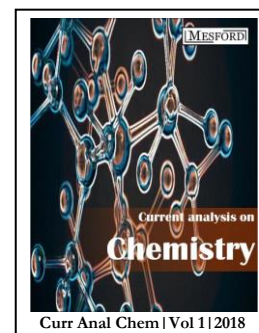


A Streamlined Method for Determination of Total and Individual Glucosinolates in Rapeseed Using Spectrophotometry and High Performance Liquid Chromatography

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Abstract:

A streamlined method for determination of total and individual glucosinolates in rapeseeds using spectrophotometry and high performance liquid chromatography was developed in this study. The rapeseeds were extracted using 70% methanol solution and purified by a strong anion exchange column. The sample solution was divided into two aliquots. One part was hydrolyzed to determine the total glucosinolates content by evaluating the loss of chromogenic ferricyanide at 420 nm. The quantitative analytical method of total glucosinolates was fully validated with respect to the linearity ($r^2 > 0.995$), sensitivity, precision, accuracy (the recovery between 91.7-108%), and robustness (the relative standard deviation $< 5.23\%$). The other part was enzymatic hydrolyzed by sulfatase, with optimized reaction time of one hour to establish the desulfoglucosinolate profiles by high performance liquid chromatography. The concentrations of the individual glucosinolates were calculated based on the total glucosinolate value and their response factors. The developed method was successfully applied to determine the content of individual glucosinolates in rapeseeds without an internal standard. The newly established method is simple, fast and reliable on the quantitation of glucosinolates in rapeseeds.

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Keywords:

Total Glucosinolates; Individual glucosinolates; Rapeseed; Desulfo-Glucosinolates; HPLC; Spectrophotometry.

1. INTRODUCTION

Rapeseed is a bright-yellow flowering member of family Brassicaceae, widely used to produce vegetable oils. The southern provinces in China are known for their taste preferences of fragrant rapeseed oil in cooking food, because of the special flavour and smooth mouth feel. Rapeseeds contain a high level of glucosinolates (9-207 $\mu\text{mol/g}$ dry defatted seed meal) [1]. The main volatile flavor compounds of fragrant rapeseed oil were degraded glucosinolate products. The glucosinolates and their enzymatic degradation products such as isothiocyanates [2] were reported to possess anticarcinogenic activity [3], also contributing to the flavour characteristic and pungency of rapeseeds [4, 5]. Hence, there is an increasing demand for reliable and validated methods for analysis and quantification of glucosinolates in rapeseed.

The determination of total and individual glucosinolates is challenging for analysts due to their high polarity, ionic character and lack of standards. Glucosinolates have been evaluated indirectly by determining the contents of glucose, sulfate ions or goitrin released by myrosinase [6]. Total glucosinolates have been evaluated indirectly by determining

the accurate contents by colorimetric techniques such as thiourea [7], thymol [8], benzenedithiol cyclocondensation [9], palladium chloride [10], and sulfate ion release [11]. However, these previous analytical methods are time-consuming and require additional clean-up step. Near infrared reflectance spectroscopy (NIRS) is a rapid and non-destructive analysis method for detection of glucosinolates in rapeseeds [12]. The matrix interference protein and oil contents were determined simultaneously along with the glucosinolates [13]. Jezek et al. developed a simple and sensitive method to determine total glucosinolate content based on their alkaline hydrolysis and subsequent reaction of released 1-thioglucoose with ferricyanide [14]. Recently, Gallaher et al reported the modification and validation of the ferricyanide assay and its application in quantifying the total glucosinolates in cruciferous vegetables [15]. In the past, analysis of the individual glucosinolates via myrosinase and GC-MS of the isothiocyanates [16] or derivatisation of the desulfo-GLS (desulfo-Glucosinolates) to trimethylsilyl (TMS) [17] were used. LC-MS/MS has recently been applied as a technique for the determination of individual glucosinolates [18]. The shortage of reference standards for most identified glucosinolates has hampered the quantification

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of individual intact glucosinolate by the mass spectrometry [19].

Up to now, the HPLC analysis of desulfo-GLS is most widely used to determine the glucosinolate content in brassica plant samples. In general, GL (Glucosinolate) extracts are uploaded on DEAE Sephadex A-25 anion-exchange resin columns, where GLs (Glucosinolates) are bounded due to their sulphate group. The columns are incubated with sulphatase overnight at ambient temperature, removing the sulphate group and subsequently the desulfo-GLs can be eluted with water for HPLC analysis. The content of individual glucosinolate was determined with multiplying the response factors of corresponding glucosinolate by the content of internal standard (sinigrin or glucotropaeolin). However, the desulfation step on ion-exchanger in this method is time-consuming and the degradation risks of indolglucosinolates will significantly increase after a long exposure [20, 21]. Furthermore, two internal standards (sinigrin and glucotropaeolin) are frequently found in brassica plant samples, which will negatively affect the accuracy of quantification on individual glucosinolates.

We here reported on the optimization and validation of a streamlined method for determination of total and individual glucosinolates. Our study is based on alkaline and enzymatic hydrolysis to obtain the total glucosinolates and individual desulpho-glucosinolates. The concentration of the total glucosinolates was determined by UV method with ferricyanide reaction; while the contents of individual glucosinolates were analyzed via calculating from both the total and composition glucosinolates value (Fig. 1). We also demonstrated the applicability of the assay with the analysis of glucosinolates in 12 rapeseeds collected from different regions in China.

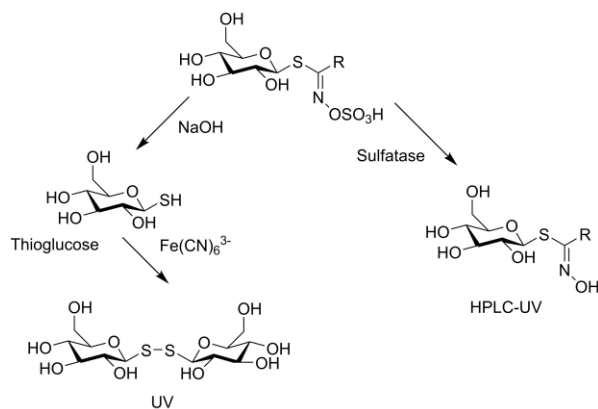


Fig. (1). Chemical workflow of hydrolysis methods for GLS analysis.

MATERIALS AND METHODS

Chemical Reagent and Materials

12 rapeseed samples were collected from different locations in China by Wilmar Biotechnology Research & Development Center Co. Ltd (Shanghai, China) and stored at ambient temperature before analysis.

Sinigrin hydrate potassium salt, sulfatase (from *Helix pomatia*, 10 KU) and sephadex A-25 were purchased from

Sigma-Aldrich (St. Louis, MO, USA). Glucotropaeolin potassium salt was obtained from TCI Company (Tokyo, Japan). Strong anion exchange solid phase extraction (SPE) column (LC-SAX, 500 mg) was purchased from Supelco (Park Bellefonte, PA, USA). Imidazole was purchased from Aladdin (Shanghai, China). All other chemical used were reagent grade. Ultrapure water was obtained from a Milli-QRG purification unit (Millipore, Bedford, MA, USA).

Methods

Extraction of glucosinolate from rapeseeds. The extraction method of rapeseeds were carried out according to the ISO official procedure [6] with some modifications. The dried rapeseeds were grinded in the microgrinder for two times (20 s and 5 s, respectively) and passed through the 100 mesh screen. 200 mg rapeseeds meal was transferred to 15 mL centrifuge tube. After that, the centrifuge tube was placed in a water-bath at 75 °C for 1 minutes, 2 mL of boiling 70% methanol aqueous solution was added and heated for a further 10 minutes by manual shaking at regular intervals. The solution was centrifuged at 5000 g for 3 minutes. The extraction process was repeated one more time and the supernatants were combined, and diluted with 3 mL of water. The sample solution can be kept for 2 weeks if stored in the dark in a freezer.

Purification of glucosinolate. A strong anion exchange columns (LC-SAX, 500 mg) was conditioned with 3 mL of methanol, followed by 3 mL of water, 2.5 mL of 0.5 M sodium acetate (pH = 4.6), and 2.5 mL of water, all of sample solution was loaded and eluted with 2.5 mL water. Then, the analytes were eluted using 4 mL of 0.5 M sodium chloride. The elutes were diluted with 6 mL of water and divided into two aliquots for the quantification of total and individual glucosinolates, respectively.

Quantification of Total Glucosinolates

Analysis of total glucosinolates. The content of total glucosinolates was determined following a previously reported method with minor modifications [14]. 2 mL of purified sample solution was mixed with 2 mL of freshly prepared 2 M sodium hydroxide. The mixture was shaken at room temperature for 50 minutes, 310 μ L of 12 M hydrochloric acid was then added to neutralize the mixture. After cooling to room temperature, 0.75 mL of 0.4 M phosphate buffer (pH = 7.0) was transferred into the mixture, and the reaction was initiated by addition of 0.4 mL of a solution of 20 mM potassium ferricyanide in 0.4 M phosphate buffer (pH = 7.0). After 2 minutes, the absorbance of the solution was measured using a microplate reader with UV absorbance mode at 420 nm immediately (FLUOstar Omega, BMG Labtech, Offenburg, Germany).

Calculation of Total Glucosinolate Concentration. The concentration of total glucosinolates in the rapeseed sample was calculated from the absorbance reading using a calibration curve of sinigrin and was expressed as μ mol of glucosinolates /g. The blank sample was prepared following the same preparation process without the rapeseed sample.

Quantification of Individual Glucosinolate

Preparation of desulpho-glucosinolate. 200 μL of sample solution, 20 μL sulfatase solution (5.0 mg/mL in water) and 400 μL of 200 mM sodium acetate buffer (pH 5.0 at 37 °C with 5 M HCl) were mixed in a 2 mL conical tube. The mixture was shaken at 37 °C to obtain desulpho-GLs. All experimental conditions were based on the manufacturer's recommendations.

A simple homemade column for purifying desulpho-GLs was prepared as according to the ISO official procedure [6] with some modifications to remove the sulfatase and unreacted GLs: 1 mL of DEAE-Sephadex A-25 anion-exchange resin was filled in a 5 mL injection syringe connected with a 0.45 μm filter (25 mm), and was washed successively with 2 mL of imidazole formate (6 M), 2 mL of water, 1 mL of 0.02 M sodium acetate buffer (pH=4.0) and 2 mL of water. The mixture containing desulpho-GLs was loaded on the homemade column to elute desulpho-GLs at room temperature.

HPLC analysis of desulpho-glucosinolate. HPLC analysis of desulpho-glucosinolate was performed on an Agilent 1260 HPLC system (Palo Alto, CA, USA), equipped with a quaternary solvent delivery system, an autosampler and a DAD detector. The qualitative analysis was carried out on an Agilent Zorbax Eclipse XDB-C18 column (150 \times 4.6 mm i.d., 5 μm). The elution gradient (mobile phase A: water; mobile phase B: 25% acetonitrile in water) started from 5% (mobile phase B) and kept in 4 minutes, increased to 15% (mobile phase B) in 7 minutes, then increased to 100% (mobile phase B) in 25 minutes, then kept for 1 minute. UV absorption was monitored at 229 nm. The flow rate was 1.0 mL/min and the column oven temperature was set at 30 °C. The injection volume was 20 μL .

Qualitative analysis of desulpho-glucosinolate using HPLC-MS/MS. Identification of individual desulpho-glucosinolate in rapeseed samples were analyzed by a HPLC-MS/MS 1290-6460 system (Agilent Technologies, USA) equipped with an ESI ion source. The reversed-phase was conducted using an Agilent Zorbax Eclipse XDB-C18 column (150 \times 4.6 mm i.d., 5 μm). 20 μL of solution was injected. The elution gradient (mobile phase A: water, mobile phase B: 25% acetonitrile in water) started from 5% (mobile phase B) and was kept for 4 minutes, it was then increased to 15% (mobile phase B) at 7 minute, then increased to 100% (mobile phase B) at 25 minute, and then kept for 1 minute. The flow rate was 0.75 mL/min and the column oven temperature was set at 30 °C. The typical optimized values for the source parameters were: gas temperature 300 °C, gas flow 5 L/min, nebulizer 45 Psi, sheath gas temperature 250 °C, sheath gas flow 11 L/min, capillary voltage 3500 V, nozzle voltage 500 V, fragmentor 70 V, cell accelerator voltage 7 V, respectively. The ESI was in positive mode and the mass range was set at 80-800 amu in MS scan type. The relative concentrations of the individual glucosinolates were calculated from equation as below:

$$CIG = CTG \times RFIG \times PIG / \sum_1^n (RFIG \times PIG)$$

Where CTG is the total glucosinolate concentration obtained by our developed UV method; CIG is the individual glucosinolate concentration obtained by our developed method; PIG is the peak area percentage of individual glucosinolate in total glucosinolates; RFIG is the response factor of individual glucosinolate adopted from ISO official method, are shown in Fig. (4). N is the number of individual glucosinolates in the chromatogram. The samples were used for the analysis and were treated according to the procedure described in the flowchart (Fig. 2).

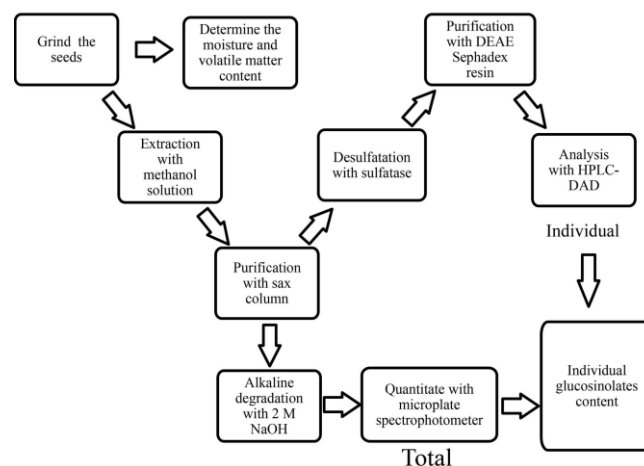


Fig. (2). Flowchart of the experimental procedure.

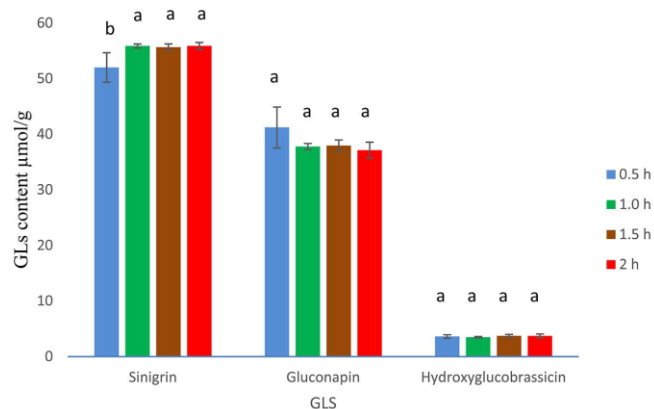


Fig. (3). The GLS contents in different reaction time in desulphation reaction (n=3).

Statistical analysis. Data were reported as the mean \pm SD for triplicate determinations. One-way ANOVA and Tukey's test were employed for different independent samples. Data evaluation was performed with SPSS for Windows (version rel. 16.0, SPSS Inc., Chicago, IL, USA). Significant differences were declared at $p < 0.05$.

RESULTS AND DISCUSSION

Analysis of Individual Glucosinolate

Method validation. The UV method was validated for linearity, precision, repeatability, accuracy and stability as follows: The standard compound sinigrin was dissolved in water to a final concentration of 2.0 mg/mL (5.03 $\mu\text{mol/L}$). A set of standard solutions were prepared by appropriate dilution of the stock

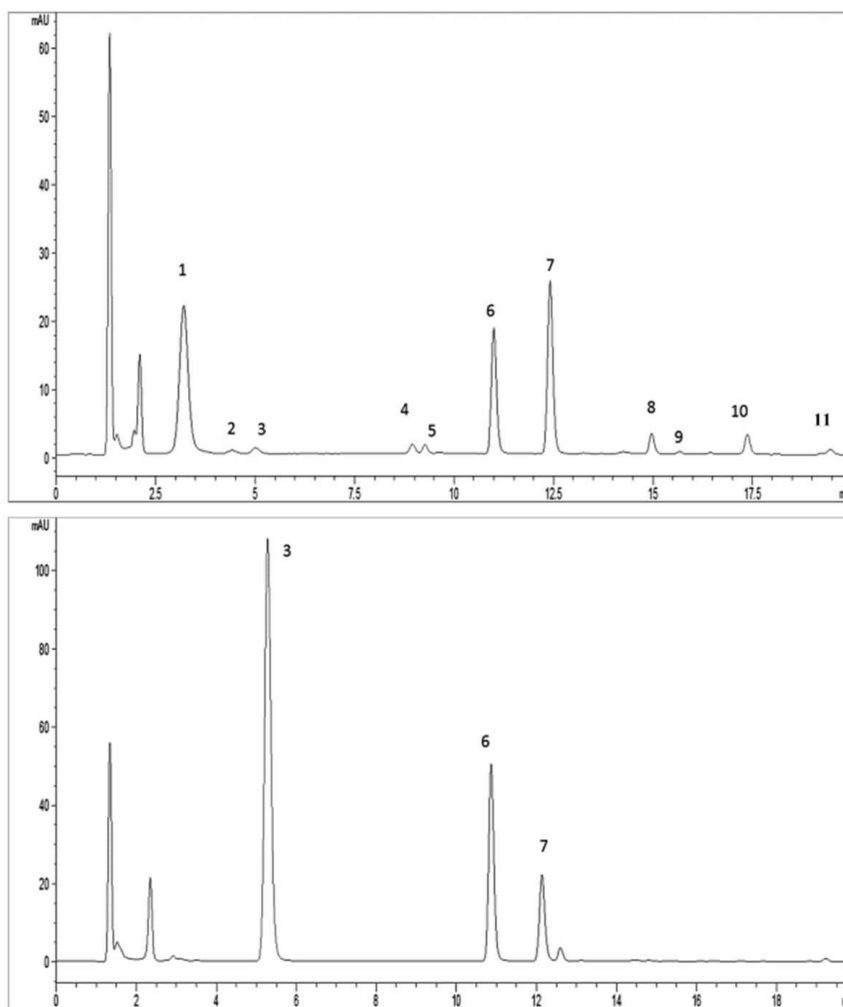


Fig. (4). HPLC-UV chromatogram of two typical rapeseed samples.

solution, containing 0-2000 $\mu\text{g}/\text{mL}$ of sinigrin. Calibration curves were constructed by plotting the ultraviolet spectral response versus the concentrations of analytes. The calibration curves for total glucosinolates showed excellent linear regression ($R^2 = 0.9957$). The limit of detection (LOD) and limit of quantification (LOQ) for the standard compounds were determined as 3- and 10-fold of the standard deviation (SD, $n=6$) of a blank value, respectively. The LOD and LOQ was 10.80 and 32.18 $\mu\text{mol}/\text{g}$, respectively. The intra-day precision was calculated using data collected at five different time points within a day, while inter-day precision was examined over five days at the concentration of 85.5 $\mu\text{mol}/\text{g}$. Method repeatability was estimated by analysis of six independently prepared samples. The established method showed good reproducibility for the quantification of total glucosinolates with intra- and inter-day variations less than the number of 5.23 and 5.04% respectively. Repeatability was examined with six sample solutions. The accuracy of analytical method was evaluated by a rapeseed sample spiked with 26.4 μmol of sinigrin. Spike recoveries of sinigrin ranged from 103 to 108%, and the RSD value was 3.57%. The accuracy of the analytical method was evaluated using the recovery test. The concentration level of sinigrin (26.4 μmol) was spiked in 0.2 g of sample, and the percentage recovery was calculated by the difference between the spiked and non-spiked sample using the

calibration curve. All the testing solution was prepared using real sample. For the stability test, the sample solution was analyzed over a period of two weeks. The content of total glucosinolates were found to be stable if it was kept within two weeks at -20°C .

To further validate the established method, the results of total glucosinolates in three rapeseed samples were determined by the newly developed method and ISO method based on HPLC analysis. 2 rapeseeds samples from different origins of China (An Hui, Si Chuan) were tested with both the method in this paper and the ISO method respectively. For rapeseeds sample tested with the method in this paper, the results were: 135.56 ± 6.25 $\mu\text{mol}/\text{g}$ (An Hui), 75.58 ± 4.16 $\mu\text{mol}/\text{g}$ (Si Chuan). For rapeseeds sample tested with the ISO method, the results were: 125.64 ± 5.67 $\mu\text{mol}/\text{g}$ (An Hui), 75.87 ± 3.64 $\mu\text{mol}/\text{g}$ (Si Chuan). The deviation of the two analysis methods as mean were 7.89% (An Hui), 0.38% (Si Chuan) on $n = 3$. It suggests that the UV spectrophotometric method was reliable and accurate for quantifying total glucosinolates.

Optimization reaction times with ferricyanide in rapeseed. In the step of quantification of total glucosinolates, the reduction reaction is rapid and the degradation of the glucosinolates will also react with ferricyanide. To further validate the established method in real sample, reaction time

Table 1. The trivial name, type of side chain, molecular formula, mass of glucosinolate, mass values of quasi-molecular ions of desulfo- glucosinolates (DS-GLs) at positive mode and response factors in UV

No.	Trivial name	Type of side chain	Molecular formula	M	M _{DS+H}	M _{DS+Na}	Response factors
1	Progoitrin	2-Hydroxy-3-butenyl	C ₁₁ H ₁₉ NO ₁₀ S ₂	389	310.0	332.0	1.09
2	Glucoraphanin	4-Methylsulfinylbutyl	C ₁₂ H ₂₃ NO ₁₀ S ₃	437	358.0	380.1	1.07
3	Sinigrin	2-Propenyl	C ₁₀ H ₁₇ NO ₉ S ₂	359	279.1	302.1	1.00
4	Gluconapoleiferin	2-Hydroxy-pent-4-enyl	C ₁₂ H ₂₁ NO ₁₀ S ₂	403	324.0	346.0	1.00
5	Glucoalyssin	5-Methylsulfinylpentyl	C ₁₃ H ₂₅ NO ₁₀ S ₃	451	372.1	394.0	1.07
6	Gluconapin	3-Butenyl	C ₁₁ H ₁₉ NO ₉ S ₂	373	294.0	316.0	1.11
7	Hydroxyglucobrassicin	4-Hydroxy-3-indolylmethyl	C ₁₆ H ₂₀ N ₂ O ₁₀ S ₂	464	385.1	407.0	0.28
8	Glucobrassicinapin	Pent-4-enyl	C ₁₂ H ₂₁ NO ₉ S ₂	387	308.1	330.0	1.15
9	Glucoerucin	4-Methylthiobutyl	C ₁₂ H ₂₃ NO ₉ S ₃	421	342.0	364.0	1.04
10	Glucobrassicin	3-Indolylmethyl	C ₁₆ H ₂₀ N ₂ O ₉ S ₂	448	369.0	391.0	0.29
11	Gluconasturtiin	2-Phenethyl	C ₁₅ H ₂₁ NO ₉ S ₂	423	344.1	366.1	0.95

with ferricyanide were optimized in the best conditions without side reactions. The effect of reaction time on the total glucosinolates content in 4 different batches of rapeseeds was obtained when compared to the spiked blank samples. The rapeseeds that were detected to have glucosinolates less than 5 $\mu\text{mol/g}$ were selected to be used as blank samples. 4 blank rapeseed samples were spiked with GLS (37-78 $\mu\text{mol/g}$) and the best reaction time was selected when the GLS estimate and the GLS content from the calibration curve in the spiked samples were equal. A 2 minutes (± 10 s) reaction time is selected.

Analysis of Individual Glucosinolate

Effect of time on the glucosinolate desulphation by sulfatase. Previous studies showed that the glucosinolate desulphation can be completed within several minutes in solution. However, in most widely accepted standards, desulphation step is time-consuming (usually more than 6 h). This heavily hampered the analysis of desulfo glucosinolates [20]. In addition, the glucosinolate solution may not sufficiently react with the sulfatase that is absorbed on the column due to its small volume. Meanwhile, indolglucosinolates tend to be unstable during a long time of exposure [22].

Considering all of the above factors, our current study presented a simple and effective method to prepare desulfo glucosinolates by directly mixing the glucosinolate solution with sulfatase. To evaluate the efficiency of desulfation, different reaction time including 0.5, 1, 1.5 and 2 hours were chosen to compare the concentrations of three representative desulfo glucosinolates in a rapeseed sample. Our results (Fig. 3) indicated that there is no significant increase on the content of three different desulfo glucosinolates in rapeseed samples after 1h. We selected 1 h for desulfation in subsequent experiments. Furthermore, considering the advantages of improved accuracy, the individual GLS were determined with internal standard using this rapid method.

Identification of desulfo glucosinolates (DS-GLs) by HPLC-MS/MS. The identification of desulfo glucosinolates (DS-GLs) was achieved using HPLC-MS/MS in positive ion mode [23]. In total, 11 desulfo glucosinolates were identified based on their quasi-molecular ions including $[\text{MDS}+\text{H}]^+$ and $[\text{MDS}+\text{Na}]^+$ (Table 1).

Calculation on the concentration of individual glucosinolate. A HPLC method with an internal standard (sinigrin and glucotropaeolin) has been widely applied in the determination of the content of individual GLs in rapeseeds followed by multiplying a response factor. However, some type of rapeseeds inherently contained sinigrin and glucotropaeolin, which will affect the accuracy of analytical results.

To circumvent this problem, we established an indirect method for determination of individual GLs by calculating the percentage of individual GLs in total glucosinolates with an area normalization method, followed by multiplying with a corresponding response factor to obtain the concentration of individual GLs. The representative HPLC chromatograms and corresponding response factor were shown in Fig. (4) and Table 1. The method showed good reproducibility for the quantification of individual glucosinolates (more than 1 $\mu\text{mol/g}$ of individual GLs) with intra- and inter-day variations less than 14.02 and 15.04% respectively. The developed method is still undergoing improvement. Nevertheless, it provides an alternative route to quantify the individual GLs content in rapeseeds in a shorter period of time.

Rapeseed Sample Analysis

The developed method was applied to analyse the concentration of individual GLs in 12 rapeseeds collected from different origins in China. The GLs contents in rapeseed samples were listed in Table 2. Therefore, the total and individual method reported here for analyzing the sample can be applied to a broad range of rapeseeds. Total glucosinolate concentration of rapeseeds varies from 70-140 $\mu\text{mol/g}$. The

Table 2. The contents of Investigated Glucosinolates in Rapeseed Samples ($\mu\text{mol/g}$)^a

Samples	Origin	1	2	3	4	5	6	7	8	9	10	11
A	An Hui 1	44.68±1.25	1.06±0.26	1.37±0.08	1.74±0.23	1.48±0.20	21.51±0.39	9.06±0.85	3.73±0.23	-	-	1.08±0.06
B	Si Chuan 1	41.55±0.52	1.34±0.06	2.92±0.01	1.56±0.10	2.29±0.04	20.24±0.12	7.25±0.31	4.82±0.35	-	-	-
C	Yun Nan	41.96±1.16	-	-	2.24±0.04	1.99±0.28	14.47±0.27	9.02±0.75	4.61±0.01	-	-	-
D	Gui Zhou	-	-	92.62±0.27	-	-	37.93±0.16	5.01±0.40	-	-	-	-
E	Shang Hai	43.63±0.65	1.54±0.06	-	1.19±0.02	1.31±0.39	25.33±0.19	5.78±0.53	2.30±0.14	1.01±0.02	-	-
F	Si Chuan 2	60.92±1.77	-	2.22±0.64	2.47±0.16	2.65±0.10	19.78±0.73	8.64±0.24	7.94±3.44	-	-	1.04±0.04
G	He Bei	9.52±0.05	-	-	1.74±0.02	2.75±0.08	52.26±0.49	5.75±0.38	16.48±0.09	-	-	3.42±0.07
H	Zhe Jiang 1	39.09±0.40	1.51±0.10	-	1.19±0.07	1.30±0.26	22.14±0.28	3.35±0.24	2.75±0.07	-	-	-
I	Zhe Jiang 2	-	-	42.03±0.37			35.40±0.25	3.93±0.14	2.47±0.45	-	-	-
J	Zhe Jiang 3	49.76±1.80	-	2.06±0.20	1.45±0.07	1.37±0.04	29.69±0.45	8.63±0.85	4.73±0.83	1.02±0.12	-	1.06±0.08
K	An Hui 2	38.38±0.32	1.89±0.53	1.91±1.36	1.36±0.19	1.94±0.15	20.02±0.71	7.93±0.94	3.79±0.78	-	-	-
L	Si Chuan 3	50.35±1.58	2.71±1.51	-	2.28±0.45	2.79±0.33	3.92±0.14	6.04±0.16	-	6.10±0.13	-	1.39±0.02

^aData are reported on per gram of rapeseeds basis as mean on n = 3.

major rapeseed lines in China are *Brassica napus* L and *B campestris* L. The individual glucosinolates content are different in different type of rapeseed. In the high glucosinolate *B napus* lines progoitrin and gluconapin are the major contents with the present of small amounts of other glucosinolates. While the high glucosinolate *B campestris* L lines comprise sinigrin and gluconapin as dominant components. The *B napus* and *B campestris* L varieties were classified into types according to the amounts of individual glucosinolates content [24].

CONCLUSIONS

Our study developed a streamline method that determines the total and individual glucosinolates. This method is easily handled compared to previous methods, which could provide a great help to rapeseed oil industries for monitoring the contents of glucosinolates in raw materials. This method obtain the total glucosinolates contents using UV spectrophotometry in factory without HPLC, while it can also determine the individual glucosinolates without internal standard and shorter desulfation of glucosinolates incubation periods than official methods in laboratory. Thereof, it provides novel convenience strategy for in-house analytical laboratory that capable to perform comprehensive analysis of glucosinolates transferred from corresponding factory by refrigeration transport, with adherence to similar extraction and purification solution.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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