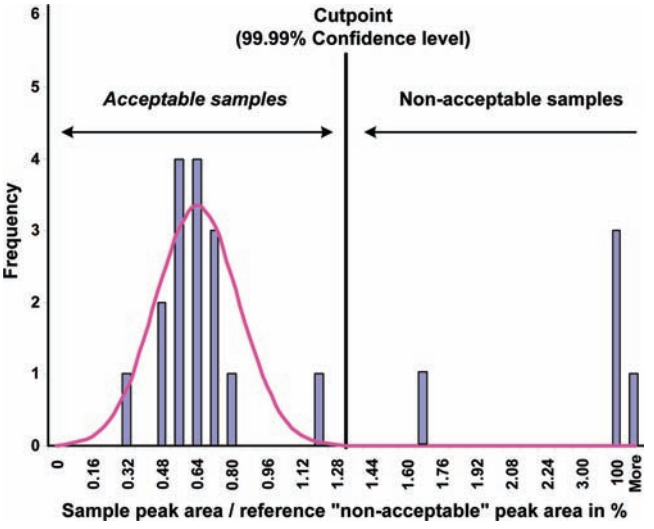


Figure 3. Distribution of 3-butenyl isothiocyanate content of day 1 data and the corresponding theoretical normal distribution given by statistical determination of acceptable samples.

nonacceptable. In this case the result was expressed as the ratio between the peak area of the 3-butenyl isothiocyanate in the sample and the one of the yellow mustard seed nonacceptable reference sample. To ensure an adequate limit of detection is retained among different future analytical series, a quality control sample must be analyzed with each batch, and quality criteria should be met (in our case a dedicated sample of yellow mustard leading to a S/N of 30 for the ion m/z 113).

In the case of brown mustard seeds, 3-butenyl isothiocyanate was always detected, even at a very low level for samples considered as acceptable. It was thus decided to follow an approach described by Mire-Sluis et al. (*5*), which considers that the content of an acceptable sample class is normally distributed around a mean value. This approach is based on the determination of an assay cut point, that is, the level of response above which a sample is defined as nonacceptable (**Figure 3**). This was obtained statistically using 22 samples (21 sample and 1 reference sample) and a normalization approach based on an interday analysis of variability. To do so, a population representing acceptable samples was first identified, then a cut point over which samples may be considered as nonacceptable at a preset confidence interval was defined. Finally, a normalization factor K was calculated by dividing the cut point with the value of a reference sample, previously selected from the acceptable samples. This reference sample is then analyzed with each new analytical batch and an updated cut point is obtained by multiplying the reference sample value by the factor K. Therefore, for each new analytical batch a normalized cut point, which does not depend on day-to-day variation of the HS-GC-MS response, is obtained.

The brown mustard samples were sorted on the basis of their 3-butenyl isothiocyanate content, starting with the sample with the smaller amount of 3-butenyl isothiocyanate (considered as being acceptable). The largest family that could still be considered as normally distributed was generated on the basis of the Shapiro Francia test (5% significance, acceptable population, see **Table 5**). Three slightly different acceptable populations were identified for the runs performed on three different days, and the results obtained on the three different days were not directly comparable. This is obviously due to slight differences in the detector interday drift. As the analytical cut point is probably much lower than the sensory one, the same normal population

Table 5. Determination of Cut-Point Values and Normalization Factor *K* for the Brown Mustard Samples Analyzed on Three Different Days

		3-butenyl isothiocyanate ratio sample/reference[a] (%)		
	sample	day 1	day 2	day 3
acceptable	brown mustard 1	0.32	0.22	0.59
	brown mustard 2	0.70	0.51	0.90
	brown mustard 3	0.51	0.36	0.66
	brown mustard 4	0.74	1.28[b]	1.29
	brown mustard 5	0.61	0.41	0.73
	brown mustard 6	0.58	0.54	0.86
	brown mustard 7	0.53	0.42	0.62
	brown mustard 8	0.57	0.33	0.84
	brown mustard 9	0.52	0.36	0.53
	brown mustard 13	0.68	0.84[b]	0.84
	brown mustard 14[c]	0.70	0.81[b]	1.06
	brown mustard 15	0.60	0.65	0.60
	brown mustard 16 (Canada)	0.42	0.48	0.76
	brown mustard 18	0.54	0.33	0.50
	Oriental mustard seed 1	1.16[b]	0.34	0.69
	Oriental mustard seed 2	0.41	0.36	0.17
nonacceptable	brown mustard 10	55.44	71.34	61.41
	brown mustard 11	23.19	25.93	29.26
	brown mustard 12	9.09	12.20	11.48
	brown mustard 17	107.40	135.34	97.65
	Indian origin 4	1.62	2.08	2.41
av (acceptable population)		0.60	0.52	0.73
SD (acceptable population)		0.19	0.27	0.25
cut point at 99.99%		1.30	1.51	1.67
factor *K* (cut point/brown mustard seed 14)		1.86	1.87	1.57
factor *K* av			1.77	

[a] Nonacceptable reference sample after sensory test. [b] Samples added to the acceptable population based on the largest one, i.e., that of day 3. [c] Reference sample used to update the cut point in each new analytical batch.

was considered for the three runs, that is, the larger one (= day 3). Therefore, some samples from days 1 and 2 rejected from the normal distribution test (Shapiro Francia test as mentioned above) were added to the acceptable population (see **Table 5**). Then, the corresponding cut point for each day was calculated on the basis of a confidence level of 99.99% (0.01% risk of false positive), above which any sample would be considered as different from the acceptable population. To ensure a 99.99% level of confidence, the cut point is calculated for each day as the average (normal population) + 3.719 × standard deviation (normal population).

For calculation of the normalization factor *K*, based on the experiments from the three days, the sample for which the standard deviation of factor *K* over the three days was the smallest was selected as reference (i.e., brown mustard 14, in bold in **Table 5**). The mean factor *K* over the three days was 1.77 (CV % = 9.53%), which represents a variability lower than that obtained for the interday cut points. All future analyses will have to be assayed using this normalized factor in association with the value obtained for the reference sample at the day of analysis.

As it is not conceivable to analyze with each new analytical batch the whole acceptable population to define the cut point, the cut point of any new analytical batch can now be obtained by always analyzing the same acceptable sample as a reference sample (i.e., brown mustard 14 in **Table 5**) and by multiplying the value obtained for this reference by the average normalized factor *K* (= 1.77).

During this study, 37 different yellow mustard seed batches and 18 brown mustard seed batches were analyzed. Two yellow batches were easily detected as positive with the test as the signal-to-noise ratio for 3-butenyl isothiocyanate of 30 and 40 for these two positives samples. With regard to the brown mustard batches, the distribution of 3-butenyl isothiocyanate was more spread as summarized in **Table 5**. Among the different mustard seeds originated from various geographic areas and tested within the framework of this study, gluconapin, and thus 3-butenyl isothiocyanate, were mainly found in the mustard seeds originating from India. The presence of 3-butenyl isothiocyanate in yellow mustard seed batches was surprising as it has never been reported in the literature, indicating a possible contamination of our batches with seeds from other origins. Considering the heterogeneity in the 3-butenyl isothiocyanate content and its unexpected presence in some yellow mustard seed batches, we decided to investigate the precursor of isothiocyanates by a large glucosinolate screening performed by LC-TOF-MS to detect characteristic glucosinolates of other species specific to geographic production area.

Screening of Glucosinolates in Various Geographic Origin Seeds. The screening capacity of the LC-TOF-MS instrument was utilized to screen the different batches of mustard seeds for glucosinolates already reported by Cataldi et al. (*6*), Bennett et al. (*4*), and Lee et al. (*7*) (**Table 2**). In the absence of a glucosinolate standard for each compound in the database, confirmation of detected compounds was carried out by using in-source CID fragmentation at a higher fragmentor voltage (230 V) and checking for characteristic fragments of glucosinolates. The mass accuracy obtained on the deprotonated intact glucosinolates (generally < 2 ppm) combined with the structural information given by fragmentation patterns (also with a high mass accuracy) allowed the unambiguous identification of the glucosinolate. Some fragments already reported by Cataldi et al. (*6*) and Lee et al. (*7*) have been observed in all detected glucosinolates, such as ions with *m*/*z* at 195.0333, 227.0231, 241.0024, 259.0129, and 274.9901, corresponding to glycone fragmentation (see **Table 3** and **Figure 4a**,**c**). Other characteristic fragments were always observed such as SO_3^-, SO_4^-, and HSO_4^- at *m*/*z* 79.9574, 95.9523, and 96.96011 as depicted in **Figure 4b** corresponding to in-source CID fragmentation of gluconapin. As no parent ion had been selected for these in-source fragmentation experiments, attribution of these fragments to a particular glucosinolate was ensured by checking the exact superposition of glucosinolate chromatographic peak and extracted ion chromatograms of fragments. Other ions never previously reported were observed for all of the glucosinolates such as ions at *m*/*z* 74.991 and 290.986. As no standard has been used for analysis, no information was available about the response factor and the differences in ionization efficiencies for the different glucosinolates detected. Hence, only an interseed batch comparison for a given glucosinolate can be made, and the present results cannot be used to calculate an accurate glucosinolate composition in a seed batch.

All of the glucosinolates reported in **Table 2** were screened in the different samples, but seven glucosinolates were predominantly detected with intensities depending on the seed: sinigrin, gluconapin, and glucosinalbin with a very high response and progoithrin/epiprogoithrin, 4-hydroxyglucobrassicin, glucobrassicanapin, and gluconasturtiin with a lower response (see **Figure 5**). Among the analyzed seed batches, *S. alba* (yellow

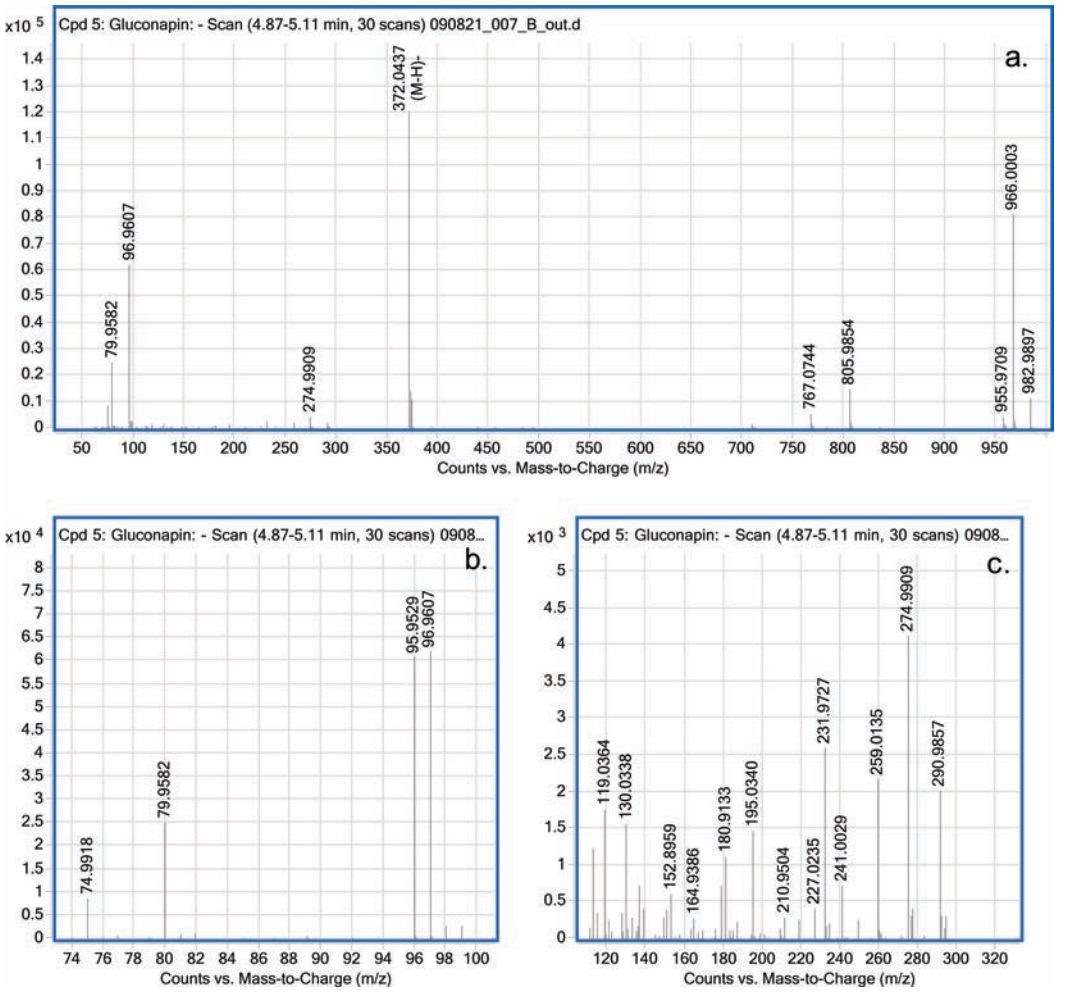


Figure 4. Intrasource fragmentation of gluconapin (theoretical *m*/*z* 372.0429; fragmentor, 230 V) extracted from *Brassica juncea* in a seed batch considered to be nonacceptable after sensory trials: full spectra (**a**) and zoomed spectra showing ions of SO_3^-, SO_4^-, HSO_4^- at theoretical *m*/*z* 79.9574, 95.9523, 96.96011 (**b**), and glycone fragments (see **Table 3**) at theoretical *m*/*z* 195.0333, 227.0231, 241.0024, 259.0129, and 274.9901 (**c**).

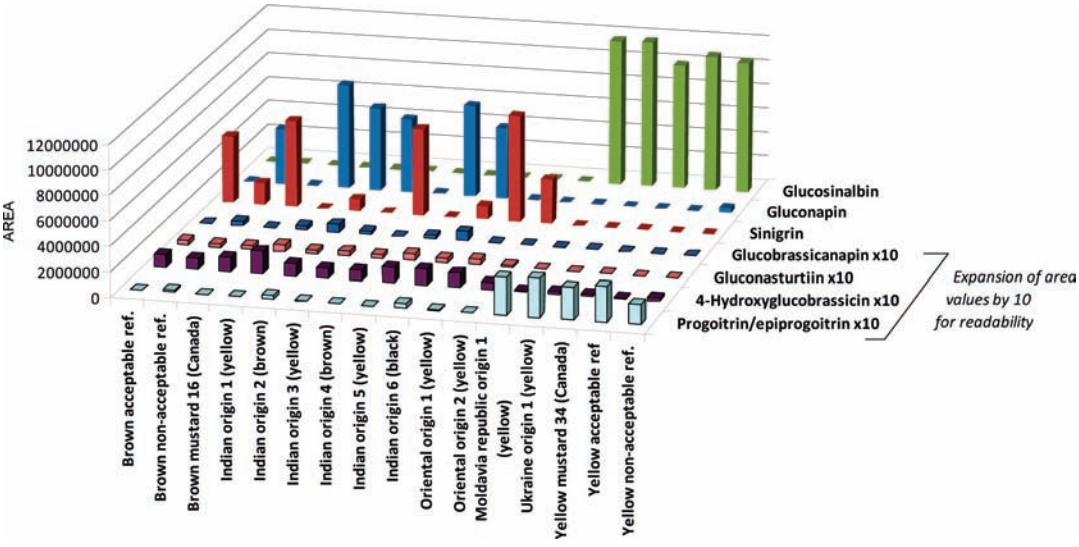


Figure 5. Glucosinolate composition of mustard samples from different geographical origins. The area of the three glucosinolates, glucosinalbin, sinigrin, and gluconapin, leading to the highest intensity are reported as such, whereas the four glucosinolates glucobrassicanapin, gluconasturtiin, 4-hydroxyglucobrassicin, and progoitrin/epiprogoitrin detected with a lower intensity are reported with an area multiplied by 10 to make comparisons easier.

mustard) samples originating from Canada contained glucosinalbin (yellow mustard seed originating from Canada, yellow reference acceptable and yellow reference nonacceptable as shown in **Figure 5**) as the major glucosinolate. The ones from

Moldavia and Ukraine mainly contained glucosinalbin, showing that these two kinds of mustards belong to the species *S. alba* as expected. Similarly, brown mustard batches from Canada (brown acceptable and brown mustard 16 originating from Canada, shown in **Figure 5**) contained mainly sinigrin as expected for *B. juncea* species. Surprisingly, some seed batches originating from India (Indian origin 1, 3, and 5) contained mainly gluconapin without any glucosinalbin and sinigrin. Therefore, on the basis of the glucosinolate profile alone, it is not possible to classify them as yellow or brown mustard. Moreover, another interesting point is that some seeds could be misclassified as they looked yellow in appearance but were actually brown according to their glucosinolate content and their botanical classification as *B. juncea* (Oriental mustard seeds 1 and 2 in **Figure 5**). The high abundance of gluconapin content as well as their yellow appearance could explain the unexpected detection of gluconapin in some yellow mustard batches, as one could easily imagine that a mix of such seeds with *S. alba* seeds would be hardly detectable visually. The black mustard seed batch originating from India has also a glucosinolate profile similar to the "Indian origin 2" batch (brown seeds), with a high content of gluconapin. Concerning the other glucosinolates, their response is far less intense and only the progoitrin or its enantiomer epiprogoitrin can be easily linked to glucosinalbin content. Apart from gluconapin, none of the other glucosinolates screened can be used as a good marker of mustard off-flavor. These results highlight the huge diversity of mustard seed glucosinolate contents related to the area of production and sometimes even within the same area of production. One of them especially can lead to undesirable flavors for some consumers due to their cultural background and culinary preferences, hence the importance in our case of monitoring gluconapin and 3-butenyl isothiocyanate content linked to the cabbage-like off-flavor identified here.

In conclusion, a semiquantitative headspace−gas chromatography−mass spectrometry (HS-GC-MS) method has been developed for the rapid analysis of 3-butenyl isothiocyanate in mustard seeds providing a quality control tool for this raw material. In addition, LC-TOF-MS screening of mustard seeds from different geographic origins showed the heterogeneity of the glucosinolate profile and the difficulty of finding good origin markers.

LITERATURE CITED

(1) Hemingway, J. S. Mustard and condiment products. In *Encyclopedia of Food Sciences and Nutrition*, 2nd ed.; Trugo, L., Finglas, P. M., Caballero, B., Eds.; Academic Press (Elsevier Science and Technology Books): Oxford, U.K., 2003; pp 4055−4058.

(2) Smith, V.; Jimmerson, J. Mustard seed. *AMPC Briefing 59*; Agricultural Marketing Policy Center: Bozeman, MT, **2005**.

(3) Thomas, J.; Kuruvilla, K. M.; Hrideek, T. K. Mustard. In *Handbook of Herbs and Spices*; ICRI Spices Board/Woodhead Publishing: Cambridge, U.K., 2004; pp 12.

(4) Bennett, R. N.; Mellon, F. A.; Kroon, P. A. Screening crucifer seeds as sources of specific intact glucosinolates using ion-pair high-performance liquid chromatography negative ion electrospray mass spectrometry. *J. Agric. Food Chem.* **2004**, *52*, 428–438.

(5) Mire-Sluis, A. R.; Barrett, Y. C.; Devanarayan, V.; Koren, E.; Liu, H.; Maia, M.; Parish, T.; Scott, G.; Shankar, G.; Shores, E.; Swanson, S. J.; Taniguchi, G.; Wierda, D.; Zuckerman, L. A. Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J. Immunol. Methods* **2004**, *289*, 1–16.

(6) Cataldi, T. R.; Rubino, A.; Lelario, F.; Bufo, S. A. Naturally occurring glucosinolates in plant extracts of rocket salad (*Eruca sativa* L.) identified by liquid chromatography coupled with negative ion electrospray ionization and quadrupole ion-trap mass spectrometry. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 2374–2388.

(7) Lee, K. C.; Chan, W.; Liang, Z.; Liu, N.; Zhao, Z.; Lee, A. W.; Cai, Z. Rapid screening method for intact glucosinolates in Chinese medicinal herbs by using liquid chromatography coupled with electrospray ionization ion trap mass spectrometry in negative ion mode. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 2825–2834.

(8) Romeo, V.; Ziino, M.; Giuffrida, D.; Condurso, C.; Verzera, A. Flavour profile of capers (*Capparis spinosa* L.) from the Elian Archipelago by HS-SPME/GC-MS. *Food Chem.* **2005**, *101*, 1272–1278.

(9) D'auria, M.; Mauriello, G.; Racioppi, R. SPME-GC-MS analysis of horseradisch (*Armoracia rusticana*). *Ital. J. Food Sci.* **2004**, *16*, 487–490.

(10) Mellon, F. A.; Bennett, R. N.; Holst, B.; Williamson, G. Intact glucosinolate analysis in plant extracts by programmed cone voltage electrospray LC/MS: performance and comparison with LC/MS/MS methods. *Anal. Biochem.* **2002**, *306*, 83–91.

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