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High-yield production of secoisolariciresinol diglucoside from flaxseed hull by extraction with alcoholic ammonium hydroxide and chromatography on microporous resin

Cong-Cong Zhuang[1,2†], Chun-Rui Liu[1,2†], Cheng-Bin Shan[1,2], Zheng Liu[3], Ling Liu[3] and Chao-Mei Ma[1,2*]

Abstract

This study used alcoholic ammonium hydroxide to directly hydrolyze and extract secoisolariciresinol diglucoside (SDG) from flaxseed hull in a one pot reaction. The optimal extraction conditions, including the concentration of ammonium hydroxide, extraction time, and temperature, were examined in single factor experiments, followed by response surface methodology (RSM) with 3-level, 3-factor Box-Behnken experiments. As a result, the optimal extraction conditions were determined as follows: material-liquid ratio 1:20, percentage of reagent ammonium hydroxide (25–28% of NH_3 in water) in ethanol 33.7% (pH = 12.9), extraction time 4.9 h, and extraction temperature 75.3 °C. Under these conditions, the yield of SDG, as measured by ultra-high-performance liquid chromatography-mass spectrometry, was 23.3 mg/g, consistent with the predicted content of SDG in flaxseed hull (23.0 mg/g). Further, 30.0 g of pulverized flaxseed hull was extracted under the optimal conditions, and the extract was subjected to a single run of macroporous resin chromatography to obtain 772.1 mg of a fraction with an SDG content exceeding 76.1%. Subsequent chromatography on Sephadex LH20, yielded 602.8 mg SDG of 98.0% purity, and the yield was 20.1 mg/g (2.0%) from flaxseed hulls. Thus, one-pot hydrolysis and extraction of SDG using alcoholic ammonium hydroxide is simple, and of high-yield.

Keywords: Flaxseed hull, SDG, Extraction, Alkaline hydrolysis, RSM

* Correspondence: cmma@imu.edu.cn
†Cong-Cong Zhuang and Chun-Rui Liu contributed equally to this work.
[1]State Key Laboratory of Reproductive Regulation & Breeding of Grassland Livestock, Hohhot, China
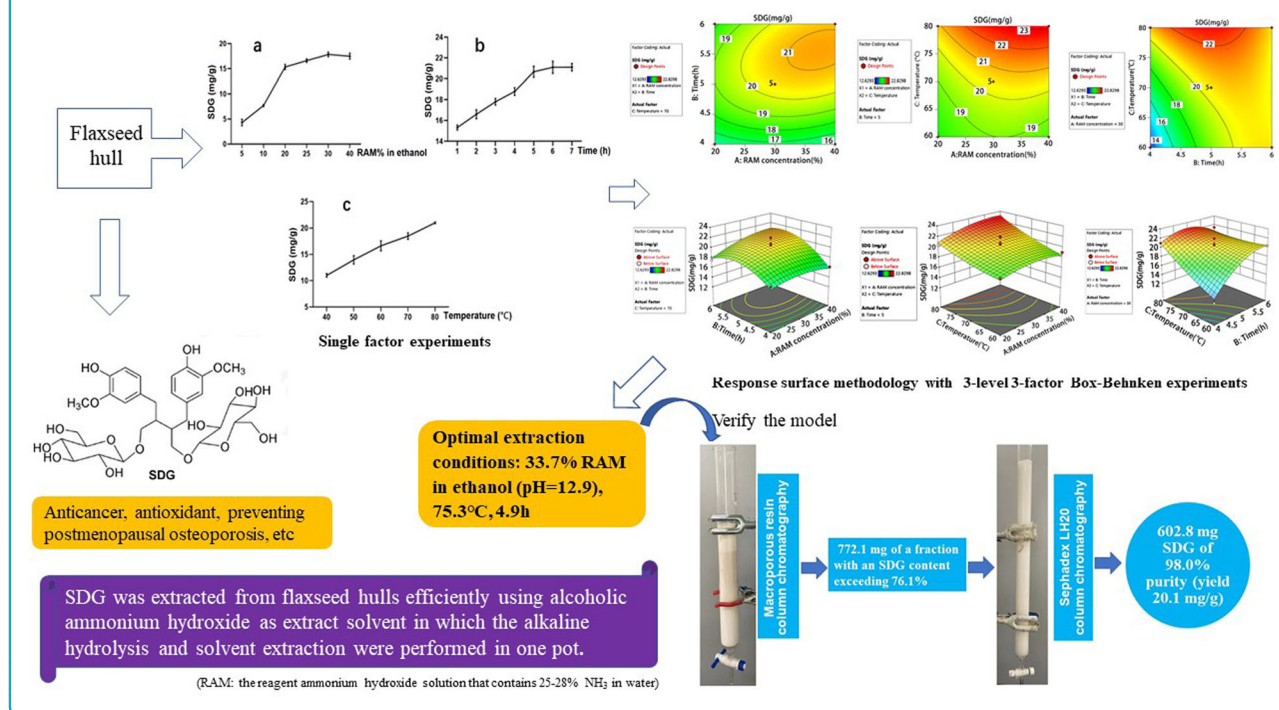[2]Key Laboratory of Herbage & Endemic Crop Biology of Ministry of Education, School of Life Sciences, Inner Mongolia University, Hohhot, P.R. China 010070
Full list of author information is available at the end of the article

Graphical abstract

Introduction

Linum usitatissimum L. is an important oil crop. Its seed, commonly known as flaxseed, contains high levels of the omega-3 unsaturated fatty acid alpha-linolenic acid (Shim et al. 2014; Abuzaytoun, & Shahidi 2006), which has positive effects on human health (Goyal et al. 2014; Lu et al. 2020). Flaxseed also contains significant amounts of lignans, especially secoisolariciresinol diglucoside (SDG), which is concentrated in the hull (Milder et al. 2005; Imran et al. 2015). SDG has attracted increasing attention due its consistently proven pharmacological activities (Kezimana et al. 2018), including its anticancer (Ayella et al. 2010; Tannous et al. 2020), antioxidant (Hu, Yuan, & Kitts 2007; Kitts et al. 1999), antidiabetes (Moree, Kavishankar, & Rajesha 2013; Prasad 2001; Sherif 2014), and cholesterol-lowering effects (Prasad 2005); prevention of postmenopausal osteoporosis (Sacco et al. 2011); protection of myocardial cells from apoptosis (Huang et al. 2018); and inhibition of melanoma (Li et al. 1999), colon cancer (Ayella et al. 2010), and breast tumor cell growth (Chen et al. 2009; Bowers et al. 2019). SDG is converted into the mammalian lignans enterodiol and enterolactone with the involvement of intestinal bacteria (Jin, Kakiuchi, & Hattori 2007a; Jin et al. 2007b). These mammalian lignans are phytoestrogens that showed inhibition on the development of hormone-related breast and prostate cancers (Begum et al. 2004; Chen, & Thompson 2003).

An efficient, safe and scalable extraction and separation technology to produce pure SDG has tremendous economic significance. In flaxseed, SDG exists in macromolecules that contain ester bonds that form through the carboxyl groups of hydroxymethyl glutaric acid and the hydroxyl groups of glucose residues in SDG (Eliasson et al. 2003; Ford et al. 2001) as depicted in Fig. 1. Many studies have attempted to optimize the extraction of SDG, most of which used aqueous methanol, aqueous ethanol, and water as solvents for extraction (Degenhardt, Habben, & Winterhalter 2002; Hosseinian, & Beta 2009). 1,4-dioxane: ethanol has also been reported as a solvent (Johnsson et al. 2000, 2002). Most extractions have been performed by sequential solvent extraction and alkaline hydrolysis and, to a lesser extent, simultaneous extraction and hydrolysis (Eliasson et al. 2003; Hosseinian, & Beta, 2009). Flaxseed crude extract and its hydrolysate were usually purified and analyzed on reverse-phase chromatography (Hao & Beta 2012; Johnsson et al. 2000; Eliasson et al. 2003). High-speed counter-current chromatography (Degenhardt, A., Habben, & Winterhalter 2002) and macroporous resin chromatography (Wang et al. 2019) were also reported for the purification of SDG.
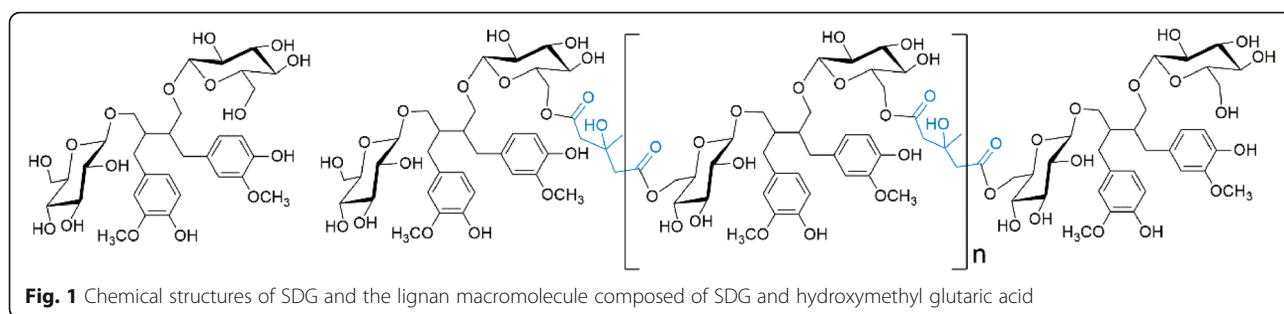
Fig. 1 Chemical structures of SDG and the lignan macromolecule composed of SDG and hydroxymethyl glutaric acid

The yield and purity of SDG depend strongly on the solvents and reagents that are used in the extraction. Strong acids break ester bonds and glycosidic bonds (Charlet et al. 2002), generating deglycosylated SDG, or secoisolariciresinol (SECO), whereas alkaline hydrolysis severs only the ester bond; thus, alkali extraction is preferred for extracting intact SDG. Higher yields of SDG have been predicted by direct extraction with 1 M NaOH versus hydrolysis of the alcohol extracts (Eliasson et al. 2003). Sodium hydroxide and calcium hydroxide are usually used in alkaline hydrolysis to release SDG (Fuentealba et al. 2015; Hosseinian, & Beta 2009).

In this study, ammonium hydroxide in aqueous ethanol was used to release and extract SDG from flax hull in a one pot reaction. Ammonium hydroxide has weak alkalinity, rendering it more amenable to adjusting the pH, and the waste ammonium hydroxide solution can be used as fertilizer. Parameters of the extraction, including the concentration of ammonium hydroxide that was calculated as the percentage of reagent ammonium hydroxide (25–28% of NH_3 in water, briefly, RAM), extraction time, and temperature, were examined and optimized in single-factor experiments, followed by response surface methodology (RSM) with 3-level, 3-factor Box-Behnken experiments. As a result, an efficient SDG extraction procedure was established, and preparative extraction and purification of SDG were performed to verify the efficacy of this method. With this approach, flaxseed hull waste in the oil industry could be used to manufacture products with health benefits.

Materials and methods
Plant material and chemicals
Flaxseed hulls were provided by Yishanyuan Biotechnology Co., Ltd. (Hohhot, China). Reagent ammonium hydroxide (25–28% of NH_3 in water), Xilong Scientific Co., Ltd. (Shantou, China). 95% Ethanol, Kemao Chemical Reagents Co., Ltd. (Tianjin, China). Ethanol and methanol of analytical grade for extraction and isolation, Xilong Scientific Co., Ltd. (Shantou, China). Methanol and formic acid of HPLC grade for analysis, Fisher Scientific Company (Fair Lawn, NJ, USA). Dimethyl sulfoxide (DMSO), Coolaber Co., Ltd. (Beijing, China).

Column chromatography adsorbents were macroporous resin (particle size: 250 μm, Mitsubishi Chemical Co., Japan), Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Sweden)., and octadecyl silane (ODS, 38–63 μm, Wako Pure Chemical Industries, Ltd.). Standard SDG was purified in previous work (Jin et al., 2007b) and was kindly provide by Professor Hattori in University of Toyama, Japan.

Quantification of SDG
Ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) analyses were performed on an Agilent 1290 Infinity UPLC coupled with an Agilent 6430 Triple Quadrupole MS [Agilent Technologies Sin-78, Singapore (International) Pte. Ltd.] with a chromatographic column of Agilent ZORBAX Eclipse Plus C18 (2.1 mm × 50 mm; particle size, 1.8 μm). The temperature of the column was kept at 30 °C. The UHPLC mobile phase contained A (0.1% formic acid-water) and B (methanol). Elution program was set as follows: 0 min, 10% B; 4 min, 100% B; 6 min, 100% B. The flow rate was 0.3 ml/min and the injection volume was 1 μl. The voltage of the capillary of the MS was 3 kV; the temperature of the ion source was 325 °C. Using ESI-MS in negative ionization mode, SDG displayed molecular ion at m/z 685 and product ion at m/z 361 at fragmentor of 230 V and collision energy of 40 V. The calibration curve was obtained by multiple-reaction-monitory (MRM) analysis of the standards at 50.0000, 25.0000, 6.2500, 1.5625, 0.3906, 0.0977, 0.0244, 0.0061 and 0.0015 μg/ml. The linear regression equation and determination coefficient of the calibration curve were Y = 65.98x + 6.44, and R^2 = 0.9997, respectively. The accuracy representing the ratio of the measured concentration to the theoretical concentration from three levels was in the range of 84–104%. All samples were diluted 40 times with DMSO before UHPLC-MS analysis.

Single factor experiments
At first, crushed (20 mesh) and non-crushed flaxseed hull were compared for the SDG yield. Flaxseed hull (1 g) was extracted at 60 °C for 2 h with alcohol containing different RAM concentrations, 10, 20, 30, and 40%. The

experiments were carried out in triplicates. As the crushed flaxseed hull displayed higher SDG yield than the non-crushed flaxseed hull, the following experiments were carried out using crushed flaxseed hull.

A number of factors, including RAM concentration, extraction time, and extraction temperature that could affect the SDG yield were examined. At first, the RAM concentration was examined. One gram of crushed flaxseed hull was extracted with 5, 10, 20, 25, 30, and 40% RAM alcohol at 60 °C for 2 h, and the SDG content was analyzed by UHPLC-MS; Next, the extraction time was examined with the hull being extracted with 20% RAM in alcohol at 60 °C for 1, 2, 3, 4, 5, 6, and 7 h, and the SDG content was analyzed; Thirdly, the extraction temperature was examined by fixing the extraction time at 2 h, and the RAM concentration at 20%, and varying the water bath temperature at 40 °C (reaction temperature 38.9 °C), 50 °C (reaction temperature 49.1 °C), 60 °C (reaction temperature 58.9 °C), 70 °C (reaction temperature 67.8 °C), and 80 °C (reaction temperature 76.0 °C), and the corresponding SDG content was analyzed.

RSM experiment

RSM uses mathematical and statistical techniques to fit experimental data with polynomial equations. It is especially useful to optimize multiple variables at the same time to obtain the best system performance (Bezerra et al. 2008). RSM could offer large amounts of information from relatively small number of experiments, having important applications in design new process and improving the efficacy of known process, especially when the data were fit to a second order polynomial (Baş, & Boyacı 2007). When smaller ranges of independent parameters were chosen, the accuracy could be increased even for systems that were hardly explained by second order models, on the basis that preliminary works determined the independent parameter ranges. There are several response surface designs, among which, Box-Behnken design is more efficient for the study of three factors and could avoid experiments in extreme conditions (Ferreira et al. 2007).

On the basis of single factor experiments, RSM was carried out to optimize the SDG extraction condition. The concentration of ammonium hydroxide, extraction time and extraction temperature were considered as the investigation factors, and Design-Expert 12.0 software was used for the Box-Behnken three-factor and three-level design optimization. Taking the yield of SDG as the response value, the optimum technological parameters for extracting SDG from flaxseed hulls were studied. The response surface level is shown in Table 1.

Optimization of material-liquid ratio was carried out by extracting 1 g of crushed flaxseed hull under optimal extraction conditions of the three factors, while varying material-liquid ratios at 1:10, 1:20, 1:30, 1:40 and 1:50, and the corresponding contents of SDG were analyzed.

Separation and purification of SDG

Flaxseed hulls was pulverized to pass through 20 mesh and 30 g of the powder was extracted at 75.3 °C with 1200 ml of 33.7% RAM for 4.9 h. The mixture was filtered through filter paper and the filtrate was vacuum evaporated followed with freeze drying to obtain 5.7 g of an extract.

The extract was separated by a macroporous resin column (23.00 cm × 3.50 cm) chromatography eluted with ethanol-water to obtain crude SDG (772.12 mg) with purity of at least 76.08% from 10 to 20% ethanol eluted part (yield 2.57%). The crude SDG was further purified by Sephadex LH20 column (45.00 cm × 3.00 cm) chromatography eluted with water to obtain SDG (602.76 mg, yield 2.0%) at purity of 98%.
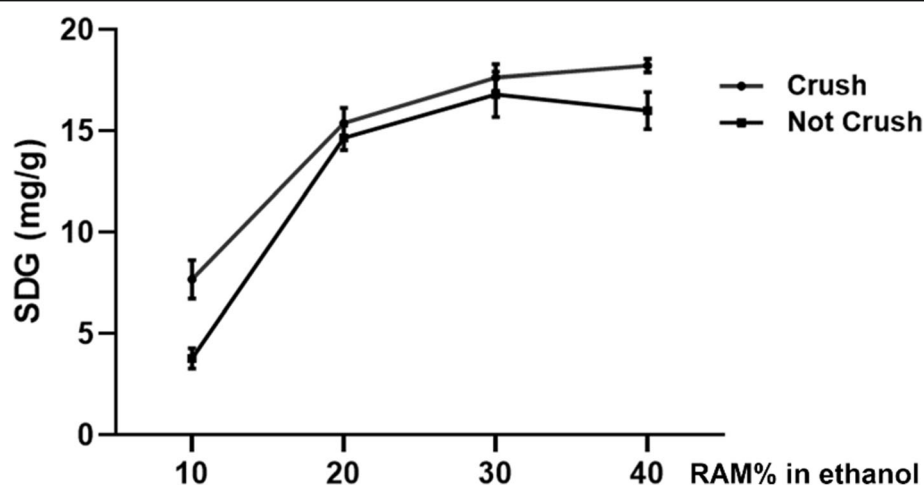
Statistical analysis

The RSM were performed using Design-Expert 12.0 software (Stat-ease Inc., Minneapolis, MN, USA). Data were subjected to a two-way analysis of variance (ANOVA) with the program GraphPad Prim 8.02 software.

Results

Effects of different extraction factors on the yield of SDG

Crushed (20 mesh) and non-crushed flaxseed hull were compared for the SDG yield. The results are shown in Fig. 2. The SDG yield of flaxseed hulls after crushing was higher.

In order to obtain the maximum yield of SDG, the influence of different factors on the extraction rate was explored. Alcoholic ammonium hydroxide is used as the reagent to alkali hydrolyze and extract SDG from flaxseed hulls in one pot. Therefore, the effects of different concentration of RAM on the yield of SDG were firstly studied. When the RAM concentration increased from 5 to 20%, the yield of SDG increased significantly. However, when the RAM concentration increased from 20 to 40%, the yield of SDG only changed slightly (Fig. 3a). As the extraction time increased, the yield of SDG continues to increase, and in 1−5 h, the yield showed a linear increase. After 5 h of extraction, there is little change

Table 1 Responses experimental factor level table

Name	Units	Low	High
RAM concentration	%	20(−1)	40(1)
Time	h	4(−1)	6(1)
Temperature	°C	60(−1)	80(1)

RAM: the reagent ammonium hydroxide solution that contains 25–28% NH₃ in water.

Fig. 2 The effect of crush on SDG yield. RAM: the reagent ammonium hydroxide solution that contains 25–28% NH_3 in water

in the yield of SDG (Fig. 3b). As shown in Fig. 3c, as the bath temperature increased from 40 °C to 80 °C (reaction temperature 38.9–76.0 °C), the yield of SDG increased from 11.01 mg/g to 21.03 mg/g. When the water bath temperature reached 80 °C, the alcoholic ammonium hydroxide was boiling, and the highest temperature was set at 80 °C.

Optimized SDG extraction conditions by RSM

The Box-Behnken three-factor and three-level design in RSM was used to optimize the experimental conditions. The RAM concentration in alcohol (A), time (B) and temperature (C) were taken as independent variables, and the SDG yield (Y) as the response value (Table 2). Multiple regression analysis was conducted to analyze the experimental data to obtain a full quadratic polynomial equation in terms of coded factors:

$$Y = 20.47 + 0.7032A + 1.67B + 1.89C + 0.8311AB + 0.4436\,AC - 1.86\,BC - 0.7845A^2 - 2.02B^2 + 0.4075C^2.$$

The analysis variance results of the regression model are shown in Table 3. *P*-values less than 0.0500 indicated the model terms are significant. In this case, B, C, BC, B^2 are significant model terms. $R^2 = 0.9326$, indicating that the regression model can be used to predict the variation of SDG yield with extraction conditions (RAM concentration, extraction time, extraction temperature). The Lack of Fit F-value of 0.88 implies the Lack of Fit is not significant relative to the pure error. Furthermore, the p-value of the lack of fit was 0.52, suggesting the occurrence of such a model due to noise is unlikely. The
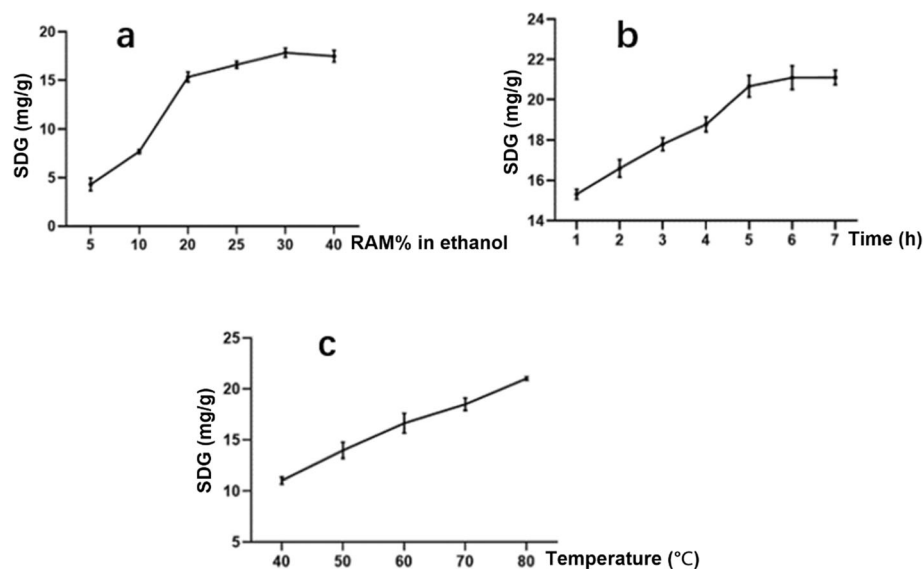
Fig. 3 The effect of different factors on SDG yield. RAM: the reagent ammonium hydroxide solution that contains 25–28% NH_3 in water

Table 2 Box-Behnken design and results

	Factor 1	Factor 2	Factor 3	Response 1
Run	A: RAM concentration	B: Time	C: Water bath temperature (Reaction temperature)	SDG
	(%)	(h)	(°C)	(mg/g)
1	40	5	60 (56.0)	18.98
2	30	4	60 (58.8)	12.63
3	30	5	70 (66.7)	19.84
4	30	5	70 (66.7)	19.32
5	30	5	70 (66.7)	20.50
6	20	6	70 (67.8)	17.51
7	20	5	80 (76.0)	20.32
8	20	4	70 (67.8)	16.63
9	30	4	80 (75.4)	20.95
10	40	6	70 (64.8)	20.36
11	40	4	70 (64.8)	16.15
12	30	6	80 (75.4)	21.35
13	30	5	70 (66.7)	20.71
14	30	5	70 (66.7)	21.98
15	20	5	60 (58.9)	18.24
16	30	6	60 (58.8)	20.49
17	40	5	80 (75.2)	22.83

RAM: the reagent ammonium hydroxide solution that contains 25–28% NH_3 in water.

analysis of variance in the regression equation shows that in the experimental range, the order of the three influencing factors on the yield of SDG is: extraction temperature > extraction time > RAM concentration.

Response surface plots and contour plots can intuitively reflect the interaction between the two factors. The steeper the response surface slope and the sharper the color change, the more obvious the interaction between the factors are. As can be seen in Fig. 4, the gradient of extraction time and extraction temperature is steep, and the *P* value of interaction is 0.0067 (Table 3), indicating that the interaction between the two is significant. Among the 100 optimal extraction process parameters obtained through response surface optimization

Table 3 Analysis of variance of regression equation

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	93.09	9	10.34	10.77	0.0024	significant
A- RAM concentration	3.96	1	3.96	4.12	0.0820	
B-Time	22.30	1	22.30	23.22	0.0019	**
C-Temperature	28.58	1	28.58	29.76	0.0010	**
AB	2.76	1	2.76	2.88	0.1337	
AC	0.7871	1	0.7871	0.8196	0.3954	
BC	13.89	1	13.89	14.47	0.0067	**
A^2	2.59	1	2.59	2.70	0.1444	
B^2	17.19	1	17.19	17.89	0.0039	**
C^2	0.6992	1	0.6992	0.7281	0.4217	
Residual	6.72	7	0.9604			
Lack of Fit	2.67	3	0.8906	0.8795	0.5230	not significant
Pure Error	4.05	4	1.01			
Cor Total	99.81	16				

*Significant $p < 0.05$; **Extremely significant $p < 0.01$
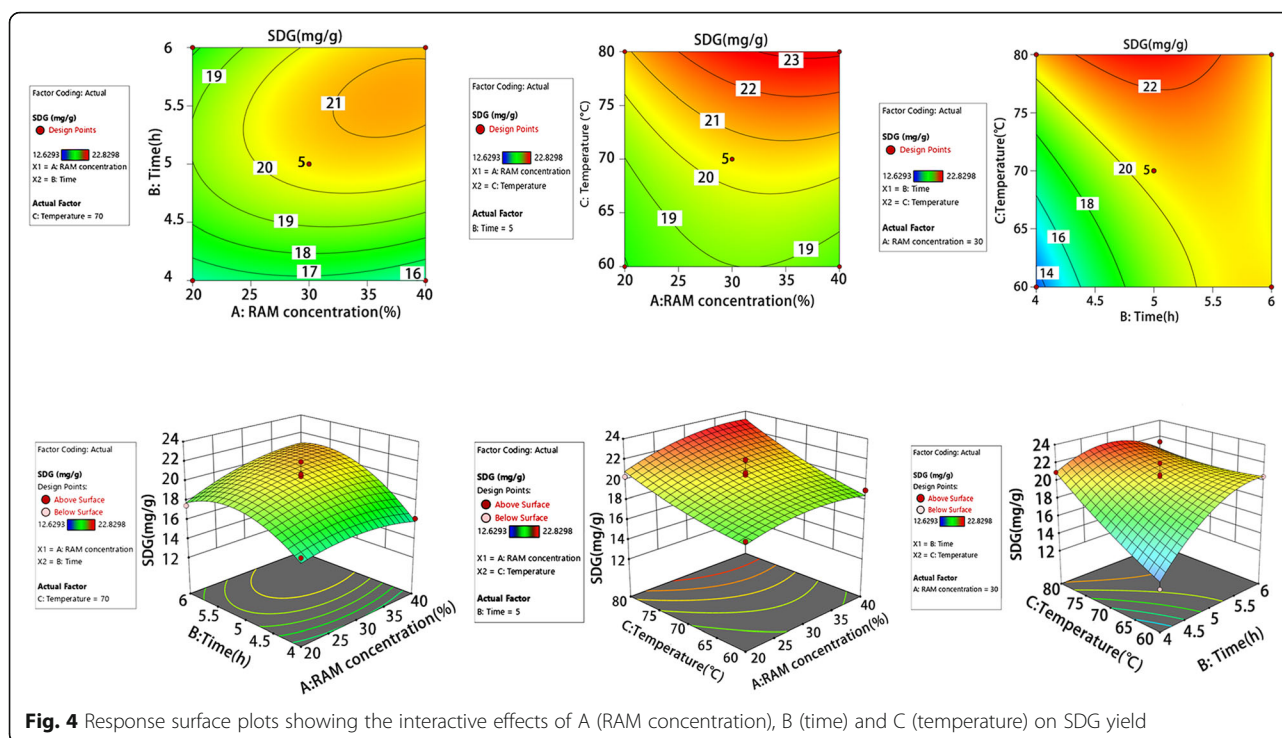
Fig. 4 Response surface plots showing the interactive effects of A (RAM concentration), B (time) and C (temperature) on SDG yield

analysis with the Design-Expert 12.0 software, the optimum extraction conditions with the shortest extraction time among the yield of SDG greater than 23 mg/g were found as: RAM concentration 33.722%, time 4.91 h, temperature 79.934 °C (reaction temperature 75.3 °C), and the predicted yield of SDG was 23.04 mg/g.

In order to verify the correctness of the model, SDG was extracted under the optimal extraction conditions and quantified by UPLC-MS. The SDG yield was found to be 23.28 mg/g, which was in consistent with the predicted results using RSM optimization analysis, indicating that the SDG extraction parameters optimized by Box-Behnken combination design were accurate and reliable. In addition, preparative extraction and isolation of SDG was performed from 30 g of flaxseed hulls powder which was extracted under the optimal extraction condition, followed by separation on macroporous resin to obtain SDG in several purity levels and amounts: 20–50% SDG 76.59 mg, 50–70% SDG 66.73 mg, 70–90% SDG 135.74 mg, and 90% SDG 493.06 mg. These fractions containing SDG were combined (772.12 mg, SDG > 76.08%) and further purified with Sephadex-LH20 column chromatography to obtain 602.76 mg SDG of 98% purity, with the yield of 20.09 mg/g, which was similar to the predicted yield.

Effect of material-liquid ratio on the SDG yield
The material-liquid ratio is also an important parameter influencing the extraction yield. The high material-liquid ratio would lead to waste of ethanol and increase of cost,

while the low material-liquid ratio would lead to incomplete SDG extraction. The material-liquid ratios of 1:10, 1:20, 1:30, 1:40, and 1:50 was studied to evaluate the influence on SDG yield. As shown in Fig. 5, the maximal SDG yield was obtained when the material-liquid ratio was 1:20.

Identification of SDG
SDG was identified and confirmed by comparison with standard compound of their retention times, UV spectra, and *m/z* values (Fig. 6).

Discussion
As the physiological activities of SDG are researched more extensively, the effects of SDG on human health have gained increasing attention. SDG can be used as a natural antioxidant additive in food to provide health benefits (Hosseinian et al. 2006; Prasad 1997).

Fuentealba et al. (2015), & Johnsson et al. (2000) studied the SDG contents in four flaxseed varieties, reporting levels of 1.13–2.41% in defatted flaxseed and 0.61–1.33% in whole flaxseed. Hao, & Beta (2012) reported that the SDG content in flaxseed hulls was 1.64–3.39%. There are many extraction techniques for flax lignans (Fuentealba et al. 2015; Hosseinian, & Beta 2009), most of which use whole flaxseed or defatted flaxseed to extract SDG. Wei et al. (2019) purified SDG from flaxseed by n-hexane defatting, ethanol extraction, sodium hydroxide hydrolysis, and AB-8 macroporous resin separation,
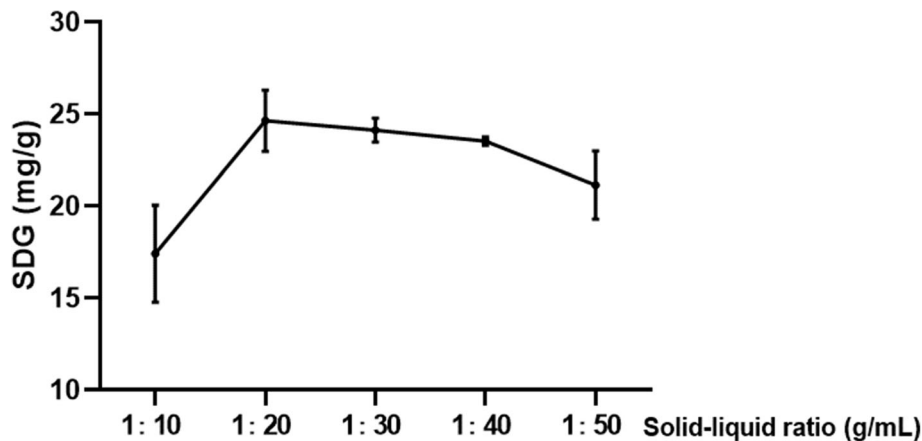
**Fig. 5** The effect of solid-liquid ratio on SDG yield

obtaining 90.1% pure SDG at a yield of 0.108%. High-speed countercurrent chromatography purification has been shown to yield SDG with 99.4% purity at a yield of 0.058% (Wang et al. 2019).

SDG is concentrated in flaxseed hulls, accounting for 22.6% of the weight of the entire flaxseed (Hosseinian, & Beta 2009). The method in this study used flaxseed hull to extract SDG. Optimizing the extract method followed by column chromatography, we obtained SDG with over 98% purity and a yield of 2.01%. This approach, using

one-pot hydrolysis and extraction, is simple, time saving, efficient, low-cost, and easy to scale up for the production of SDG.

Conclusion

SDG was extracted from flaxseed hulls efficiently using alcoholic ammonium hydroxide as the extract solvent, wherein the alkaline hydrolysis and solvent extraction were performed in one pot. Under optimized conditions—33.7% RAM in ethanol at 75.3 °C for 4.9 h—the
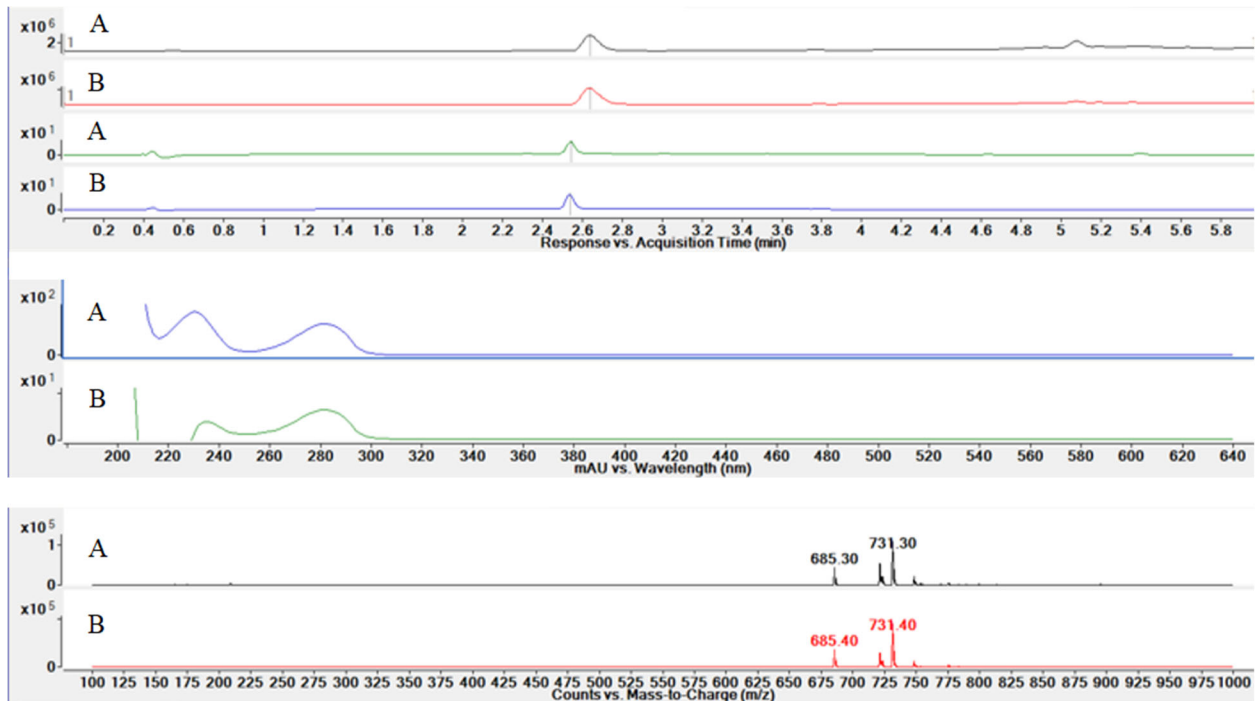
Fig. 6 UHPLC-MS comparison of the isolated SDG with standard SDG. (**A**) standard SDG, (**B**) SDG isolated in this research. Lines 1–2, UHPLC chromatograms detected with (–) ESI MS; Lines 3–4, UHPLC chromatograms detected with UV at 280 nm; Lines 5–6, UV spectra; Lines 7–8, MS spectra

yield of SDG was 23.3 mg/g. A subsequent run of microporous resin column chromatography, eluted with edible solvent (ethanol-water), generated high-purity SDG in high yield that is suitable for large-scale production of SDG in factories.

Abbreviations
SDG: secoisolariciresinol diglucoside; RAM: reagent ammonium hydroxide (25–28% of NH$_3$ in water); RSM: response surface methodology; UHPLC-MS: ultra-high performance liquid chromatography-mass;
SECO: secoisolariciresinol; DMSO: Dimethyl sulfoxide; ODS: octadecyl silane; MRM: multiple-reaction-monitory

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Authors' contributions
Cong-Cong Zhuang, methodology, investigation, data analysis and draft writing. Chun-Rui Liu: investigation, data analysis. Chen-Bin Shan, methodology, computing. Zheng Liu and Ling Liu: resources. Chao-Mei Ma: conceptualization, methodology, supervision, review and editing. All authors read and approved the final manuscript.

Availability of data and materials
All data needed to evaluate the conclusions are present in the paper. Additional data related to this paper may be available upon request.

Declarations

Ethical approval and consent to participate
Not applicable.

Consent for publication
All authors consent to the publication.

Competing interests
The authors declare that they have no competing interest.

Author details
[1]State Key Laboratory of Reproductive Regulation & Breeding of Grassland Livestock, Hohhot, China. [2]Key Laboratory of Herbage & Endemic Crop Biology of Ministry of Education, School of Life Sciences, Inner Mongolia University, Hohhot, P.R. China 010070. [3]Inner Mongolia Yishanyuan Biotechnology Co., Ltd, Hohhot, People's Republic of China.

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